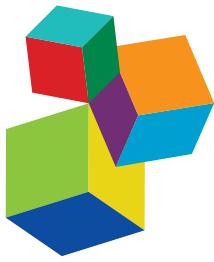


CROP TRAITS FOR DEFENSE AGAINST PESTS AND DISEASE: DURABILITY, BREAKDOWN AND FUTURE PROSPECTS, 2nd Edition

EDITED BY: Alison J. Karley, Scott N. Johnson, Rex Brennan and Peter J. Gregory
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CROP TRAITS FOR DEFENSE AGAINST PESTS AND DISEASE: DURABILITY, BREAKDOWN AND FUTURE PROSPECTS, 2nd Edition

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Defensive traits will play an important role in future crop protection: trichomes on the leaf surface can deter feeding by arthropod pests such as aphids.

Photo credit: Stuart Malecki (The James Hutton Institute, UK).

With global populations expected to exceed 9.2 billion by 2050 and available land and water resources devoted to crop production dwindling, we face significant challenges to secure global food security. Only 12 plant species feed 80% of the world's population, with just three crop species (wheat, rice and maize) accounting for food consumed by 50% of the global population. Annual losses to crop pests and pathogens are significant, thought to be equivalent to that required to feed a billion people, at a time when crop productivity has plateaued. With pesticide applications becoming increasingly unfeasible on cost, efficacy and environmental grounds,

there is growing interest in exploiting plant resistance and tolerance traits for crop protection. Indeed, mankind has been selectively breeding plants for desirable traits for thousands of years. However, resistance and tolerance traits have not always been those most desired, and in many cases have been inadvertently lost during the domestication process: crops have been effectively 'disarmed by domestication'. Moreover, mechanistic understanding of how resistance and tolerance traits operate is often incomplete, which makes identifying the right combination for crop protection difficult.

We aimed to address this Research Topic by inviting authors to contribute their knowledge of appropriate resistance and tolerance traits, explore what is known about durability and breakdown of defensive traits and, finally, asking what are the prospects for exploiting these traits for crop protection. The research topic summarised in this book addresses some of the most important issues in the future sustainability of global crop production.

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Editorial: Crop Traits for Defense against Pests and Disease: Durability, Breakdown and Future Prospects

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Keywords: climate change, crops, food security, pathogens, pests, resistance

Editorial on the Research Topic

Crop Traits for Defense against Pests and Disease: Durability, Breakdown and Future Prospects

With the Earth's population expected to reach 11.2 billion by 2100 there is a pressing need to maximize food production at a time when productivity of many crops is reaching a plateau. Minimizing losses to pests and diseases is therefore a crucial means of meeting this challenge and securing food supply. This research topic addresses some of the most important issues in the future sustainability of global crop production. The topic consists of 20 papers, of which 13 describe original research. There are six review papers, plus one hypothesis and theory contribution.

The development of plants with resistance to the most damaging pests and diseases is increasingly important in the face of growing pressure to reduce synthetic chemical inputs, including pesticides, fungicides, and herbicides used for crop protection. This reduction is partly underwritten by legislative directives, consumer demands and indeed an overall reduction in available chemical controls for most crops (Hillocks, 2012), especially those outwith the main arable species. Additionally, losses due to pest and disease attacks represent a major financial cost in crop production and throughout the subsequent supply chain, even before the costs of control measures are considered. The move toward more sustainable production systems, based on integrated pest and disease management approaches, is gaining momentum, and the development of more resistant cultivars is a major factor contributing to the success of these systems. In addition, there is a recognized need for frameworks (e.g., Birch et al., 2011) which combine natural enemies with resistant cultivars and other management practices to reduce reliance on crop protection chemicals and maintain viable and sustainable future crop production.

The development of resistant cultivars is crucial to the future of sustainable crop production practices, and there is a substantial need for the continued introgression of specific resistance genes or physical and structural traits, from existing or extended genetic resources. Recent advances in knowledge can aid this process, even in many minor crop species, where increased understanding of trait heritability and technological advances in genomics and bioinformatics are enabling the identification of genes controlling resistance, providing a framework for improved selection efficiency. Moreover, the advent of new technologies can provide significant benefits, as exemplified by the opportunities afforded by CRISPR-based tools in understanding plant-pathogen interactions; these are addressed in the review by Barakate and Stephens. The need for new resistance genes is crucial within many pathogen/crop systems, and Van Weymers et al. describe the application of a range of "omics" technologies in potato to identify novel resistances to potato blight within a large germplasm collection. This approach is one that can be adapted for other species and pathogens, as the future role of resistance genes from existing but underexploited genetic

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resources is likely to be significant. This is not limited to inherent crop resistance, however, and Reynolds et al. also highlight the role of “omics” approaches for augmenting plant resistance to invertebrates using application of silicon.

Breeding strategies focused on the identification and incorporation of specific resistance genes, both individually or in effective groups, are being developed and implemented in many active breeding programmes. The use of marker-assisted backcross breeding in rice to pyramid resistance genes for bacterial blight and blast diseases is the subject of the paper by Abhilash Kumar et al. An investigation of genes involved in the infection processes by *Phytophthora capsici* in *Capsicum* spp. by Zhang et al. through genome-wide identification highlights the role of SQUAMOSA promoter binding protein (SBP)-box genes that can be utilized in future breeding and development research. Genetic mapping of host plants, such as *Capsicum*, was used by Barbary et al. to identify QTLs linked to resistance to *Meloidogyne* nematode species, and from this the underlying genes can eventually be identified and utilized in the breeding of resistant plants. Similarly, wild *Vitis* genotypes studied by Wan et al. to find sources of resistance to *Botrytis*, based on antioxidant activity linked to resistance, will be deployed in future breeding strategies. Further work on wild *Vitis* by Wen et al. identified a gene linked to powdery mildew resistance and confirmed its effect through ectopic expression in *Arabidopsis*; again, this provides a resource for future breeding.

The effects of pest attacks on host plant metabolism was investigated by Liu et al., using the wild brassica species *Barbarea vulgaris* and the global pest diamondback moth, *Plutella xylostella*. By examining changes in glucosinolate biosynthesis induced by larval infestation, inferences can be made about the mechanisms underpinning defense induction. A quantitative approach to the breeding of resistant cultivars is described by Mohammed et al. in *Sorghum*, where the use of diallel progeny enabled the identification of a significant interaction between resistance to shoot fly and morphological traits such as grain yield and seed size. The information on the quantitative genetics of *Sorghum* can be used to inform decisions on parental choice for resistance breeding.

Many host-pathogen systems are subject to rapid evolution, for example in pathogen race structure or breakdown of host resistance. The paper by Gómez-Cortecero et al. describes the use of SNPs and SSRs to study diversity in global populations of *Neonectria ditissima* affecting apple trees, and also present evidence of a relatively simple pattern of host response which is not influenced by any race structure in the pathogen population.

Integrated Pest and Disease Management (IPDM) approaches to pest and disease control require the development of resistant varieties and also monitoring and management strategies that can be applied effectively within crops. The paper by Joshi et al. deals with the development of a susceptibility index for codling moth on apple, based on oviposition preferences. The review by Peterson et al. considers the wide range of plant defense traits that can influence the responses of natural enemies used in IPDM systems, and also includes the potential impacts of transgenic crops on trophic levels and arthropod communities.

Plant defenses can be exploited to enhance resistance to pest attack and also to confer tolerance of pest infestations. Plant physical defenses can offer particularly durable resistance to pests and pathogens (Johnson et al., 2016; Moore and Johnson, 2017); ecological studies, for example, have shown plant physical traits to be more effective deterrents to insect herbivory than plant secondary metabolites (Peeters et al., 2007; Cooke and Leishman, 2012). As outlined in the review by Mitchell et al., plant structural traits such as trichomes, spines, and cuticles can provide a physical barrier to arthropod pest attachment, feeding and oviposition, while plant vigor and altered phenology can increase tolerance of pest damage and reduce the incidence of pest attacks. This paper highlights new avenues for discovery of plant defensive traits, particularly through research to understand pest-induced changes in plant chemistry and mechanisms of plant defense “priming”.

The effects of a changing climate are likely to alter both severity of pest and pathogen attacks, and also the spectrum of organisms that will cause damage to crop plants (Johnson and Jones, 2017). Two papers in this research topic consider changes under conditions of elevated CO₂; the first by McKenzie et al. presents data illustrating the changes in herbivory by two pests of raspberry, the European large raspberry aphid (*Amorphophoia idaei*) and the root-feeding vine weevil (*Otiorrhynchus sulcatus*). The second contribution, a review by Sun et al., examines changes in host plant metabolism and water-use efficiency instigated under elevated CO₂ conditions, and how these changes impact on the outcome of plant-aphid interactions.

Host plant resistance can have significant effects on organisms and trophic levels beyond the target pest or pathogen, and this has implications for integrated management of crop systems. This area is discussed by Peterson et al. in their review, which also considers the use of transgenic crops. Genetic modification technology has potential application in the development of pest-resistant cultivars of some arable species, especially where there are limited sources of resistance within the genus, and this is discussed in the context of cotton production systems by Trapero et al. and de Oliveira et al. The identification by the latter authors of resistances covering multiple pests may offer particular opportunities.

Making use of multiple resistance traits might also offer an alternative approach toward pathogen control. The theory paper by Newton suggests that the harmful effects of fungal disease outbreaks in cereal crops could be limited by using mixed genotype plantings; cultivar mixtures often show higher tolerance of, or resistance to, disease. The impact of this approach on other microbial species in the crop environment is also considered.

The development of environmentally friendly control mechanisms for pests is a further aspect of future crop production systems, and there are various less damaging ingredients under consideration. These can include the use of plant mutualists or manipulation of soil conditions, for example by application of chemical constituents such as silicon (Johnson et al., 2016). In terms of plant mutualists, arbuscular mycorrhizal fungi can improve crop productivity, as reported by Robinson-Boyer et al., which may result in better tolerance of pest and disease attack Mitchell et al. and also help plants

resist attack by root herbivores (Johnson et al., 2016). Moreover, the role of silicon application to the soil and resistance to herbivory is considered in the review by Reynolds et al. They present evidence for direct and indirect defenses (e.g., recruitment of natural enemies of pests via volatile emissions) by silicon.

We hope this research topic will provide a valuable resource for workers and researchers in the field of crop protection by providing a source of information about existing and novel sources of crop resistance and tolerance traits, their incorporation into breeding programmes, how they can be deployed for maximum efficacy under field conditions when integrated with other pest and disease control measures, and their potential to provide durable and sustainable crop protection under a changing environment.

TRIBUTE TO ALAIN PALLOIX

It is with sadness that we note the death of Alain Palloix, whose manuscript on resistance to root-knot nematodes Barbary et al.

is included in this research topic. Alain's work over many years made a significant contribution to the understanding of pepper-pathogen interactions and resistance breeding, and we extend our sympathy to his family and colleagues.

AUTHOR CONTRIBUTIONS

All authors listed have made substantial, direct and intellectual contribution to the work and approved it for publication.

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REFERENCES

- Birch, A. N. E., Begg, G. S., and Squire, G. R. (2011). How agro-ecological research helps to address food security issues under new IPM and pesticide reduction policies for global crop production systems. *J. Exp. Bot.* 62, 3251–3261. doi: 10.1093/jxb/err064
- Cooke, J., and Leishman, M. R. (2012). Tradeoffs between foliar silicon and carbon-based defences: evidence from vegetation communities of contrasting soil types. *Oikos* 121, 2052–2060. doi: 10.1111/j.1600-0706.2012.20057.x
- Hillocks, R. J. (2012). Farming with fewer pesticides: EU pesticide review and resulting challenges for UK agriculture. *Crop Prot.* 31, 85–93. doi: 10.1016/j.cropro.2011.08.008
- Johnson, S. N., Benefer, C. M., Frew, A., Griffiths, B. S., Hartley, S. E., Karley, A. J., et al. (2016). New frontiers in belowground ecology for plant protection from root-feeding insects. *Appl. Soil Ecol.* 108, 96–107. doi: 10.1016/j.apsoil.2016.07.017
- Johnson, S. N., and Jones, T. H. (2017). *Global Climate Change and Terrestrial Invertebrates*. Chichester: John Wiley & Son Ltd.
- Moore, B. D., and Johnson, S. N. (2017). Get tough, get toxic, or get a bodyguard: identifying candidate traits conferring belowground resistance to herbivores in grasses. *Front. Plant Sci.* 7:1925. doi: 10.3389/fpls.2016.01925
- Peeters, P. J., Sanson, G., and Read, J. (2007). Leaf biomechanical properties and the densities of herbivorous insect guilds. *Funct. Ecol.* 21, 246–255. doi: 10.1111/j.1365-2435.2006.01223.x

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Utilizing “Omic” Technologies to Identify and Prioritize Novel Sources of Resistance to the Oomycete Pathogen *Phytophthora infestans* in Potato Germplasm Collections

Pauline S. M. Van Weymers^{1†}, Katie Baker^{2†}, Xinwei Chen^{1†}, Brian Harrower¹, David E. L. Cooke¹, Eleanor M. Gilroy¹, Paul R. J. Birch¹, Gaëtan J. A. Thilliez¹, Alison K. Lees¹, James S. Lynott¹, Miles R. Armstrong¹, Gaynor McKenzie¹, Glenn J. Bryan^{1*} and Ingo Hein^{1*}

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The greatest threat to potato production world-wide is late blight, caused by the oomycete pathogen *Phytophthora infestans*. A screen of 126 wild diploid *Solanum* accessions from the Commonwealth Potato Collection (CPC) with *P. infestans* isolates belonging to the genotype 13-A2 identified resistances in the species *S. bulbocastanum*, *S. capsicibaccatum*, *S. microdontum*, *S. mochiquense*, *S. okadae*, *S. pinnatisectum*, *S. polyadenium*, *S. tarijense*, and *S. verrucosum*. Effector-omics, allele mining, and diagnostic RenSeq (dRenSeq) were utilized to investigate the nature of resistances in *S. okadae* accessions. dRenSeq in resistant *S. okadae* accessions 7129, 7625, 3762, and a bulk of 20 resistant progeny confirmed the presence of full-length *Rpi-vnt1.1* under stringent mapping conditions and corroborated allele mining results in the accessions 7129 and 7625 as well as Avr-vnt1 recognition in transient expression assays. In contrast, susceptible *S. okadae* accession 3761 and a bulk of 20 susceptible progeny lacked sequence homology in the 5' end compared to the functional *Rpi-vnt1.1* gene. Further evaluation of *S. okadae* accessions with *P. infestans* isolates that have a broad spectrum of virulence demonstrated that, although *S. okadae* accessions 7129, 7625, and 7629 contain functional *Rpi-vnt1.1*, they also carry a novel resistance gene. We provide evidence that existing germplasm collections are important sources of novel resistances and that “omic” technologies such as dRenSeq-based genomics and effector-omics are efficacious tools to rapidly explore the diversity within these collections.

Keywords: germplasm collection, Commonwealth potato collection, diagnostic, RenSeq, *Phytophthora infestans*, oomycete, RXLR effectors

INTRODUCTION

Potato is the most important non-cereal food crop worldwide and is consumed by more than a billion people (Birch et al., 2012). Global potato production between 1991 and 2007 has shown an increase of 21% that is driven by a 48% rise of potato production in the developing world, where the growing area has increased alongside yield. Pests and pathogens represent a serious and continuing

threat to potato production, and the most widespread and economically significant of these is late blight, caused by the oomycete pathogen *Phytophthora infestans*. In agricultural systems major population changes of *P. infestans* lineages have been observed that often impact negatively on crop production. For example, in the European *P. infestans* population a new clonal lineage referred to as 13-A2 or “blue 13” was first detected in 2004 and, upon its arrival in Great Britain, came to dominate the population within 3 years (Cooke et al., 2012). Previously resistant potato cultivars such as Lady Balfour and Stirling were susceptible to the 13-A2 lineage and are consequently no longer suitable for the organically grown potato market. A conservative estimate of the chemical control costs and yield losses associated with late blight exceeds €6.7 Billion (Haverkort et al., 2009). In many parts of the world fungicide application is the only means to prevent disease. Predictions suggest that global potato production could exceed 400 Mt per year if diseases that reduce yields by ~25% could be controlled (Agrios, 1997).

The ability to withstand multiple biotic and abiotic stresses is critical for wild potato species, suggesting that many untapped, natural sources of resistance exist for exploitation in breeding programs. With the availability of extensive germplasm resources, including the Commonwealth Potato Collection (CPC) at the James Hutton Institute (Bradshaw et al., 2006), and improved genomics tools, the potential to exploit this natural biodiversity is considerable. Newly identified and deployed resistances could provide an environmentally benign opportunity to secure potatoes as a major food source in the future (Birch et al., 2012). Critical for the success of such disease control is, however, a detailed knowledge of the underlying mechanisms of defense to facilitate complementary deployment of resistances.

Inducible resistance responses in plants require the direct or indirect detection of pathogen molecules such as defense elicitors or effector molecules via plant receptors (Jones and Dangl, 2006; Wiesel et al., 2014). Effectors, once recognized, are known as avirulence (*Avr*) genes as their recognition often yields incompatibility for the pathogen on plants that carry the cognate resistance (R) protein. Genome-wide analysis of *P. infestans* and other oomycetes has shown that all identified *Avr* genes contain a canonical RXLR motif, which has led to coining of the term RXLR effectors (Armstrong et al., 2005; Hein et al., 2009; Raffaele et al., 2010; Cooke et al., 2012). Heterologous expression of these effectors is used as a novel tool for the identification of resistances and for disease resistance breeding (Birch et al., 2008; Vleeshouwers and Oliver, 2014; Lenman et al., 2016). The recognition of effectors is often dependent on R proteins that contain nucleotide binding (NB) and leucine-rich repeat (LRR) domains and are collectively known as NB-LRRs (Meyers et al., 1999). In the innate plant immune system this process is known as effector-triggered immunity (ETI; Jones and Dangl, 2006). NB-LRR genes are key to plant immunity and their presence, absence or allelic diversity is decisive for disease resistance. At least seven distinct potato NB-LRRs effective toward *P. infestans* have been cloned so far and their cognate effectors are well described (reviewed in Vleeshouwers and Oliver, 2014). Furthermore, allele mining for late blight resistance genes such as *Rpi-blb1*, *Rpi-blb2*, and *Rpi-blb3* from the diploid Mexican species *S. bulbocastanum*

has identified functional orthologs in other species (Lokossou et al., 2009, 2010). For example, *Rpi-blb1* orthologous genes were identified in the Mexican diploid species *S. cardiophyllum*, the allotetraploid species *S. papita* and *S. polytrichon* as well as in *S. stoloniferum* amongst others (Wang et al., 2008; Lokossou et al., 2010). When seeking novel resistances in germplasm collections, it is thus imperative to exclude accessions that contain already characterized resistances as the sole means of defense against the pathogen in question.

Recent advances in genome sequencing technologies enable rapid analysis of entire crop genomes and have accelerated the identification of functional R genes. Indeed, 11 years since sequencing the model plant *Arabidopsis thaliana*, the genomes of two important Solanaceae crop plants, potato, and tomato, were reported (Potato Genome Sequencing Consortium (PGSC), 2011; Tomato Genome Consortium (TGC), 2012). These genomes provide a blueprint for identification of genes coding for important traits such as disease resistance. In the sequenced *Solanum tuberosum* group Phureja clone DM1-3 516 R44 (DM), 755 NB-LRR genes have been identified and their phylogenetic relationships as well as their physical locations in the 12 potato chromosomes described (Jupe et al., 2012, 2013). These studies formed the basis of a novel R gene enrichment and sequencing platform (dRenSeq) that enables the improved annotation of resistance genes in sequenced genomes and facilitates rapid mapping and cloning of resistances via bulked-segregant analysis (Jupe et al., 2013).

In this study we utilized a combination of late blight infections, effector-omics, allele mining, and dRenSeq to identify and/or prioritize novel sources of resistance toward the *P. infestans* lineage 13-A2. As a proof of concept, dRenSeq was applied as a diagnostic tool to two accessions of the diploid potato species *S. okadae* and confirmed the presence of *Rpi-vnt1.1* in this species.

MATERIALS AND METHODS

Late Blight Screening of Diploid CPC Accessions

Isolates of *P. infestans* were established *in vivo* on leaves of the late blight susceptible cultivar Craig’s Royal and passaged through several generations according to Andrivon et al. (2011). Detached leaf tests were carried out as described by Whisson et al. (2007) and seedling and whole plant tests (two replicates) as described by Stewart et al. (1983) and Bradshaw et al. (2006), respectively. Disease was scored between 5 and 8 days post infection (dpi) on a scale of resistance ranging from 1 = very susceptible to 5 = very resistant for seedling and detached leaf tests and 1 = very susceptible to 9 = very resistant; symptomless plants, for whole plants according to the Malcolmson scale (Cruickshank et al., 1982).

Transient Expression of *P. infestans* Effectors in *S. okadae* Accessions

P. infestans effectors were cloned into the binary vector pGRAB and transformed into the *A. tumefaciens* strain Agl1 with VirG

and pSoup. An empty vector was used as a negative control. Infiltrations and analysis of infiltration sites were conducted as described previously (Gilroy et al., 2011).

Rpi-vnt1 Allele Mining in *S. okadae* Accessions

Rpi-vnt1-like genes have been amplified from the *S. okadae* accessions 7129, 7625, and 7629 through PCRs with the *Rpi-vnt1* specific primers Rpi-vnt1_F_full: 5'ATGAATTATTGTGTTACAAGACTTGG3' and Rpi-vnt1_R_full: 5'TTATAGTACCTGTGATATTCTCAACTTTGC3'. To assess the diversity of the *Rpi-vnt1-like* sequences PCR products were cloned into the vector pGEM-T easy for Sanger sequencing, according to the manufacturer's recommendations (pGEM®-T Easy Vector System—Promega). Recombinant clones were selected following transformation of the constructs into electro competent *Escherichia coli* DH10B and DH5α cells (Invitrogen) using colony PCR with the gene specific primers mentioned above. Sequencing products were subjected to a BLASTn analysis and compared to functional *Rpi-vnt1* variants (Pel et al., 2009) using Geneious v5.6.3 (Biomatters).

RenSeq Analysis

RenSeq target enrichment and sequencing was performed according to Jupe et al. (2013, 2014) with minor modifications. The covaris sonicator M220 (Covaris), was used for the fragmentation of DNA to ~500 bp in length, with the following settings: 50 W Peak Incident Power, 20% Duty Factor, 200 cycles per burst, 60 s treatment time and 50 μL volume with 1 μg starting amount. The fragments sizes were checked using a Bioanalyser (Agilent) and no upper size selection was conducted. The samples were quantified using Qubit (Thermofisher) and the enrichment was started with 750 ng of indexed libraries. The Agilent SureSelect enrichment library utilized was designed to include all NB-LRRs identified by Jupe et al. (2013) and the sequences of the corresponding 46,220 probes can be accessed at <http://solanum.hutton.ac.uk>. Added to the hybridization was 1 μL of 1000 mM universal blocking primer, containing six inosines in place of the six nucleotide index sequence and a 3' spacer C3 modification to prevent the primer from participating in any subsequent PCR amplification. The post capture amplification was performed with the Herculase II polymerase (Agilent). Sequencing was conducted on an Illumina MiSeq platform using the 2x 300 bp kit. The raw sequence reads were deposited at the European Nucleotide Archive under accession number PRJEB12834.

Paired-end Illumina MiSeq reads were first checked with FastQC (v0.10.0; Andrews, 2010) and then quality and adapter trimmed with cutadapt (v1.9; Martin, 2011) to a minimum length of 100 bp and minimum base quality of 20. The trimmed reads were then mapped to the potato DM reference genome (v4.03; Potato Genome Sequencing Consortium (PGSC), 2011; Sharma et al., 2013) or a FASTA file containing 12 cloned R genes using Bowtie2 (v2.2.1; Langmead and Salzberg, 2012) in very-sensitive end-to-end mode.

The known R genes comprise: *R1* (GenBank: AF447489.1; Ballvora et al., 2002), *R2* (GenBank: FJ536325.1; Lokossou et al., 2009), *R2-like* (GenBank: FJ536323.1; Lokossou et al., 2009),

R3a (GenBank: AY849382.1; Huang et al., 2005), *R3b* (GenBank: JF900492.1; Li et al., 2011), *Rpi-sto1* (GenBank: EU884421.1; Vleeshouwers et al., 2008), *Rpi-pt1* (GenBank: EU884422.1; Vleeshouwers et al., 2008), *Rpi-blb1* (GenBank: AY426259.1; van der Vossen et al., 2003), *Rpi-blb2* (GenBank: DQ122125.1; van der Vossen et al., 2005), *Rpi-blb3* (GenBank: FJ536346.1; Lokossou et al., 2010), *Rpi-abpt* (GenBank: FJ536324.1; Lokossou et al., 2009), and *Rpi-vnt1.1* (GenBank: FJ423044.1; Foster et al., 2009).

For read mapping, discordant and mixed mappings were disabled and maximum insert was set to 1000 bp. Four score-min parameters were used in different mapping runs: "L,-0.03,-0.03," "L,-0.06,-0.06," "L,-0.3,-0.3," and "L,-0.6,-0.6," approximately equal to 0.5, 1, 5, and 10% mismatch rates, respectively. The resulting BAM files were sorted and indexed using SAMtools (v0.1.18; Li et al., 2009).

The percentage of mapped reads on target was calculated as the proportion of reads mapping to an annotated, targeted RenSeq region in the DM genome reference. Intersecting these RenSeq regions (plus 1000 bp up- and down-stream) against the mapped reads using BEDTools (v2.20.1; Quinlan and Hall, 2010) gave the number of on-target reads. The reads on target was then calculated as a proportion of the total number of mapped reads. Read coverage to on-target regions was estimated by dividing the number of base pairs mapped to the 704 R genes (plus 1000 bp up- and down-stream) on chromosomes 1–12 by their total length (plus 2000 bp per gene). Read coverage was also estimated for the 12 R gene reference set by dividing the total length of mapped reads by the total length of the reference set.

RESULTS

Identification of Diploid CPC Accessions Resistant to *P. infestans* Genotype 13-A2

Seedlings and selected whole plants of 126 diploid CPC accessions belonging to 34 species (Supplementary Table S1) were tested with the *P. infestans* isolates 2006-3928A and/or 2009-7654A belonging to the *P. infestans* clonal lineage 13-A2. Resistance was observed within 29 of those accessions, belonging to the species *S. bulbocastanum*, *S. capsicibaccatum*, *S. microdontum*, *S. mochiquense*, *S. okadae*, *S. pinnatisectum*, *S. polyadenium*, *S. tarjense*, and *S. verrucosum* (Table 1). There was a strong correlation in the resistance phenotypes observed with both isolates and in the seedling vs. whole plant assays.

To determine if the resistances in these species are based on novel or already characterized resistance genes, a number of complementary assays were performed. In this study we report only on accessions of *S. okadae* and tested for the presence of *Rpi-vnt1.1* amongst other characterized R genes. The resistance gene *Rpi-vnt1.1* was initially cloned from *S. venturii* and *S. okadae* as well as *S. phureja* accessions and is a homolog of the tomato mosaic virus gene TM-2(2) (Foster et al., 2009).

S. okadae Accessions Respond to Avr-vnt1 in Heterologous Transient Expression Assays

A set of over 90 *P. infestans* RXLR effectors has been cloned into binary expression systems to allow the heterologous expression

via *Agrobacterium tumefaciens*. A subset of 82 effectors that includes known *Avr* genes (Supplementary Table S2) such as *Avr-vnt1* (Pel, 2010) was screened on accessions of *S. okadae* including susceptible plants *S. okadae* 7775 and 3761. In at least seven independent replicates with more than 14 individual infiltration sites in total, *Avr-vnt1* was recognized reproducibly in *S. okadae* accessions 7129, 7625, and 7629 but not in susceptible plants 7775 or 3761 (Figure 1). *S. okadae* accession 3762 was not responsive to *Agrobacterium*-based expression of effectors and controls (data not shown).

Allele Mining and dRenSeq Confirm that *S. okadae* Accessions Contain *Rpi-vnt1.1*

Rpi-vnt1.1 gene specific PCR primers were designed and utilized to ascertain if the *S. okadae* accessions 7129, 7625, and 7629 contain the 2676 bp long gene *Rpi-vnt1.1* (Foster et al., 2009) that is also present in *S. okadae* accession 3762 (Hein et al., unpublished). PCR products were cloned and Sanger sequenced to establish the sequences of individual clones. Alignment of PCR product sequences with *Rpi-vnt1.1* indicates that all three accessions contain a sequence identical to *Rpi-vnt1.1* alongside additional gene variations and truncated sequences (Figure 2).

RenSeq-based sequence analysis was conducted to corroborate the allele mining results and to establish whether RenSeq could be used as a diagnostic tool for validating the presence of functional NB-LRR genes. Genomic potato DNA samples from *S. okadae* accessions 7129 and 7625 were indexed, enriched for NB-LRR genes, and sequenced on a single lane of Illumina MiSeq. Each sample took a 12th of the MiSeq lane. Following quality control, 1,814,975 paired-end reads were obtained for *S. okadae* accession 7129 and 1,518,349 for 7625. Mapping against the sequenced potato clone DM, which has 704 NB-LRRs with known positions on chromosomes 1–12 (Jupe et al., 2013) was conducted at 0.5, 1, 5, and 10% mismatch rates. At 0.5 and 1% mismatch rates the systematic differences between *S. okadae* and *S. phureja* were apparent and a maximum of 6.49% of all reads could be mapped, of which more than 50% were on target. However, when allowing for a 5 or 10% mismatch rate, more than 46 or 70% of all reads could be mapped, respectively. Furthermore, the on-target rate increased to a maximum of 69.5% and mean coverage of NB-LRRs reached 108x (Table 2). Importantly, more of the 704 NB-LRR reference genes from DM were covered by reads from *S. okadae* accessions with conditions allowing for 5% or higher mismatch rates (Figure 3A, Supplementary Figure S1A, Table 3) indicating that the enrichment was successful.

Sequences derived from 7129 and 7625 were also mapped to a reference set of 12 characterized potato late blight NB-LRR sequences including *R1*, *R2*, *R2-like*, *Rpi-abpt*, *Rpi-blb3*, *R3a*, *R3b*, *Rpi-blb1*, *Rpi-ptal*, *Rpi-sto1*, *Rpi-blb2*, and *Rpi-vnt1.1* in a dRenSeq analysis. At 1% mismatch rate, only functional *Rpi-vnt1.1* was completely represented by dRenSeq reads (Figure 3B, Supplementary Figure S1B). Similar specific results were observed at 0.5% mismatch rate but not at 5 or 10% (Supplementary Figure S2). Indeed, at 5 and 10% mismatch rates, the mean read coverage of *Rpi-vnt1.1* was comparable to other characterized R genes (Supplementary Figure S2).

TABLE 1 | Seedling and whole plant late blight resistance screening results for 29 diploid accessions from the CPC.

Species	CPC accession	Seedling tests with 2006_3928A		Whole plant test with 2009_7654A	
		[1 = S to 5 = R] Mean of 2 replicates	[1 = S to 9 = R] Mean of 2 replicates	[1 = S to 9 = R] Mean of 2 replicates	[1 = S to 9 = R] Mean of 2 replicates
<i>S. bulbocastanum</i>	7636	4		9	
	7637	5		—	
	7639			9	
	7641	5		9	
	7642	—		9	
	7643	—		9	
	7644	4		9	
	7645	—		9	
	7646	—		9	
	7647	—		9	
	7650	5		9	
	7651	4		9	
<i>S. capsicibaccatum</i>	7760	4.5		8.5	
<i>S. microdonatum</i>	3724	—		9	
	3764	—		8.5	
<i>S. mochiquense</i>	6021	5		—	
<i>S. okadae</i>	7129	5		9	
	7625	5		9	
	7629	5		9	
	3762*	5			
<i>S. pinnatisectum</i>	7521	5		—	
	7659	5		—	
<i>S. polyadenium</i>	7665	—		9	
	7777	4.9		9	
	7778	4.4		9	
	7786	4.6		8	
	7795	3.7		7.5	
<i>S. tarjense</i>	7515	5		—	
<i>S. verrucosum</i>	54	4		8	

Late blight resistance was assessed on 25 4–5 week old seedlings (two replicates per test) or 9–10 weeks old selected plants from the accession (two replicates per plant) with the isolates 2006-3928A or 2009-7654A (both 13-A2), respectively. Results were recorded at 8 dpi, using a sliding scale of resistance ranging from 1 = very susceptible to 5 = very resistant for seedling tests and 1 = very susceptible to 9 = very resistant; symptomless plants, for whole plants according to the Malcomson scale (Cruickshank et al., 1982). The resistance in accession 3762 (denoted with a *) is known to be based on the presence of *Rpi-vnt1.1* only.

Importantly, dRenSeq was also applied to resistant *S. okadae* accession 3762 (containing *Rpi-vnt1.1*) and susceptible *S. okadae* 3761 (without functional *Rpi-vnt1.1*) to validate the concept and to discern between resistant and susceptible plants from the same species. Included were also a pool of 20 resistant and 20 susceptible plants that are derived from a cross between both accessions (Figure 4, Supplementary Table S3). At a mismatch rate of either 0.5% (data not shown) or 1%, full-length *Rpi-vnt1.1* was recovered from accession 3762 and the resistant pool. However, an *Rpi-vnt1.1*-like sequence with a truncated 5' end, compared to the functional gene, was recovered from both the susceptible accession 3761 and the susceptible pool. Indeed, the lack of sequence conservation in

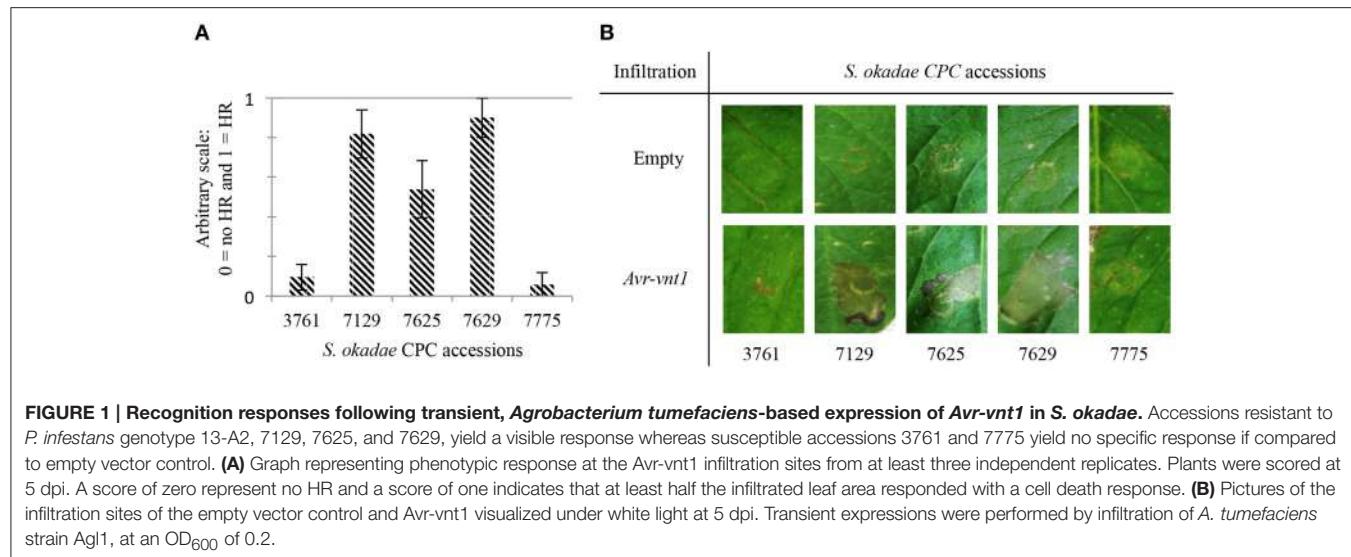


TABLE 2 | RenSeq reads were mapped to DM genome v4.03 or a reference set of 12 R genes at various mismatch rates (% MM).

CPC	% MM	Reads mapped to DM genome v4.03					Reads mapped to 12 functional NB-LRRs		
		Total	% Mapped	On target	% On target	Mean coverage (x)	Total	% Mapped	Mean coverage (x)
7129	0.5	87,842	2.42	33,585	38.23	1.93	1386	0.04	9.07
	1	203,384	5.60	108,583	53.39	6.49	2034	0.06	13.36
	5	1,685,852	46.44	114,7209	68.05	72.83	50,442	1.39	328.75
	10	2,554,646	70.38	1,696,516	66.41	108.23	234404	6.46	1568.62
7625	0.5	85,054	2.80	39,880	46.89	2.22	736	0.02	4.57
	1	197,172	6.49	118,332	60.01	6.83	1214	0.04	7.26
	5	1,460,566	48.10	1,015,151	69.5	62.63	60,442	1.99	384.19
	10	2,170,588	71.48	1,472,915	67.86	91.58	256,646	8.45	1683.09

The resulting alignments were intersected (± 1000 bp) against the 704 R genes from DM with known locations on chromosomes 1–12 to give the proportion of on target reads. The on target reads were then assessed for mean read coverage against the 704 genes, whilst for the 12 R gene set all the mapped reads were used to calculate the read depth.

this region was consistently detected in both susceptible samples (Figure 4).

S. okadae Accessions Contain Additional Resistance that is Independent of Rpi-vnt1.1

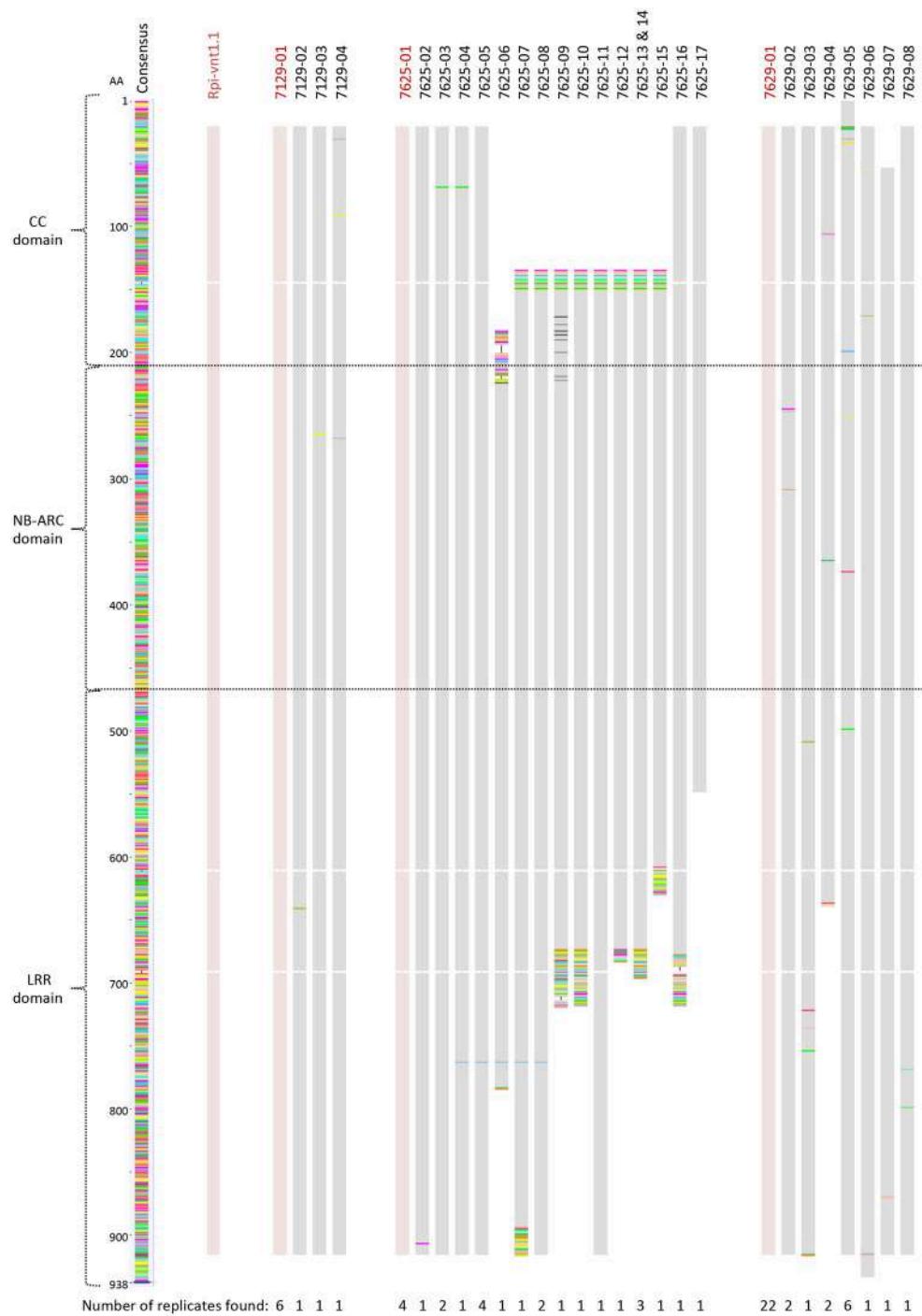
Selected *S. okadae* accessions were screened with five additional *P. infestans* isolates that display broad race specificity (Supplementary Table S4). Importantly, the isolate EC1, which overcomes *Rpi-vnt1.1* resistance, was included to discern between resistances that are exclusively based on the presence of *Rpi-vnt1.1*. The potato clone *Rpi-vnt1.1_R6*, which is an F1 clone derived from the cross between *S. okadae* accessions 3762 (containing *Rpi-vnt1.1*) and 3761 (susceptible), was used as a control.

In line with previous results, the clone *Rpi-vnt1.1_R6* was resistant to the 13-A2 isolate 2009-7654A and other isolates but susceptible to EC1 (Table 4). The *S. okadae* accession 7775 was susceptible to the 13-A2 isolate but partially resistant to EC1. The three *S. okadae* accessions (7129, 7625, and 7629) recognizing

Avr-vnt1 (Figure 1), however, were resistant to all isolates including EC1 (Figure 5, Table 4). This provides evidence that these accessions, unlike clone *Rpi-vnt1.1_R6*, carry at least one additional, novel resistance gene that functions independently of *Rpi-vnt1.1*.

DISCUSSION

Potato production is constantly threatened by late blight. The risk of infection is further exacerbated by the rapidly evolving nature of the pathogen, marked by rapid expansion of population size through asexual multiplication or increased genetic diversity through sexual reproduction. Controlling late blight by host resistance requires the continuous development of cultivars by introgression of new resistance from wild species. Breeding strategies in the 1950s largely relied on the deployment of resistances from the hexaploid species *S. demissum*, which resulted in the release of cultivars carrying one or more resistance genes. Pentland Dell, for example, a potato cultivar released in Great Britain in 1963, contained three resistance genes *R1*,



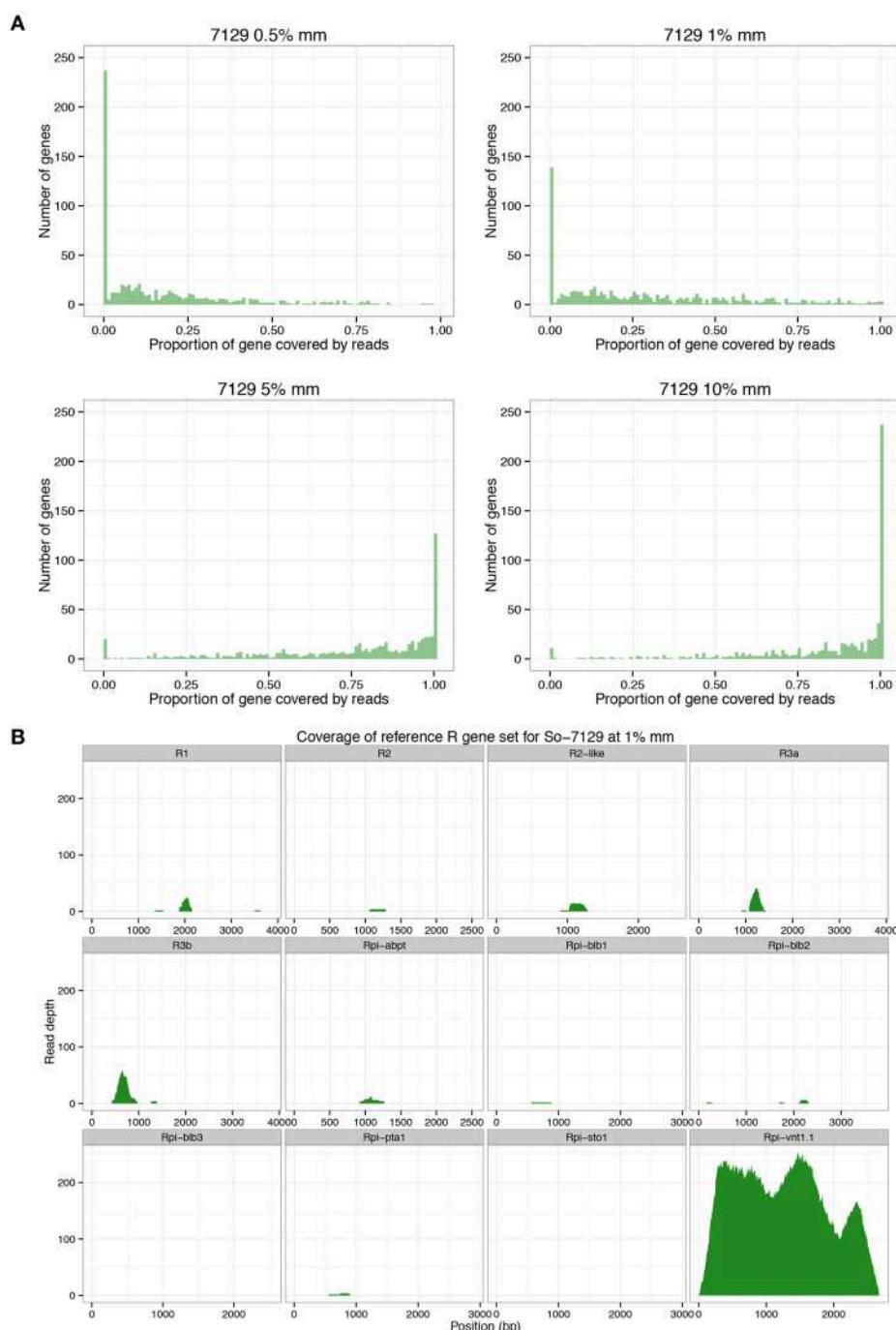


FIGURE 3 | RenSeq analysis for *S. okadae* accession 7129. (A) The number of 704 R genes from DM with known locations on chromosomes 1–12 that are not covered (0.00), partially covered or fully covered (1.00) following RenSeq analysis in *S. okadae* accession 7129 is shown. Mismatch rates (%mm) ranging from stringent 0.5 or 1% to more relaxed 5 or 10% are displayed. **(B)** The read depth and coverage of 12 functional R genes with homologous sequences isolated from *S. okadae* accession 7129 following RenSeq analysis and mapping under stringent conditions (1% mismatch rate) are depicted.

showing the importance of pyramiding resistances. However, introgression of resistance genes is a long and laborious process. For example, *Rpi-blb2* has been successfully introgressed into cultivars such as Toluca and Bionica that were developed after more than 30 years of breeding and selection efforts (Havertkort et al., 2009).

In light of these observations, the need for rapid and reliable diagnostic R gene tools is apparent. Effector-omics has proven useful for breeding and the identification of orthologous R gene in wild species (Vleeshouwers et al., 2008; Vleeshouwers and Oliver, 2014; Lenman et al., 2016). However, for this system to be successful, a detailed knowledge of the recognized effector

is required alongside responsive plants that yield reproducible recognition response upon transient effector expression. We have obtained reproducible Avr-vnt1 recognition responses in *S. okadae* accessions 7129, 7625, and 7629 (**Figure 1**) but not

TABLE 3 | RenSeq reads were mapped to DM genome v4.03 at 0.5, 1, 5, and 10% mismatch rates (%MM).

Sample	% MM	Number of genes with % coverage			
		0%	≤5%	≥95%	100%
7129	0.5	236	278	3	0
	1	138	167	14	3
	5	20	22	231	127
	10	11	12	340	237
7625	0.5	211	259	3	0
	1	121	156	15	3
	5	25	26	200	123
	10	15	17	318	208

The resulting alignments were cross-referenced against the 704 R genes from DM with known locations on chromosomes 1–12 to determine how many R genes were covered extensively (≥95%), completely (100%), minimally (≤5%), or not at all (0%).

for 3762 that contains the cognate R gene *Rpi-vnt1.1*. The latter proved non-responsive to the transient *Agrobacterium*-based expression system.

In line with the Avr-vnt1 recognition, PCR-based allele mining and Sanger sequencing confirmed the presence of *Rpi-vnt1.1* in *S. okadae* accessions 7129, 7625, and 7629 (**Figure 2**). A similar approach has been utilized successfully to identify orthologous genes in wild potato species (Lokossou et al., 2009, 2010). A PCR-based screening for full-length R genes alone could, however, be prone to false-positives and/or false-negative results. Furthermore, the cloning and sequencing of PCR products, which is required to discriminate highly similar sequences (**Figure 2**), renders this process low to medium throughput.

This study has shown that mapping RenSeq reads with stringent mismatch rates against reference R genes, results in a quick and easy way to screen plants for the presence or absence of known R genes (**Figures 3, 4**, as well as Supplementary Figures S1, S2). Indeed, dRenSeq is specific enough that it could distinguish between functional *Rpi-vnt1.1* in resistant accessions and its homologs in susceptible accessions as well as bulks. As such, dRenSeq could also be used for allele mining under various stringent mapping conditions and also aid evolutionary studies. Importantly, the obtained RenSeq sequence from plants that do

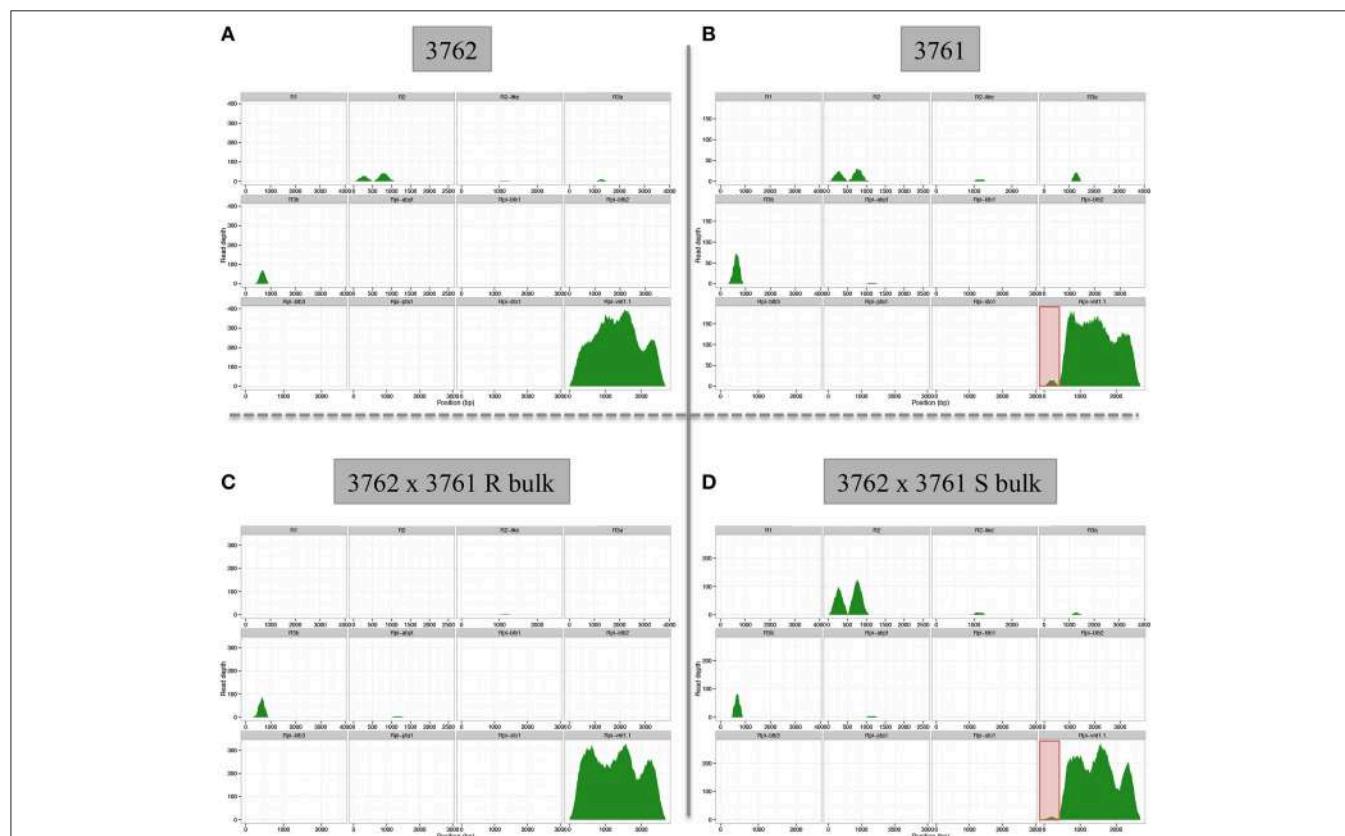


FIGURE 4 | dRenSeq analysis for resistant and susceptible *S. okadae* accession and bulked progeny. The read depth and coverage of 12 functional R genes with homologous sequences isolated from *S. okadae* accession **(A)** 3762 carrying *Rpi_vnt1.1*, **(B)** 3761 (susceptible), **(C)** bulk of 20 resistant plants derived from a cross between 3762 and 3761, and **(D)** bulk of 20 susceptible plants derived from a cross between 3762 and 3761 following RenSeq analysis and mapping under stringent conditions (1% mismatch rate) are depicted.

TABLE 4 | Late blight screen of five diploid *S. okadae* accessions from the CPC.

CPC accession number	Species or cultivars	<i>P. infestans</i> isolates (genotype)					
		2009-7654A (13 A2)	2010-7822 (6A1)	2010-7814 (23A1)	2010-8122D (8 2 A1)	2010-7838A (Misc')	EC1 (non-characterized)
3761	<i>S. okadae</i>	1.0	1.5	4.0	2.0	1.5	—
Rpi-vnt1.1_R6	JHI cross	5.0	5.0	5.0	5.0	5.0	1.0
7129	<i>S. okadae</i>	5.0	5.0	5.0	5.0	5.0	5.0
7625	<i>S. okadae</i>	5.0	5.0	5.0	5.0	5.0	4.0
7629	<i>S. okadae</i>	5.0	5.0	5.0	5.0	5.0	5.0
7775	<i>S. okadae</i>	1.0	—	—	—	—	3.0

The isolate names and genotypes are shown where known. The blight tests were performed on detached leaves using different isolates of *P. infestans*. Results were scored at 8 dpi, from 1 = susceptible to 5 = resistant; symptomless leaf. The scores shown are the average of at least two independent replicates. Highlighted in gray are compatible and intermediate compatible interactions.

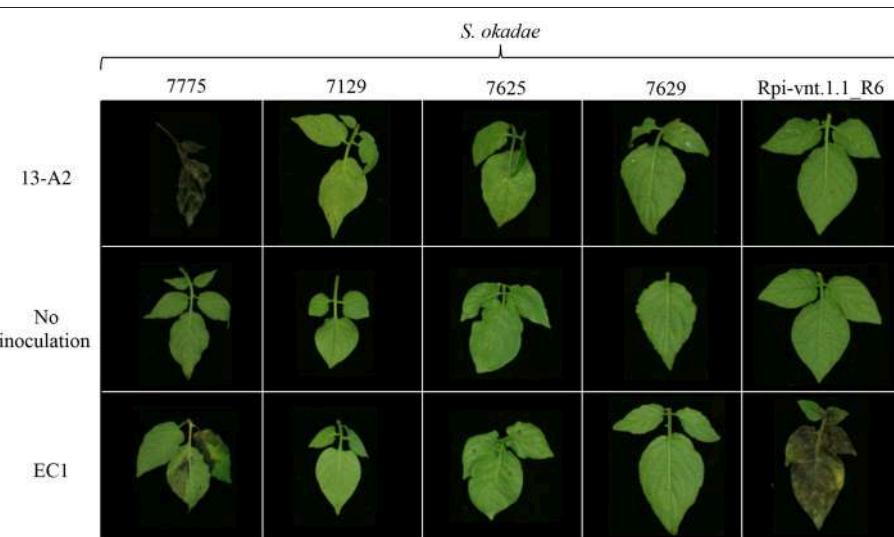


FIGURE 5 | Late blight screen of *S. okadae* accessions with EC1, a Rpi-vnt1.1 race specific isolate of *P. infestans*, and 13-A2. Isolates of *P. infestans* were drop-inoculated on detached leaves and symptoms assessed at 8 dpi. The *S. okadae* clone 3762-R6 has been independently characterized and only contains Rpi-vnt1.1, and was used as a control.

contain novel resistances can subsequently be used as a reference in a bulked-segregant analysis if genetic crosses can be achieved (Jupe et al., 2013). Therefore, sequence data can be used to answer different biological questions.

Interestingly, the *S. okadae* accessions 7129, 7625, and 7629 all contain functional Rpi-vnt1.1 as demonstrated by effector recognition, allele mining and, in the case of 7129 and 7625, dRenSeq. However, they also contain a resistance that operates independent of Rpi-vnt1.1 as demonstrated by additional late blight screening (Figure 5). The clone Rpi-vnt1.1_R6 carries Rpi-vnt1.1 and is, as expected, resistant to blue 13 but susceptible to the isolate EC1 (Foster et al., 2009), whereas 7129, 7625, and 7629 were all resistant to both isolates (Figure 5). RenSeq-derived reads are of dual utility and the additional resistance(s) could be mapped via a bulked segregant RenSeq analysis as described in Jupe et al. (2013). In this case, the RenSeq reads that have been used for the dRenSeq analysis described here could be

utilized to represent the resistant/susceptible parents. Using DM as a reference for the mapping, RenSeq reads are typically mapped at a 5% mismatch rate to allow for systematic differences between species which contrasts with dRenSeq where a 0.5 or 1% mismatch rate is used to establish the presence/absence of already known NB-LRRs.

Future efforts to identify resistances toward major pathogens in germplasm collection can quickly identify plants that contain novel resistances by taking advantage of target enrichment and sequencing technologies. For example, traditional allele mining based on PCR amplification, cloning of amplicons, and Sanger sequencing of individual clones can be omitted with dRenSeq application. Furthermore, a combination of late blight screening that includes isolates with a broad virulence spectrum followed by dRenSeq could be utilized to first prioritize plants that could subsequently be subjected to effector-omic analysis prior to a detailed genetic study. In breeding programs, dRenSeq (or similar

enrichment strategies for additional genes) could be utilized to aid R gene pyramiding and/or to follow multiple important traits on a sequence-based level.

AUTHOR CONTRIBUTIONS

PV, XC, BH, GT, AL, JL were involved in late blight screening and effector recognition. DC characterised *P. infestans* isolates. GM provided high health CPC accessions for the experiments. PV conducted allele mining and RenSeq analysis with MA. EG and PB provided effectors and were involved in analysing effector recognition. KB conducted computational analysis of RenSeq and DRenSeq. PV, GB, XC, and IH wrote the manuscript. IH directed the work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00672>

REFERENCES

- Agrios, G. N. (1997). *Plant Pathology*, 4th Edn. London: Elsevier.
- Andrews, S. (2010). *FastQC: A Quality Control Tool for High Throughput Sequence Data*. Available online at: <http://www.bioinformatics.babraham.ac.uk/projects/fastqc>
- Andrion, D., Avendano-Corcoles, J., Cameron, A. M., Carnegie, S. F., Cooke, L. R., Corbiere, R., et al. (2011). Stability and variability of virulence of *Phytophthora infestans* assessed in a ring test across European laboratories. *Plant Pathol.* 60, 556–565. doi: 10.1111/j.1365-3059.2010.02392.x
- Armstrong, M. R., Whisson, S. C., Pritchard, L., Bos, J. I. B., Venter, E., Avrova, A. O., et al. (2005). An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. *Proc. Natl. Acad. Sci. U.S.A.* 102, 7766–7771. doi: 10.1073/pnas.0500113102
- Ballvora, A., Ercolano, M. R., Weiü, J., Meksem, K., Bormann, C. A., Oberhagemann, P., et al. (2002). The R1 gene for potato resistance to late blight (*Phytophthora infestans*) belongs to the leucine zipper/NBS/LRR class of plant resistance genes. *Plant J.* 30, 361–371. doi: 10.1046/j.1365-313X.2001.01292.x
- Birch, P. R. J., Boevink, P. C., Gilroy, E. M., Hein, I., Pritchard, L., and Whisson, S. C. (2008). Oomycete RxLR effectors: delivery, functional redundancy and durable disease resistance. *Curr. Opin. Plant Biol.* 11, 373–379. doi: 10.1016/j.pbi.2008.04.005
- Birch, P. R. J., Bryan, G., Fenton, B., Gilroy, E. M., Hein, I., Jones, J. T., et al. (2012). Crops that feed the world 8: potato: are the trends of increased global production sustainable? *Food Secur.* 4, 477–508. doi: 10.1007/s12571-012-0220-1
- Bradshaw, J. E., Bryan, G. J., and Ramsay, G. (2006). Genetic resources (Including Wild and Cultivated Solanum Species) and progress in their utilisation in potato breeding. *Potato Res.* 49, 49–65. doi: 10.1007/s11540-006-9002-5
- Bradshaw, J. E., and Ramsay, G. (2005). Utilisation of the Commonwealth Potato Collection in potato breeding. *Euphytica* 146, 9–19. doi: 10.1007/s10681-005-3881-4
- Cooke, D. E. L., Cano, L. M., Raffaele, S., Bain, R. A., Cooke, L. R., Etherington, G. J., et al. (2012). Genome analyses of an aggressive and invasive lineage of the Irish potato famine pathogen. *PLOS Pathog.* 8:e1002940. doi: 10.1371/journal.ppat.1002940
- Cruickshank, G., Stewart, H. E., and Wastie, R. L. (1982). An illustrated assessment key for foliage blight of potatoes. *Potato Res.* 25, 213–214. doi: 10.1007/BF02359807
- Foster, S. J., Park, T.-H., Pel, M., Brigneti, G., Sliwka, J., Jagger, L., et al. (2009). *Rpi-vnt1.1*, a Tm-22 Homolog from *Solanum venturii*, confers resistance to potato late blight. *Mol. Plant Microbe Interact.* 22, 598–600. doi: 10.1094/MPMI-22-5-0589
- Gilroy, E. M., Breen, S., Whisson, S. C., Squires, J., Hein, I., Kaczmarek, M., et al. (2011). Presence/absence, differential expression and sequence polymorphisms between PiAVR2 and PiAVR2-like in *Phytophthora infestans* determine virulence on R2 plants. *New Phytol.* 191, 763–776. doi: 10.1111/j.1469-8137.2011.03736.x
- Haverkort, A., Struijk, P., Visser, R., and Jacobsen, E. (2009). Applied biotechnology to combat late blight in potato caused by *Phytophthora infestans*. *Potato Res.* 52, 249–264. doi: 10.1007/s11540-009-9136-3
- Hein, I., Gilroy, E. M., Armstrong, M. R., and Birch, P. R. J. (2009). The zig-zag-zig in oomycete-plant interactions. *Mol. Plant Pathol.* 10, 547–562. doi: 10.1111/j.1364-3703.2009.00547.x
- Huang, S., van der Vossen, E. A., Kuang, H., Vleeshouwers, V. G., Zhang, N., Borm, T. J., et al. (2005). Comparative genomics enabled the isolation of the R3a late blight resistance gene in potato. *Plant J.* 42, 251–261. doi: 10.1111/j.1365-313X.2005.02365.x
- Jones, J. D. G., Witek, K., Verweij, W., Jupe, F., Cooke, D., Dorling, S., et al. (2014). Elevating crop disease resistance with cloned genes. *Philos. Trans. R. Soc. B* 369, 20130087. doi: 10.1098/rstb.2013.0087
- Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286
- Jupe, F., Chen, X., Verweij, W., Witek, K., Jones, J. D., and Hein, I. (2014). Genomic DNA library preparation for resistance gene enrichment and sequencing (RenSeq) in plants. *Methods Mol. Biol.* 1127, 291–303. doi: 10.1007/978-1-62703-986-4_22
- Jupe, F., Pritchard, L., Etherington, G. J., MacKenzie, K., Cock, P. J. A., Wright, F., et al. (2012). Identification and localisation of the NB-LRR gene family within the potato genome. *BMC Genomics* 13:75. doi: 10.1186/1471-2164-13-75
- Jupe, F., Witek, K., Verweij, W., Sliwka, J., Pritchard, L., Etherington, G. J., et al. (2013). Resistance gene enrichment sequencing (RenSeq) enables reannotation of the NB-LRR gene family from sequenced plant genomes and rapid mapping of resistance loci in segregating populations. *Plant J.* 76, 530–544. doi: 10.1111/tpj.12307
- Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923
- Lenman, M., Ali, A., Mühlenbock, P., Carlson-Nilsson, U., Liljeroth, E., Champouret, N., et al. (2016). Effector-driven marker development and cloning of resistance genes against *Phytophthora infestans* in potato breeding clone SW93-1015. *Theor. Appl. Genet.* 129, 105–115. doi: 10.1007/s00122-015-2613-y
- Li, G., Huang, S., Guo, X., Li, Y., Yang, Y., Guo, Z., et al. (2011). Cloning and characterization of R3b; members of the R3 superfamily of late blight resistance

- genes show sequence and functional divergence. *Mol. Plant Microbe Interact.* 24, 1132–1142. doi: 10.1094/MPMI-11-10-0276
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009). The Sequence alignment/map (SAM) format and SAMtools. *Bioinformatics* 25, 2078–2079. doi: 10.1093/bioinformatics/btp352
- Lokossou, A. A., Park, T. H., van Arkel, G., Arens, M., Ruyter-Spira, C., Morales, J., et al. (2009). Exploiting knowledge of R/Avr genes to rapidly clone a new LZ-NBS-LRR family of late blight resistance genes from potato linkage group IV. *Mol. Plant-Microbe Interact.* 22, 630–641. doi: 10.1094/MPMI-22-6-0630
- Lokossou, A. A., Rietman, H., Wang, M., Krenek, P., van der Schoot, H., Henken, B., et al. (2010). Diversity, distribution, and evolution of Solanum bulbocastanum late blight resistance genes. *Mol. Plant-Microbe Interact.* 23, 1206–1216. doi: 10.1094/MPMI-23-9-1206
- Malcolmson, J. F. (1969). Races of *Phytophthora infestans* occurring in Great Britain. *Trans. Br. Mycol. Soc.* 53, 417–423. doi: 10.1016/S0007-1536(69)80099-9
- Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBNet J.* 17, 10–12. doi: 10.14806/ej.17.1.200
- Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., and Young, N. D. (1999). Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant J.* 20, 317–332. doi: 10.1046/j.1365-313X.1999.t01-1-00606.x
- Pel, M. A., Foster, S. J., Park, T. H., Rietman, H., van Arkel, G., Jones, J. D. G., et al. (2009). Mapping and cloning of late blight resistance genes from Solanum venturii using an interspecific candidate gene approach. *Mol. Plant-Microbe Interact.* 22, 601–615. doi: 10.1094/MPMI-22-5-0601
- Pel, M. A. (2010). *Mapping, Isolation and Characterization of Genes Responsible for Late Blight Resistance in Potato*. Ph.D. thesis, Wageningen University, Wageningen, Netherlands.
- Potato Genome Sequencing Consortium (2011). Genome sequence analysis of the tuber crop potato. *Nature* 475, 189–197. doi: 10.1038/nature10158
- Quinlan, A. R., and Hall, I. M. (2010). BEDTools: a flexible suite of utilities for comparing genomic features. *Bioinformatics* 26, 841–842. doi: 10.1093/bioinformatics/btq033
- Raffaele, S., Farrer, R. A., Cano, L. M., Studholme, D. J., MacLean, D., Thines, M., et al. (2010). Genome evolution following host jumps in the Irish potato famine pathogen lineage. *Science* 330, 1540–1543. doi: 10.1126/science.1193070
- Sharma, S. K., Bolser, D., de Boer, J., Sønderkær, M., Amoros, W., Carboni, M. F., et al. (2013). Construction of reference chromosome-scale pseudomolecules for potato: integrating the potato genome with genetic and physical maps. *G3* 3, 2031–2047. doi: 10.1534/g3.113.007153
- Stewart, H. E., Taylor, K., and Wastie, R. L. (1983). Resistance to late blight in foliage (*Phytophthora infestans*) of potatoes assessed as true seedlings and as adult plants in the glasshouse. *Potato Res.* 26, 363. doi: 10.1007/BF02356155
- Tomato Genome Consortium (2012). The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* 485, 635–641. doi: 10.1038/nature11119
- van der Vossen, E. A. G., Sikkema, A., te Lintel Hekkert, B., Gros, J., Stevens, P., Muskens, M., et al. (2003). An ancient R gene from the wild potato species Solanum bulbocastanum confers broad-spectrum resistance to *Phytophthora infestans* in cultivated potato and tomato. *Plant J.* 36, 867–882. doi: 10.1046/j.1365-313X.2003.01934.x
- van der Vossen, E. A. G., Gros, J., Sikkema, A., Muskens, M., Wouters, D., Wolters, P., et al. (2005). The Rpi-blb2 gene from Solanum bulbocastanum is an Mi-1 gene homolog conferring broad-spectrum late blight resistance in potato. *Plant J.* 44, 208–222. doi: 10.1111/j.1365-313X.2005.02527.x
- Vleeshouwers, V. G. G. A., Rietman, H., Krenek, P., Champouret, N., Young, C., Oh, S.-K., et al. (2008). Effector genomics accelerates discovery and functional profiling of potato disease resistance and *Phytophthora infestans* avirulence genes. *PLoS ONE* 3:e2875. doi: 10.1371/journal.pone.0002875
- Vleeshouwers, V. G. A. A., and Oliver, R. P. (2014). Effectors as tools in disease resistance breeding against biotrophic, hemibiotrophic, and necrotrophic plant pathogens. *Mol. Plant Microbe Interact.* 27, 196–206. doi: 10.1094/mpmi-13-0313-ia
- Wang, M., Allefs, S., van den Berg, R. G., Vleeshouwers, V. G., van der Vossen, E. A., and Vosman, B. (2008). Allele mining in *Solanum*: conserved homologues of *Rpi-blb1* are identified in *Solanum stoloniferum*. *Theor. Appl. Genet.* 116, 933–943. doi: 10.1007/s00122-008-0725-3
- Whisson, S. C., Boevink, P. C., Moleleki, L., Avrova, A. O., Morales, J. G., Gilroy, E. M., et al. (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. *Nature* 450, 115–118. doi: 10.1038/nature06203
- Wiesel, L., Newton, A. C., Elliott, I., Booty, D., Gilroy, E. M., Birch, P. R. J., et al. (2014). Molecular effects of resistance elicitors from biological origin and their potential for crop protection. *Front. Plant Sci.* 5:655. doi: 10.3389/fpls.2014.00655

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An Overview of CRISPR-Based Tools and Their Improvements: New Opportunities in Understanding Plant–Pathogen Interactions for Better Crop Protection

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Modern omics platforms have made the determination of susceptible/resistance genes feasible in any species generating huge numbers of potential targets for crop protection. However, the efforts to validate these targets have been hampered by the lack of a fast, precise, and efficient gene targeting system in plants. Now, the repurposing of clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9) system has solved this problem. CRISPR/Cas9 is the latest synthetic endonuclease that has revolutionized basic research by allowing facile genome editing in prokaryotes and eukaryotes. Gene knockout is now feasible at an unprecedented efficiency with the possibility of multiplexing several targets and even genome-wide mutagenesis screening. In a short time, this powerful tool has been engineered for an array of applications beyond gene editing. Here, we briefly describe the CRISPR/Cas9 system, its recent improvements and applications in gene manipulation and single DNA/RNA molecule analysis. We summarize a few recent tests targeting plant pathogens and discuss further potential applications in pest control and plant–pathogen interactions that will inform plant breeding for crop protection.

Keywords: CRISPR/Cas9, gene editing, plant–pathogen interactions, DNA double-stranded break, homologous recombination, non-homologous end joining

INTRODUCTION

Genetic crosses and mutagenesis based breeding are time consuming and laborious. The recent development of next generation sequencing is making available fast and cost effective genomic platforms of an increasing number of species including pests, plant models and crops. Now it is easier than ever to perform genome-wide association studies and determine the genes and pathways involved in any particular aspect of pathogen resistance (Olukolu et al., 2014), and pipelines are now well established for genomics-informed breeding (Varshney et al., 2015). It is also quicker and cheaper to obtain the transcriptome of any crop under pathogen attack and determine the virulence and defense pathways and genes that are deployed by both (Kawahara et al., 2012; O’Connell et al., 2012). Proteomics is also starting to make a dent in understanding plant–pathogen interactions (Lodha et al., 2013). A complex network of nuclear processes regulating gene expression and function is emerging from this gene discovery phase but association of a particular pathogen effector with the corresponding host target(s) is still poorly understood (Motion et al., 2015).

Omics technologies generate a huge amount of data and require powerful computational tools to integrate these high-throughput platforms in order to fully understand the multi-layered networks of biomolecules underpinning plant-pathogen interactions. Gene silencing has been extensively used to validate the function of candidate host resistance genes (Duan et al., 2012) and pathogen virulence factors (Yin et al., 2015). However, pathogens have evolved effective suppressors against host RNA silencing, making the system unsuitable for engineering strong and durable resistance in crops (Pumplin and Voinnet, 2013). A more attractive option is gene targeting (GT) which allows any endogenous gene to be disrupted or replaced with a copy that has been manipulated *in vitro*. In GT experiments, double-stranded break (DSB) at the target gene is repaired by one of the two main competing DNA repair pathways: the more frequent non-homologous end joining (NHEJ) pathway or the rare but precise homologous recombination (HR) (Chapman et al., 2012). GT could have a huge impact as a 'clean transgenesis' technology for precise gene manipulation or transfer of novel traits into crops. Despite huge efforts, this powerful tool has been elusive in plant science for a long time as it relied on extremely rare spontaneous DSBs (Puchta and Fauser, 2013). However, this barrier has been recently overcome by the development of novel endonucleases that break DNA specifically at chosen genomic targets. Unfortunately, gene replacement by HR is still inefficient in plants and will need further improvement.

Initially, two endonucleases were engineered by fusing a programmable DNA-binding domain to the cleavage domain of the bacterial restriction enzyme *FokI*. The first endonuclease was generated by linking the DNA-binding domain of a zinc-finger transcription factor to make the first truly flexible chimeric nuclease zinc-finger nuclease (ZFN) (Smith et al., 2000). Similarly, the DNA-binding domain of a transcription activator-like effector of the plant pathogen *Xanthomonas* was used to make the second, and relatively easier to design, nuclease transcription activator-like effector nuclease (TALEN) (Christian et al., 2010). These two big breakthroughs were superseded by an even simpler system based on the clustered, regularly interspaced, short palindromic repeats (CRISPR) and CRISPR-associated genes (Cas) used by some bacteria and Archaea to destroy invading genetic material (Jinek et al., 2012). Our knowledge of CRISPR/Cas is rapidly evolving and the findings are extensively reported and reviewed. Here we will briefly describe the natural and engineered CRISPR–Cas systems followed by the latest and future applications in plant–pathogen interactions.

THE NATIVE CRISPR–Cas SYSTEM

The CRISPR–Cas system was discovered in bacterial genomes as early as 1987 (Ishino et al., 1987) but its biological role was determined only in 2007 (Barrangou et al., 2007). These evolving adaptive immune systems against invading phages and plasmids are now re-classified into five types I–V (Makarova et al., 2015). During the first invasion, the hosts capture short DNA sequences of about 20 nucleotides, known as

spacers, from the foreign genetic material and integrate them between two repeats at the CRISPR locus (Nuñez et al., 2015). Upon subsequent encounters, CRISPR arrays with the acquired spacers are transcribed and processed into small CRISPR RNAs containing the spacer (crRNAs). This chimeric molecule interacts with another auxiliary *trans*-activating CRISPR RNA (tracrRNA), forming a duplex RNA or guide RNA (gRNA) that guides the Cas nuclease to the homologous target (protospacer), resulting in an R-loop structure. The tracrRNA activates Cas nuclease activity, cleaving both strands of the target DNA upstream of a conserved protospacer-adjacent motif (PAM). Cas nuclease has two domains, RuvC and HNH, that cut the PAM-containing strand and its complementary strand, respectively, thus producing a single DSB (Heler et al., 2015). The spacer and PAM requirements depend on CRISPR–Cas type (Xue et al., 2015). In the case of the widely used type II CRISPR–Cas9 system, the last 12 ribonucleotides at the 3'-end of the RNA spacer, known as the seed sequence, dictates the specificity of the complementary target. Mismatches at its 5'-end were thought to be tolerated during gRNA–Cas9 binding to the target. However, the interaction of this region and PAM-distal sequences turned out to be necessary for the activation of Cas9 endonuclease activity (Cencic et al., 2014). PAM sequences are 2–5 bp motifs essential for spacer acquisition and target cleavage (Shah et al., 2013).

REPURPOSING OF THE CRISPR–Cas9 SYSTEM FOR GENE EDITING IN EUKARYOTES

The knowledge of the biological function and mechanism of CRISPR–Cas inspired its reprogramming to target any chosen DNA sequence. CRISPR–Cas9 of *Streptococcus pyogenes* was engineered by simply replacing the first 20 nucleotides of crRNA with the intended target sequence and fusing both crRNA and tracrRNA molecules to make a single guide RNA (sgRNA) (Jinek et al., 2012). This newly programmable system was first adopted to target eukaryotic genes in animals, followed by several successful applications in plants including crops (Bortesi and Fischer, 2015; Butler et al., 2015; Lawrenson et al., 2015). The ease of implementation of CRISPR–Cas9 by anyone with basic molecular biology skills has made it the tool of choice for gene editing in any species of interest. Upon generating a DSB at the desired site by the Cas9–gRNA complex, the host cell repairs the DNA lesion by NHEJ pathway resulting in short insertions or deletions, leading to gene knockout. The flexibility of the CRISPR–Cas9 system allows targeting of adjacent sites in a single gene for specific removal of a region, which will be extremely useful for the studies of gene and mRNA *cis*-elements and protein domains (Brooks et al., 2014). CRISPR–Cas9 can also be used in plants to knockout all or single multigene family members (Endo et al., 2015) and even multiple unrelated genes (Lowder et al., 2015).

The DSB lesion can also be repaired by the HR mechanism in the presence of a donor template, leading to precise gene replacement (knock-in). HR-based gene replacement is still

inefficient and has been demonstrated in only a few plant species (Bortesi and Fischer, 2015). The efficiency of homology directed repair (HDR) of CRISPR–Cas9 induced DSBs was recently increased by inhibiting the NHEJ pathway in mammalian cells (Chu et al., 2015; Maruyama et al., 2015). Cas9 was recently found to dissociate slowly from DSB by releasing first the 3' end of the cleaved DNA strand that is not complementary to the sgRNA. Consequently HDR was increased to 60% in human cells by using rationally designed single-stranded DNA donor template of the optimal length complementary to the strand that is released first (Richardson et al., 2016). Maize was the first crop where CRISPR–Cas9 was successfully used to generate plants with precise modifications (Svitashov et al., 2015). Precise gene modifications have been achieved at high frequency in tomato by combining the CRISPR–Cas9 nuclease with a geminivirus-based vector for donor DNA template delivery (Čermák et al., 2015). The combination of some or all of the incremental improvements in different animal and plant species could enhance gene replacement efficiency for all crops.

In pathogens, GT without DSB induction was only improved by inhibiting the NHEJ pathway, as in the ku70 mutant of *Verticillium dahliae* (Qi et al., 2015). CRISPR–Cas9 has now made gene editing possible in fungi (Matsu-ura et al., 2015; Nødvig et al., 2015). The effector Avr4/6 of the soybean pathogen *Phytophthora sojae* was efficiently knocked out or even precisely replaced by the selectable marker *nptII*, uncovering additional roles for the corresponding R gene loci *RPS4* and *RPS6* (Fang and Tyler, 2016). The establishment of gene editing tools in *P. sojae* will speed up studies for crop protection in other oomycetes.

Resistance to geminiviruses has been long sought after and was achieved recently in three independent studies using CRISPR–Cas9 in *Nicotiana benthamiana* (Ali et al., 2015; Baltes et al., 2015; Ji et al., 2015). In these works, CRISPR–Cas9 was shown to mutate the viral genome, resulting in reduced viral replication and attenuated infection symptoms. A single gRNA targeting a conserved sequence in the replication origin resulted in efficient inhibition of multiple monopartite and bipartite geminiviruses in the same host. However, further studies will be required to monitor the evolution of this resistance over generations and in more challenging environments (Chaparro-Garcia et al., 2015).

Viral vectors can also be targeted by CRISPR–Cas9 technology to abolish pathogen transmission or even reduce insect population by the so-called mutagenic chain reaction (Gantz and Bier, 2015). This system is initiated when both Cas9 and gRNA transgenes are inserted by homology directed repair at the intended target in males. The transgenes are then copied into the homologous chromosome by HR in the germ-line cells. During fertilization, the males transfer the CRISPR–Cas9 cassette into the next generation and the chain continues. This gene drive system has been demonstrated to be very efficient in manipulating two species of mosquito which are vectors for malaria (Esveld et al., 2014; Gantz et al., 2015; Hammond et al., 2016). Though attractive, gene drive will not work in self-fertilizing weeds and non-native invasive plant species but it could potentially be used against flies that are vectors of plant pathogens provided that they are amenable to transgenesis. However, safeguarding against the

unintended ecological impact of manipulated insect populations is of great importance and biosafety concerns are starting to be addressed by developing antidote systems to reverse gene drive effects (DiCarlo et al., 2015).

IMPROVEMENTS TO THE CRISPR–Cas9 SYSTEM EFFICIENCY AND SPECIFICITY

Since the conception of the CRISPR–Cas9 gene editing system, its components Cas9 and gRNA have been continuously optimized to improve the efficiency and accuracy of GT (Bolukbasi et al., 2015; Graham and Root, 2015). The repurposing of the CRISPR–Cas9 system to alter eukaryotic genes necessitated targeting the bacterial Cas9 to the nucleus by adding a nuclear localization signal at one or both termini of the protein. To improve translation efficiency, the Cas9 gene was initially codon optimized for human cells and was quickly followed by several plant versions, for both dicots and monocots (Bortesi and Fischer, 2015). The endonuclease Cas9 can easily be converted into a DNA nickase by a single amino acid change in either of its two domains (D10A in RuvC and H840A in HNH; Cong et al., 2013) to cut only one strand. A DSB can still be introduced at the target by these nickases in the presence of two gRNAs that target opposing strands at neighboring sites. This feature has been exploited to improve the specificity of CRISPR–Cas9 and reducing potential off-targets (Ran et al., 2013), a major concern with engineered endonucleases in animals (Hendel et al., 2015) and in plants (Bortesi and Fischer, 2015). Several assays for quantifying on- and off-targets have been developed and inspired strategies for minimizing off-target effects (Hendel et al., 2015; Zhang et al., 2015). In plants, the use of whole genome sequencing as the most accurate method is limited to *Arabidopsis* and rice with good genome reference (Bortesi and Fischer, 2015). Unlike in human gene therapy, off-targets are less problematic in plants where one could eliminate such events by backcrosses. The determination of Cas9 structure (Nishimasu et al., 2014, 2015) has also inspired rational engineering of new Cas9 variants with altered PAM recognition (Kleinstiver et al., 2015) and greater specificity (Slaymaker et al., 2016). Orthologs of commonly used Cas9 from *S. pyogenes* (SpCas9) have been reported to have different features and requirements. The *S. aureus* Cas9 (SaCas9) gene is 1 kbp shorter than SpCas9, improving its stability in viral vectors (Ran et al., 2015). In the screening effort for SpCas9 orthologs, another protein, Cpf1 (CRISPR from *Prevotella* and *Francisella* 1) of type V CRISPR–Cas systems, has been reported to function in a completely different way to Cas9. Cpf1 does not need a tracrRNA but requires a T-rich PAM motif upstream of the target site and generates a DSB with 5' overhangs (Zetsche et al., 2015).

The design of guide RNAs for efficient and specific gene editing has also been the focus of many studies combining experimental and computing analyses. Several user-friendly algorithms have been developed and freely shared online with the scientific community¹. Most of these bioinformatics tools

¹<http://omictools.com/crispr-cas9-category>

are designed to score the efficiency of all potential targets with a PAM motif in the input gene sequence (Wiles et al., 2015). The chance of off-target effects elsewhere in the genome can also be accounted for where the genome sequence is available. These bioinformatics tools are continuously being refined with the availability of new experimental data (Malina et al., 2015; Wong et al., 2015). The structure of the artificial single guide RNA has been revisited recently and improved by lengthening the duplex crRNA/tracrRNA and improving gRNA transcription by shortening its thymine repeat (Dang et al., 2015).

Several systems for delivering Cas9 and gRNA molecules into the cell are available, depending on the species of interest. Plasmid constructs are often used to express Cas9 from RNA polymerase II-driven promoters and gRNAs with polymerase III-mediated transcription. A new strategy based on endogenous tRNA maturation has been developed for expressing multiple gRNAs from a single pol III promoter (Lowder et al., 2015; Xie et al., 2015). While a pol II promoter can be chosen to drive tissue-specific expression of Cas9, snoRNA U3, and U6 pol III promoters are constitutive. However, newly reconstructed sgRNAs can now be expressed from pol II promoters (Wang et al., 2015). Inducible promoters can also be used to induce gene editing *in vivo*, yet reduce potential off-target effects and Cas9-associated toxicity (Dow et al., 2015). Even better, Cas9 and the gRNA can be simultaneously expressed from a single promoter allowing for more spatio-temporal control of each component (Yoshioka et al., 2015). These conditional gene editing methods present new opportunities in crop research but have not yet been tested in plants.

OTHER FACETS OF THE CRISPR–Cas9 SYSTEM

The CRISPR–Cas9 system has become the tool of choice for gene manipulation owing to its simplicity and the willingness of researchers to share the necessary plasmids and methods, including the various algorithms for designing gRNAs. Most of these ingredients are now deposited with the non-profit plasmid repository Addgene² (Harrison et al., 2014). Although most studies focus on knocking out a single gene or a combination of a few targets (multiplexing), the CRISPR–Cas9 system is so powerful that it has been successfully used for genome-wide mutagenesis in mammalian (Malina et al., 2014; Peng et al., 2015) and *Drosophila* (Bassett et al., 2015) cells. The CRISPR–Cas9 based genetic screen uses thousands of unique gRNAs covering the genome of interest and relies on efficient delivery of Cas9/gRNA cargo. This type of forward genetic screen will be very useful in studies of plant–pathogen interactions, but the transformation of plant or pathogen cells must be optimized. This goal can be achieved with at least some pathogens and plant models like *Arabidopsis* and tobacco.

When both RuvC and HNH nuclease domains are mutated, Cas9 becomes an inactive or dead endonuclease (dCas9). Qi et al.

(2013) were the first to demonstrate that dCas9 can specifically repress gene expression in *Escherichia coli* in the presence of the gene specific gRNA. This work was quickly followed by another report where dCas9 was fused to transcriptional effectors to silence or activate gene expression in eukaryotes, thereby reversibly manipulating gene expression (Gilbert et al., 2013). Similarly, the epigenome can be manipulated at a specific site by fusing dCas9 with various DNA effectors or histone methylases and acetylases (Hilton et al., 2015; Laufer and Singh, 2015). dCas9-based gene regulation platforms can be used for both genome-wide loss-of-function and gain-of-function screens and the system is amenable to controlled induction (Dominguez et al., 2016). When tagged with fluorescent proteins, dCas9 can be used instead of fluorescence *in situ* hybridization (FISH) to detect chromosomal loci in living (Chen et al., 2013) and fixed (Deng et al., 2015) cells. In this application, dCas9-fluorescent protein fusions can be targeted by a gRNA to a specific locus in the genome for cytological detection. The simultaneous detection of multiple loci in the same cell is feasible by simply fusing different dCas9 orthologs with different fluorescent proteins. Most of the dCas9-based tools will be very useful in deciphering plant–pathogen interactions. Inducible activation or inhibition of master regulators could have huge practical agronomical applications but the down-side is that the dCas9/gRNA transgenes must be kept permanently in the plant.

CONCLUDING REMARKS AND OUTLOOK

Different omics platforms have opened the flood gate of potential disease resistance genes that need a more efficient validation pipeline than earlier gene manipulation tools like gene silencing. Plant–pathogen omics data could be improved even further by reducing the background noise in the biological samples. This can now be achieved, for example, by performing cell-type specific RNA or chromatin profiling with novel tools like INTACT (Deal and Henikoff, 2010). Cell-type enrichment will help monitor the dynamics of post-translational modifications during plant–pathogen interactions (Park and Yun, 2013; Motion et al., 2015). CRISPR–Cas9 technology has revolutionized gene manipulation capabilities in many species including crops. The multitude of functions that can be performed with CRISPR–Cas9 and its many derivatives (Sander and Joung, 2014) make it a molecular tool that will open new opportunities in the complicated world of plant–pathogen interactions and help design durable crop resistance to pathogens. Only the gene editing function of CRISPR–Cas9 has so far been used in plants and pathogens. However, the future use of dCas9-based tools will also help to unmask the master regulators of disease resistance (Seo and Choi, 2015). GT tools will help integrate omics data in order to fully understand and improve crop defense mechanisms. The complexity of the plant microbiome with good and bad microbes is beginning to be unraveled (Bai et al., 2015). CRISPR–Cas9 tools will help future studies of plant–pathogen interactions to transcend individual genes or

²<https://www.addgene.org/crispr/>

pathogens and become more holistic in approaches to elucidate plant microbiome systems.

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REFERENCES

- Ali, Z., Abulfaraj, A., Idris, A., Ali, S., Tashkandi, M., and Mahfouz, M. M. (2015). CRISPR/Cas9-mediated viral interference in plants. *Genome Biol.* 16:238. doi: 10.1186/s13059-015-0799-6
- Bai, Y., Müller, D. B., Srinivas, G., Garrido-Oter, R., Potthoff, E., Rott, M., et al. (2015). Functional overlap of the *Arabidopsis* leaf and root microbiota. *Nature* 528, 364–369. doi: 10.1038/nature16192
- Baltes, N. J., Hummel, A. W., Konecna, E., Cegan, R., Bruns, A. N., Bisaro, D. M., et al. (2015). Conferring resistance to geminiviruses with the CRISPR-Cas prokaryotic immune system. *Nat. Plants* 1:15145. doi: 10.1038/nplants.2015.145
- Barrangou, R., Fremaux, C., Deveau, H., Richards, M., Boyaval, P., Moineau, S., et al. (2007). CRISPR provides acquired resistance against viruses in prokaryotes. *Science* 315, 1709–1712. doi: 10.1126/science.1138140
- Bassett, A. R., Kong, L., and Liu, J.-L. (2015). A genome-wide CRISPR library for high-throughput genetic screening in *Drosophila* cells. *J. Genet. Genomics* 42, 301–309. doi: 10.1016/j.jgg.2015.03.011
- Bolukbasi, M. F., Gupta, A., and Wolfe, S. A. (2015). Creating and evaluating accurate CRISPR-Cas9 scalpels for genomic surgery. *Nat. Methods* 13, 41–50. doi: 10.1038/nmeth.3684
- Bortesi, L., and Fischer, R. (2015). The CRISPR/Cas9 system for plant genome editing and beyond. *Biotechnol. Adv.* 33, 41–52. doi: 10.1016/j.biotechadv.2014.12.006
- Brooks, C., Nekrasov, V., Lippman, Z. B., and Van Eck, J. (2014). Efficient gene editing in tomato in the first generation using the clustered regularly interspaced short palindromic repeats/CRISPR-associated9 system. *Plant Physiol.* 166, 1292–1297. doi: 10.1104/pp.114.247577
- Butler, N. M., Atkins, P. A., Voytas, D. F., and Douches, D. S. (2015). Generation and inheritance of targeted mutations in potato (*Solanum tuberosum* L.) using the CRISPR/Cas system. *PLoS ONE* 10:e0144591. doi: 10.1371/journal.pone.0144591
- Cencic, R., Miura, H., Malina, A., Robert, F., Ethier, S., Schmeing, T. M., et al. (2014). Protospacer adjacent motif (PAM)-distal sequences engage CRISPR Cas9 DNA target cleavage. *PLoS ONE* 9:e109213. doi: 10.1371/journal.pone.0109213
- Čermák, T., Baltes, N. J., Čegan, R., Zhang, Y., and Voytas, D. F. (2015). High-frequency, precise modification of the tomato genome. *Genome Biol.* 16:232. doi: 10.1186/s13059-015-0796-9
- Chaparro-Garcia, A., Kamoun, S., and Nekrasov, V. (2015). Boosting plant immunity with CRISPR/Cas. *Genome Biol.* 16:254. doi: 10.1186/s13059-015-0829-4
- Chapman, J. R., Taylor, M. R., and Boulton, S. J. (2012). Playing the end game: DNA double-strand break repair pathway choice. *Mol. Cell* 47, 497–510. doi: 10.1016/j.molcel.2012.07.029
- Chen, B., Gilbert, L. A., Cimini, B. A., Schnitzbauer, J., Zhang, W., Li, G.-W., et al. (2013). Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. *Cell* 155, 1479–1491. doi: 10.1016/j.cell.2013.12.001
- Chu, V. T., Weber, T., Wefers, B., Wurst, W., Sander, S., Rajewsky, K., et al. (2015). Increasing the efficiency of homology-directed repair for CRISPR-Cas9-induced precise gene editing in mammalian cells. *Nat. Biotechnol.* 33, 543–548. doi: 10.1038/nbt.3198
- Christian, M., Cermak, T., Doyle, E. L., Schmidt, C., Zhang, F., et al. (2010). Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 186, 757–761. doi: 10.1534/genetics.110.120717
- Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., et al. (2013). Multiplex genome engineering using CRISPR/Cas systems. *Science* 339, 819–823. doi: 10.1126/science.1231143
- Dang, Y., Jia, G., Choi, J., Ma, H., Anaya, E., Ye, C., et al. (2015). Optimizing sgRNA structure to improve CRISPR-Cas9 knockout efficiency. *Genome Biol.* 16:280. doi: 10.1186/s13059-015-0846-3
- Deal, R. B., and Henikoff, S. (2010). A simple method for gene expression and chromatin profiling of individual cell types within a tissue. *Dev. Cell* 18, 1030–1040. doi: 10.1016/j.devcel.2010.05.013
- Deng, W., Shi, X., Tjian, R., Lionnet, T., and Singer, R. H. (2015). CASFISH: CRISPR/Cas9-mediated in situ labeling of genomic loci in fixed cells. *Proc. Natl. Acad. Sci. U.S.A.* 112, 11870–11875. doi: 10.1073/pnas.1515692112
- DiCarlo, J. E., Chavez, A., Dietz, S. L., Esvelt, K. M., and Church, G. M. (2015). Safeguarding CRISPR-Cas9 gene drives in yeast. *Nat. Biotechnol.* 33, 1250–1255. doi: 10.1038/nbt.3412
- Dominguez, A. A., Lim, W. A., and Qi, L. S. (2016). Beyond editing: repurposing CRISPR-Cas9 for precision genome regulation and interrogation. *Nat. Rev. Mol. Cell Biol.* 17, 5–15. doi: 10.1038/nrm.2015.2
- Dow, L. E., Fisher, J., O'Rourke, K. P., Muley, A., Kastenhuber, E. R., Livshits, G., et al. (2015). Inducible *in vivo* genome editing with CRISPR-Cas9. *Nat. Biotechnol.* 33, 390–394. doi: 10.1038/nbt.3155
- Duan, C.-G., Wang, C.-H., and Guo, H.-S. (2012). Application of RNA silencing to plant disease resistance. *Silence* 3:5. doi: 10.1186/1758-907X-3-5
- Endo, M., Mikami, M., and Toki, S. (2015). Multigene knockout utilizing off-target mutations of the CRISPR/Cas9 system in rice. *Plant Cell Physiol.* 56, 41–47. doi: 10.1093/pcp/pcu154
- Esvelt, K. M., Smidler, A. L., Catteruccia, F., and Church, G. M. (2014). Emerging technology: concerning RNA-guided gene drives for the alteration of wild populations. *eLife* 3:e03401. doi: 10.7554/eLife.03401
- Fang, Y., and Tyler, B. M. (2016). Efficient disruption and replacement of an effector gene in the oomycete *Phytophthora sojae* using CRISPR/Cas9. *Mol. Plant Pathol.* 17, 127–139. doi: 10.1111/mpp.12318
- Gantz, V. M., and Bier, E. (2015). The mutagenic chain reaction: a method for converting heterozygous to homozygous mutations. *Science* 348, 442–444. doi: 10.1126/science.aaa5945
- Gantz, V. M., Jasinskiene, N., Tatarenkova, O., Fazekas, A., Macias, V. M., Bier, E., et al. (2015). Highly efficient Cas9-mediated gene drive for population modification of the malaria vector mosquito *Anopheles stephensi*. *Proc. Natl. Acad. Sci. U.S.A.* 112:e6736–43. doi: 10.1073/pnas.1521077112
- Gilbert, L. A., Larson, M. H., Morsut, L., Liu, Z., Brar, G. A., Torres, S. E., et al. (2013). CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell* 154, 442–451. doi: 10.1016/j.cell.2013.06.044
- Graham, D. B., and Root, D. E. (2015). Resources for the design of CRISPR gene editing experiments. *Genome Biol.* 16, 260. doi: 10.1186/s13059-015-0823-x
- Hammond, A., Galizi, R., Kyrou, K., Simoni, A., Siniscalchi, C., Katsanos, D., et al. (2016). A CRISPR-Cas9 gene drive system targeting female reproduction in the malaria mosquito vector *Anopheles gambiae*. *Nat. Biotechnol.* 34, 78–83. doi: 10.1038/nbt.3439
- Harrison, M. M., Jenkins, B. V., O'Connor-Giles, K. M., and Wildonger, J. (2014). A CRISPR view of development. *Gene Dev.* 28, 1859–1872. doi: 10.1101/gad.248252.114
- Heler, R., Samai, P., Model, J. W., Weiner, C., Goldberg, G. W., Bikard, D., et al. (2015). Cas9 specifies functional viral targets during CRISPR-Cas adaptation. *Nature* 519, 199–202. doi: 10.1038/nature14245

- Hendel, A., Fine, E. J., Bao, G., and Porteus, M. H. (2015). Quantifying on- and off-target genome editing. *Trends Biotechnol.* 33, 132–140. doi: 10.1016/j.tibtech.2014.12.001
- Hilton, I. B., D’Ippolito, A. M., Vockley, C. M., Thakore, P. I., Crawford, G. E., Reddy, T. E., et al. (2015). Epigenome editing by a CRISPR-Cas9-based acetyltransferase activates genes from promoters and enhancers. *Nat. Biotechnol.* 33, 510–517. doi: 10.1038/nbt.3199
- Ishino, Y., Shinagawa, H., Makino, K., Amemura, M., and Nakata, A. (1987). Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J. Bacteriol.* 169, 5429–5433.
- Ji, X., Zhang, H., Zhang, Y., Wang, Y., and Gao, C. (2015). Establishing a CRISPR-Cas-like immune system conferring DNA virus resistance in plants. *Nat. Plants* 1:15144. doi: 10.1038/nplants.2015.144
- Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829
- Kawahara, Y., Oono, Y., Kanamori, H., Matsumoto, T., Itoh, T., and Minami, E. (2012). Simultaneous RNA-Seq analysis of a mixed transcriptome of rice and blast fungus interaction. *PLoS ONE* 7:e49423. doi: 10.1371/journal.pone.0049423
- Kleinsteuber, B. P., Prew, M. S., Tsai, S. Q., Topkar, V. V., Nguyen, N. T., Zheng, Z., et al. (2015). Engineered CRISPR-Cas9 nucleases with altered PAM specificities. *Nature* 523, 481–485. doi: 10.1038/nature14592
- Laufee, B. I., and Singh, S. M. (2015). Strategies for precision modulation of gene expression by epigenome editing: an overview. *Epigenetics Chromatin* 8:34. doi: 10.1186/s13072-015-0023-7
- Lawrenson, T., Shorinola, O., Stacey, N., Li, C., Østergaard, L., Patron, N., et al. (2015). Induction of targeted, heritable mutations in barley and *Brassica oleracea* using RNA-guided Cas9 nuclease. *Genome Biol.* 16:258. doi: 10.1186/s13059-015-0826-7
- Lodha, T. D., Hembram, P., Tep, N., and Basak, J. (2013). Proteomics: a successful approach to understand the molecular mechanism of plant-pathogen interaction. *Am. J. Plant Sci.* 4, 1212–1226. doi: 10.4236/ajps.2013.46149
- Lowder, L. G., Zhang, D., Baltes, N. J., Paul, J. W. III, Tang, X., Zheng, X., et al. (2015). A CRISPR/Cas9 toolbox for multiplexed plant genome editing and transcriptional regulation. *Plant Physiol.* 169, 971–985. doi: 10.1104/pp.15.00636
- Makarova, K. S., Wolf, Y. I., Alkhnbashi, O. S., Costa, F., Shah, S. A., Saunders, S. J., et al. (2015). An updated evolutionary classification of CRISPR-Cas systems. *Nat. Rev. Microbiol.* 13, 722–736. doi: 10.1038/nrmicro3569
- Malina, A., Cameron, C. J. F., Robert, F., Blanchette, M., Dostie, J., and Pelletier, J. (2015). PAM multiplicity marks genomic target sites as inhibitory to CRISPR-Cas9 editing. *Nat. Commun.* 6:10124. doi: 10.1038/ncomms10124
- Malina, A., Katigbak, A., Cencic, R., Maiga, R. I., Robert, F., Miura, H., et al. (2014). Adapting CRISPR/Cas9 for functional genomics screens. *Methods Enzymol.* 546, 193–213. doi: 10.1016/B978-0-12-801185-0.00010-6
- Maruyama, T., Dougan, S. K., Truttmann, M. C., Bilate, A. M., Ingram, J. R., and Ploegh, H. L. (2015). Increasing the efficiency of precise genome editing with CRISPR-Cas9 by inhibition of nonhomologous end joining. *Nat. Biotechnol.* 33, 538–542. doi: 10.1038/nbt.3190
- Matsu-ura, T., Baek, M., Kwon, J., and Hong C. (2015). Efficient gene editing in *Neurospora crassa* with CRISPR technology. *Fungal Biol. Biotechnol.* 2, 1–7. doi: 10.1186/s40694-015-0015-1
- Motion, G. B., Amaro, T. M., Kulagina, N., and Huitema, E. (2015). Nuclear processes associated with plant immunity and pathogen susceptibility. *Brief. Funct. Genomics* 14, 243–252. doi: 10.1093/bfgp/elfv013
- Nishimasu, H., Cong, L., Yan, W. X., Ran, F. A., Zetsche, B., Li, Y., et al. (2015). Crystal structure of *Staphylococcus aureus* Cas9. *Cell* 162, 1113–1126. doi: 10.1016/j.cell.2015.08.007
- Nishimasu, H., Ran, F. A., Hsu, P. D., Konermann, S., Shehata, S. I., Dohmae, N., et al. (2014). Crystal structure of Cas9 in complex with guide RNA and target DNA. *Cell* 156, 935–949. doi: 10.1016/j.cell.2014.02.001
- Nødvig, C. S., Nielsen, J. B., Kogle, M. E., and Mortensen, U. H. (2015). A CRISPR-Cas9 system for genetic engineering of filamentous fungi. *PLoS ONE* 10:e0133085. doi: 10.1371/journal.pone.0133085
- Nuñez, J. K., Lee, A. S. Y., Engelman, A., and Doudna, J. A. (2015). Integrase-mediated spacer acquisition during CRISPR-Cas adaptive immunity. *Nature* 519, 193–198. doi: 10.1038/nature14237
- O’Connell, R. J., Thon, M. R., Hacquard, S., Amyotte, S. G., Kleemann, J., Torres, M. F., et al. (2012). Lifestyle transitions in plant pathogenic *Colletotrichum fungi* deciphered by genome and transcriptome analyses. *Nat. Genet.* 44, 1060–1065. doi: 10.1038/ng.2372
- Olkokulu, B. A., Wang, G.-F., Vontimitta, V., Venkata, B. P., Marla, S., Ji, J., et al. (2014). A genome-wide association study of the maize hypersensitive defense response identifies genes that cluster in related pathways. *PLoS Genet.* 10:e1004562. doi: 10.1371/journal.pgen.1004562
- Park, H. J., and Yun, D. J. (2013). New insights into the role of the small ubiquitin-like modifier (SUMO) in plants. *Int. Rev. Cell. Mol. Biol.* 300, 161–209. doi: 10.1016/B978-0-12-405210-9.00005-9
- Peng, J., Zhou, Y., Zhu, S., and Wei, W. (2015). High-throughput screens in mammalian cells using the CRISPR-Cas9 system. *FEBS J.* 282, 2089–2096. doi: 10.1111/febs.13251
- Puchta, H., and Fauser, F. (2013). Gene targeting in plants: 25 years later. *Int. J. Dev. Biol.* 57, 629–637. doi: 10.1387/ijdb.130194hp
- Pumplin, N., and Voinnet, O. (2013). RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. *Nat. Rev. Microbiol.* 11, 745–760. doi: 10.1038/nrmicro3120
- Qi, L. S., Larson, M. H., Gilbert, L. A., Doudna, J. A., Weissman, J. S., Arkin, A. P., et al. (2013). Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 152, 1173–1183. doi: 10.1016/j.cell.2013.02.022
- Qi, X., Su, X., Guo, H., Qi, J., and Cheng, H. (2015). A ku70 null mutant improves gene targeting frequency in the fungal pathogen *Verticillium dahliae*. *World J. Microbiol. Biotechnol.* 31, 1889–1897. doi: 10.1007/s11274-015-1907-1
- Ran, F. A., Cong, L., Yan, W. X., Scott, D. A., Gootenberg, J. S., Kriz, A. J., et al. (2015). In vivo genome editing using *Staphylococcus aureus* Cas9. *Nature* 520, 186–191. doi: 10.1038/nature14299
- Ran, F. A., Hsu, P. D., Lin, C. Y., Gootenberg, J. S., Konermann, S., Trevino, A. E., et al. (2013). Double nicking by RNA-guided CRISPR Cas9 for enhanced genome editing specificity. *Cell* 154, 1380–1389. doi: 10.1016/j.cell.2013.08.021
- Richardson, C. D., Ray, G. J., DeWitt, M. A., Curie, G. L., and Corn, J. E. (2016). Enhancing homology-directed genome editing by catalytically active and inactive CRISPR-Cas9 using asymmetric donor DNA. *Nat. Biotechnol.* 34, 339–344. doi: 10.1038/nbt.3481
- Sander, J. D., and Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. *Nat. Biotechnol.* 32, 347–355. doi: 10.1038/nbt.2842
- Seo, E., and Choi, D. (2015). Functional studies of transcription factors involved in plant defences in the genomics era. *Brief. Funct. Genomics* 14, 260–267. doi: 10.1093/bfgp/elfv011
- Shah, S. A., Erdmann, S., Mojica, F. J., and Garrett, R. A. (2013). Protospacer recognition motifs: mixed identities and functional diversity. *RNA Biol.* 10, 891–899. doi: 10.4161/rna.23764
- Slaymaker, I. M., Gao, L., Zetsche, B., Scott, D. A., Yan, W. X., and Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. *Science* 351, 84–88. doi: 10.1126/science.aad5227
- Smith, J., Bibikova, M., Whitby, F. G., Reddy, A. R., Chandrasegaran, S., and Carroll, D. (2000). Requirements for double-strand cleavage by chimeric restriction enzymes with zinc finger DNA-recognition domains. *Nucleic Acids Res.* 28, 3361–3369. doi: 10.1093/nar/28.17.3361
- Svitashov, S., Young, J. K., Schwartz, C., Gao, H., Falco, S. C., and Cigan, A. M. (2015). Targeted mutagenesis, precise gene editing, and site-specific gene insertion in maize using Cas9 and guide RNA. *Plant Physiol.* 169, 931–945. doi: 10.1104/pp.15.00793
- Varshney, R. K., Singh, V. K., Hickey, J. M., Xun, X., Marshall, D. F., Wang, J., et al. (2015). Analytical and decision support tools for genomics-assisted breeding. *Trends Plant Sci.* doi: 10.1016/j.tplants.2015.10.018 [Epub ahead of print].
- Wang, J., Li, X., Zhao, Y., Li, J., Zhou, Q., and Liu, Z. (2015). Generation of cell-type-specific gene mutations by expressing the sgRNA of the CRISPR system from the RNA polymerase II promoters. *Protein Cell* 6, 689–692. doi: 10.1007/s13238-015-0169-x
- Wiles, M. V., Qin, W., Cheng, A. W., and Wang, H. (2015). CRISPR-Cas9-mediated genome editing and guide RNA design. *Mamm. Genome* 26, 501–510. doi: 10.1007/s00335-015-9565-z

- Wong, N., Liu, W., and Wang, X. (2015). WU-CRISPR: characteristics of functional guide RNAs for the CRISPR/Cas9 system. *Genome Biol.* 16:218. doi: 10.1186/s13059-015-0784-0
- Xie, K., Minkenberg, B., and Yang Y. (2015). Boosting CRISPR/Cas9 multiplex editing capability with the endogenous tRNA-processing system. *Proc. Natl. Acad. Sci. U.S.A.* 112, 3570–3575. doi: 10.1073/pnas.1420294112
- Xue, C., Seetharam, A. S., Musharova, O., Severinov, K., Brouns, S. J., Severin, A. J., et al. (2015). CRISPR interference and priming varies with individual spacer sequences. *Nucleic Acids Res.* 43, 10831–10847. doi: 10.1093/nar/gkv1259
- Yin, C., Downey, S. I., Klages-Mundt, N. L., Ramachandran, S., Chen, X., Szabo, L. J., et al. (2015). Identification of promising host-induced silencing targets among genes preferentially transcribed in haustoria of *Puccinia*. *BMC Genomics* 16:579. doi: 10.1186/s12864-015-1791-y
- Yoshioka, S., Fujii, W., Ogawa, T., Sugiura, K., and Naito K. (2015). Development of a mono-promoter-driven CRISPR/Cas9 system in mammalian cells. *Sci. Rep.* 5:18341. doi: 10.1038/srep18341
- Zetsche, B., Gootenberg, J. S., Abudayyeh, O. O., Slaymaker, I. M., Makarova, K. S., Essletzbichler, P., et al. (2015). Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. *Cell* 163, 759–771. doi: 10.1016/j.cell.2015.09.038
- Zhang, X. H., Tee, L. Y., Wang, X. G., Huang, Q. S., and Yang, S. H. (2015). Off-target effects in CRISPR/Cas9-mediated genome engineering. *Mol. Ther. Nucleic Acids* 4:e264. doi: 10.1038/mtna.2015.37

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Transgenic Cotton Plants Expressing Cry1Ia12 Toxin Confer Resistance to Fall Armyworm (*Spodoptera frugiperda*) and Cotton Boll Weevil (*Anthonomus grandis*)

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Gossypium hirsutum (commercial cootón) is one of the most economically important fibers sources and a commodity crop highly affected by insect pests and pathogens. Several transgenic approaches have been developed to improve cotton resistance to insect pests, through the transgenic expression of different factors, including Cry toxins, proteinase inhibitors, and toxic peptides, among others. In the present study, we developed transgenic cotton plants by fertilized floral buds injection (through the pollen-tube pathway technique) using an DNA expression cassette harboring the *cry1Ia12* gene, driven by CaMV35S promoter. The T0 transgenic cotton plants were initially selected with kanamycin and posteriorly characterized by PCR and Southern blot experiments to confirm the genetic transformation. Western blot and ELISA assays indicated the transgenic cotton plants with higher Cry1Ia12 protein expression levels to be further tested in the control of two major *G. hirsutum* insect pests. Bioassays with T1 plants revealed the Cry1Ia12 protein toxicity on *Spodoptera frugiperda* larvae, as evidenced by mortality up to 40% and a significant delay in the development of the target insects compared to untransformed controls (up to 30-fold). Also, an important reduction of *Anthonomus grandis* emerging adults (up to 60%) was observed when the insect larvae were fed on T1 floral buds. All the larvae and adult insect survivors on the transgenic lines were weaker and significantly smaller compared to the non-transformed plants. Therefore, this study provides GM cotton plant with simultaneous resistance against the Lepidopteran (*S. frugiperda*), and the Coleopteran (*A. grandis*) insect orders, and all data suggested that the Cry1Ia12 toxin could effectively enhance the cotton transgenic plants resistance to both insect pests.

Keywords: *Gossypium hirsutum*, genetic cotton transformation, pollen-tube pathway, Cry1Ia12, *Anthonomus grandis*, *Spodoptera frugiperda*

INTRODUCTION

Cotton (*Gossypium hirsutum*) is an economically important crop due to lead global source of natural fiber and also contribute in oil and seed meal production. However, this worldwide crop is affected by several biotic stresses that cause a dramatic reduction in plant productivity (Oerke, 2006). Among the most important insect pest that affecting cotton crops, we can highlight *Spodoptera frugiperda* and *Anthonomus grandis* (Gallo et al., 2002; Kriticos et al., 2015). The fall armyworm, *S. frugiperda* (J. E. Smith; Lepidoptera: Noctuidae), is an important insect pest that attacks many crops. In cotton, *S. frugiperda* prefers to oviposit on the lower surface of the leaves in most plant phenological stages, which difficult the insect control by insecticides (Pitre et al., 1983; Ali et al., 1989; Fernandes et al., 2002; Miranda, 2006; Barros et al., 2010). Immediately after the eggs hatching, fall armyworm larvae start feeding on the leaf causing significant damage to the plant. On the other hand, currently, cotton boll weevil, *A. grandis* Boheman (Coleoptera: Curculionidae) is the main pest affecting cotton production in South America. During the infestation, this insect increases cotton flower bud abscission and fruit fall, especially caused by its feed establishment, mechanic damage and oviposition, which results in a significant reduction of fiber production (Santos et al., 2003). Both *S. frugiperda* and *A. grandis* can devastate entire cotton fields and the control of both can represent 25% of cotton production cost (Brazilian Ministry of Agriculture, 2015). Therefore, the need to control *S. frugiperda* and *A. grandis* infestations in cotton fields is the main cause of development and expansion of insecticide control, as well as the efforts engagement in improve genetically modified (GM) cotton varieties resistant to these insect pests.

In an attempt to control crop insect pest populations throughout the world, several GM cotton lines were developed with considerable impact to reduce losses in cotton productivity. Considering this advance, currently cotton represents the third largest GM planted area of the world, comprising 13.7% of total worldwide (James, 2014). The main features inserted into cotton plants are resistance to lepidopterans and tolerance to herbicide or a combination of both traits (James, 2014). However, none of the commercial GM cotton varieties contribute to the control of coleopteran *A. grandis* (ISAAA, 2015).

The majority of GM cotton plants are obtained by insertion of *cry* genes, originated from *Bacillus thuringiensis*. With almost 750 *cry* genes described and grouped into 73 classes (Crickmore et al., 2014), the crystalline inclusions produced by *B. thuringiensis* have been shown to be toxic to several insects, nematodes, mites, and protozoans (Hofte and Whiteley, 1989; Feitelson et al., 1992; Schnepf et al., 1998; Hu et al., 2010; Bravo et al., 2013; Pan et al., 2014). The Cry1 toxin is the most studied toxin class, with more than 260 genes described (Crickmore et al., 2014). Despite its specificity to lepidopterans, some of the Cry1 proteins have shown activity against coleopterans (Escudero et al., 2006; Soberón et al., 2010). Previously, Grossi-de-Sa et al. (2007) demonstrated that the recombinant Cry1Ia12 protein, identified in a *B. thuringiensis* S811 strain and expressed in *Escherichia coli* cells, was toxic to both cotton boll weevil larvae and fall armyworm (*S. frugiperda*). In addition, Guimarães et al. (2010)

performed food security assays showing that Cry1Ia12 does not have any toxic effects on mice and thus could be suitable for the production of commercial GM plant varieties.

Different methods of transferring exogenous genes into cotton plants have been studied and used in recent decades. The most common techniques used for cotton transformation are *Agrobacterium*-mediated (Wu et al., 2008; Kumar et al., 2009; Mao et al., 2011; Vajhala et al., 2013) and particle bombardment (McCabe and Martinell, 1993; Rajasekaran et al., 1996; Rech et al., 2008; Rajasekaran, 2013). Other methods, including the direct delivery of DNA into protoplasts by electroporation and PEG-mediated gene transfer, have also been successfully employed (Chilton, 2005; Vain, 2007). Successful regeneration methods for cotton plants have been described (Rajasekaran et al., 1996; Leelavathi et al., 2004), although, in general, modifications are necessary when limitations to regenerate native cotton cultivars are considered (Khan et al., 2010). Plant regeneration from single transformed cells often produces somaclonal variations, which affect plant phenotypes and genotypes (Larkin, 2004). Several unwanted and unintended oscillations have been described, including point mutations, gene duplications, chromosomal rearrangements, and changes in DNA methylation (Larkin, 2004; Wilkins et al., 2004; Oh et al., 2007). These variations usually result in cotton off-types that reduce the commercial value of the generated plants. Therefore, the development of tissue-culture independent plant transformation techniques is of great interest.

To avoid these limitations, it is necessary to develop genotype-independent approaches. In this context, transformation techniques that target ovaries, meristems or other tissues, which ultimately give rise to gametes are included (Birch, 1997). The pollen-tube pathway approach represents a tissue-culture-free alternative for cotton transformation (Luo and Wu, 1989; Zhen et al., 1998; Yu et al., 1999). The genetic transformation occurs via direct delivery of foreign DNA into the pollinated and fertilized ovary (Zhou et al., 1983). This transformation method has been successfully used to introduce total exogenous genomic or plasmidial DNA into varieties of rice (Luo and Wu, 1989), soybean (Zhao et al., 1995; Liu et al., 1997), cotton (Ni et al., 1998), watermelon (Chen et al., 1998), wheat (Yu et al., 1999), onion (Peffley et al., 2003), and maize (Zhang et al., 2005; Yang et al., 2009).

In this present study, GM cotton plants with stable expression of Cry1I toxin were obtained, demonstrating toxicity to both cotton pests, *A. grandis* and *S. frugiperda*. The *cry1Ia12* gene was introduced into BRS Cedro cotton variety using the pollen-tube pathway technique. According to insect bioassays with floral buds of GM cotton events, the transgenic plants with a relatively high level of Cry1Ia12 toxin expression displayed insect-resistance to both insect-pests.

MATERIALS AND METHODS

Plant Material and Culture Conditions

The cotton (*G. hirsutum* L.) elite cultivar BRS Cedro was used as recipient of a microinjection in a greenhouse

at the Embrapa Genetic Resources and Biotechnology laboratory in Brasília, Brazil. The cultivar were planted in plastic bags containing soil as substrate and maintained in a greenhouse (average temperature $26 \pm 1^\circ\text{C}$; average humidity $70 \pm 10\%$).

Plasmid Constructs

The pCry1 vector containing the *cry1Ia12* gene under the control of 35S promoter of cauliflower mosaic virus (CaMV35S) *in tandem* with the alfalfa mosaic virus enhancer (AMV) was generated and introduced into the pCambia2300 vector. The cassette also contained the *nptII* coding sequence, which was also under the control of CaMV35S-AMV regulatory sequence. The *cry1Ia12* gene was subcloned upstream of nopaline synthase terminator (t-NOS), and the *nptII* gene, which confers kanamycin resistance, was subcloned upstream of the 35S terminator (Supplementary Figure S1A). The resistance to this antibiotic is needed to select the T0 cotton transformation events.

DNA Application via Microinjection

The DNA application procedure described by Zhou et al. (1983) was performed with some modifications. To use the pollen-tube pathway transformation technique, pollination must be completed with consequent pollen tube development and fecundation. This process is indicated by the color of the petals, which is creamy white on the flowering day when anthesis and pollination occur; the petals turn purple on the following day (Supplementary Figure S1B1). After flowering for 24 h (the day after anthesis), young ovaries located on reproductive branches were selected, identified and tagged for microinjection. Untreated flowers were removed. The flower petals, stamen and style were carefully removed to expose the young boll and microinjection was performed (Supplementary Figure S1B2). A Hamilton microsyringe was used to inject 10 μL of plasmid DNA ($0.1 \mu\text{g } \mu\text{L}^{-1}$) into the exposed style (Supplementary Figure S1B3). Five, ten, and twenty days after transformation, the branches were checked, and new flowers were removed. The first injections were performed 59 days after sowing. Several months later, mature bolls were harvested, and the T0 labeled seeds were removed by ginning.

Selection and Screening of Putative Transformants

Seeds from microinjected plants were sown in plastic bags containing soil as substrate (Supplementary Figure S1B4). Ten days after seed germination, tests to kanamycin resistance were performed to select putative transgenic plants. Briefly, a cotton swab that had been wetted with a $5 \mu\text{g } \text{mL}^{-1}$ kanamycin solution was applied to the surface of the younger leaves of both transformed and non-transformed plants once a week. After 3 weeks, the leaves were examined for signs of necrosis at the sites of antibiotic application. Those leaves that did not show signs of necrosis were selected for further analysis (Supplementary Figures S1B5–B8).

PCR Analysis of Transgenic Cotton Plants

Genomic DNA from selected cotton leaves was isolated following the procedure described by Michiels et al. (2003) with some modifications. The presence of *cry1Ia12* was confirmed using the primers *cry1Ia12* forward (5'-ACGCCAAGGTGACAAAATC-3') and *cry1Ia12* reverse (5'-AGGGAGCTCTGAACGAACA-3') to amplify a 420 bp internal fragment, denominated by *cry1Ia12* segment (ICS). The reaction was performed with 100 ng of DNA as follows: an initial denaturation at 95°C for 5 min followed by 32 cycles of denaturation at 95°C for 1 min; annealing at 55°C for 30 s; and extension at 72°C for 1 min, followed by a final extension for 10 min at 72°C . DNA from a non-transgenic *G. hirsutum* plant was used as the negative control, and the pCry1 vector used as positive control.

Evaluation of the Integrated DNA Using Southern Blot Analysis

Total genomic DNA from the leaves of non-transgenic and transformed cotton plants was isolated using a CTAB method modified from Michiels et al. (2003). Fresh leaves (1 g) were ground to a powder in liquid nitrogen, which was directly transferred into 15 mL of extraction buffer preheated to 60°C . The suspension was mixed carefully and incubated at 60°C for 60 min. After an extraction step using chloroform-isoamyl-alcohol (24:1), an overnight isopropanol precipitation step at 25°C was performed. The following washing steps were performed as described by Michiels et al. (2003). Once ethanol-free, the DNA pellet was dissolved in sterile water, which was incubated with RNase ($100 \mu\text{g } \text{mL}^{-1}$) at 37°C for 2 h. The DNA purification was performed according to the manufacturer's instructions from DNAeasy extraction Kit (QIAgen®). The genomic DNA quantification and purity ratio were determined using a NanoVue spectrophotometer (GE Healthcare Life Science®).

Twenty micrograms of genomic DNA was digested with *Nco*I and *Hind*III restriction enzymes. The digested DNA was resolved in 0.8% agarose gel electrophoresis and then transferred onto a nitrocellulose membrane (GE Healthcare Life Science®). A 2234 bp *cry1Ia12* DNA fragment was the probe used in hybridization step, which was labeled with α -[32P]-dCTP using a Random Primer DNA Labeling kit (Ready-to-Go DNA labeling beads, GE Healthcare Life Science®). Hybridization was performed at 65°C for 16 h, and the filter was washed at room temperature with 2x SSC/0.1% SDS and 1x SSC/0.1% SDS for 15 min each and at 60°C with 0.2x SSC/0.1% SDS for 15 min (Sambrook and Russell, 2001). After washing steps, the membrane was exposed to an imaging plate (BAS-MP, FujiFilm®) for 24 h. Images were acquired using a FLA3000 phosphoimage (FujiFilm®).

Qualitative Cry1Ia12 Protein Analysis in Cotton Leaves

The Cry1Ia12 protein expression was analyzed in cotton plants using Western blot assays. Approximately 3 g of leaves from transgenic and non-transgenic plants were pulverized in a

mortar in liquid nitrogen with a pestle until a fine powder was obtained. Proteins from the leaves were then homogenized in pre-chilled protein extraction buffer (50 mM Tris at pH 7.4, 150 mM NaCl, 10 mM sodium metabisulfite, 0.2% ascorbic acid, 0.1 M EDTA and 0.5% Triton) at 4°C. The extracts were centrifuged at 10,000 × g for 10 min at 4°C, and the supernatant was quantified using the Bradford protein assay (Bio-Rad®). A polyacrylamide gel (7.5%) was loaded with 100 µg of protein samples and approximately 500 ng of purified Cry1Ia12 at 20 mA. The protein gel was electroblottedted at 10 V for 30 min onto a nitrocellulose membrane (Hybond-C® Extra, Amersham Biosciences®) using a Trans-Blot SD Semi-Dry cell (Bio-Rad®). After transfer, the nitrocellulose membrane was blocked using a TBS buffer containing 1% gelatin and 0.25% PVA (polyvinyl alcohol) and then probed with an anti-Cry1Ia12 polyclonal antibody produced in rabbits (Genescrypt®). Goat anti-rabbit antibodies conjugated to alkaline phosphatase (SIGMA®) were used to detect the Cry1Ia12 protein. The reactive protein in the nitrocellulose membrane was revealed using an AP conjugate substrate kit (Bio-Rad®) according to the manufacturer's instructions.

Quantification of Expressed Cry1Ia12

To quantify the Cry1Ia12 in cotton leaves, an indirect ELISA (Engvall and Perlmann, 1971) was performed with 2 µg of the total protein extracted from each selected transgenic and a non-transgenic cotton plants. The preference for leaves was based on specific reasons: (i) in the case of *A. grandis*, the insect has its preferred feeding site in the vicinity of this tissue, precisely because this insect species is highly selective; (ii) in the case of *S. frugiperda*, the main objective was controlling this insect populations in early larval instars, where feeding occurs preferably on leaves.

The assay was performed in triplicate on a high-binding 96-well EIA/RIA microplate (Costar® 3590). A standard curve was obtained using purified Cry1Ia12. The plate was incubated with anti-Cry1Ia12 polyclonal antibody (Genescrypt®) and then incubated with goat anti-rabbit antibody conjugated to HRP. The plates were washed and the substrate solution was added to each well. The reaction was stopped after 30 min by the addition of 50 µL of 2 M sulfuric acid. The assay was read on a SpectraMax 190 microplate reader (Molecular Devices) at 450 nm.

Insect Bioassays

Transgenic cotton plants from the T1 progeny were subjected to bioassays with cotton boll weevils and fall armyworms. Eggs of *A. grandis* and *S. frugiperda* species were provided by the Bioecology and Semiochemicals of Insects Laboratory at Embrapa Genetic Resources and Biotechnology at Brasilia, DF, Brazil. Both *A. grandis* and *S. frugiperda* adults were maintained in an environmental controlled room with 26 ± 2°C with controlled humidity of 70 ± 10%. The insects were maintained with artificial diet, according to its specificity. The eggs were collected and then separated into petri dishes with the same diet as the adults were fed (Greene et al., 1976; Martins et al., 2007). All the experiments were performed with biological (each

distinct bioassay performed at different periods of time) and technical (all experimental repetition performed during each bioassay) triplicates. The data were statistically analyzed using ANOVA.

Spodoptera frugiperda Bioassay

Concerning *S. frugiperda* bioassay, the eggs were placed in non-transgenic cotton leaves. First instar larvae that hatched remained feeding on these non-transgenic leaves for 2 days when they reached the second instar stage. Ten fully expanded cotton leaves were detached from each non-transgenic and transgenic cotton plant of the T1 generation and placed in an entomology test chamber. One second instar larva was released onto each plate and allowed to feed on the leaf. The plates were kept at 25–27°C with controlled humidity of 70 ± 10%. Data on the survival and weight of each living larva were recorded on the 10th day.

Anthonomus grandis Bioassay

Plants containing 6 mm flower buds were selected for the boll weevil bioassays. A population of *A. grandis* was maintained at the Insect Rearing Platform at Embrapa Genetic Resources and Biotechnology on a standard rearing diet at 27 ± 2°C, 70 ± 10% relative humidity, and a photoperiod of 14 h (Monnerat, 2000). One *A. grandis* egg containing an active embryo was inoculated in a 6 mm cotton flower bud. Bud perforation was performed using a drill, and the orifice was sealed with vaseline to prevent egg dehydration. The experimental period was 20 days. After this, the mortality rate and the adult's weight were measured.

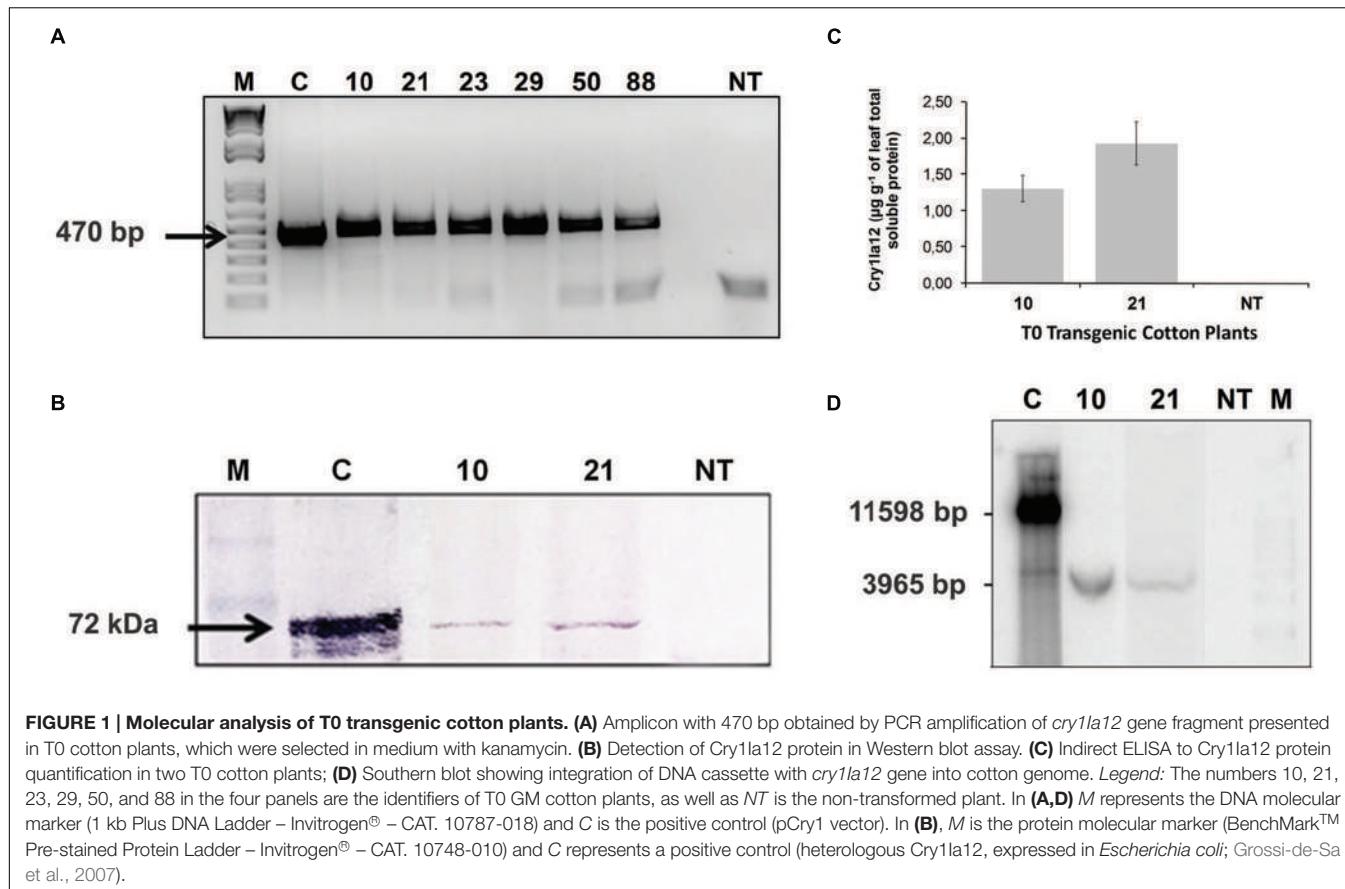
RESULTS

Pollen-Tube Pathway Transformation and Selection of Transgenic Cotton Plants

A total of 590 floral buds were microinjected, among which 315 were aborted due to the mechanical process of microinjection. The 275 remaining floral buds produced 3175 cotton seeds, which were planted in soil bags and maintained in a greenhouse. After antibiotic selection a total of 43 plants showed no signs of necrosis, indicating the presence of *nptII* gene. These plants were used in further analyses.

Molecular Characterization and Quantitation of Cry Toxin in Transgenic Cotton Plants

The *cry1Ia12* segment (ICS) was amplified by PCR technique, and according to Figure 1A, the T0 plants numbered 10, 21, 23, 29, 50, and 88 (lanes 2–3) were positive for this amplification. Regarding Cry1Ia12 protein expression in T0 cotton plants, immunoblots using an anti-Cry1Ia12 polyclonal antibody revealed that the Cry1Ia12 protein with approximately 72 kDa was expressed at significant levels only in two GM cotton plants, 10 and 21, which were 1.25 and 2.26 µg g⁻¹ of leaf respectively (Figures 1B,C). For this reason, the following molecular experiments were performed with these two GM events. Therefore, Southern blot analysis were carried out in



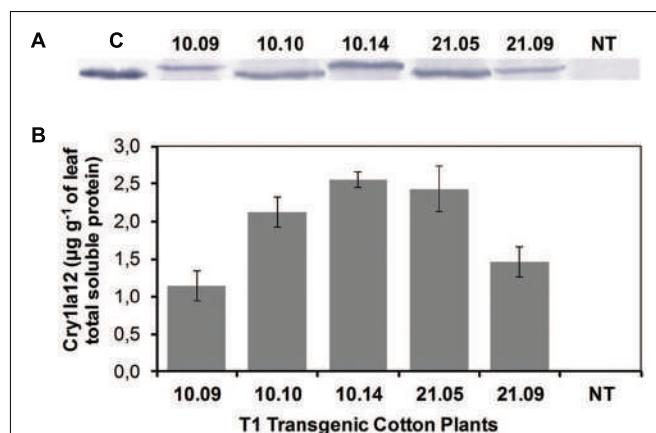
order to confirm the DNA cassette integration in the cotton genome of these two events (10 and 21; Figure 1D). The blot suggests a successful cassette genome integration in both events, which is in accordance with other molecular assays, especially with PCR experiments.

The T1 progeny of both 10 and 21 T0 GM cotton plants were molecularly evaluated and five of them (10.09, 10.10, 10.14, 21.05, and 21.09) were chosen based on both Western blot and ELISA experiments to bioassays with *S. frugiperda* and *A. grandis*. The 10.14 T1 cotton plant demonstrated higher protein level ($\sim 2.56 \mu\text{g g}^{-1}$ of leaf) when compared to respective T0 parental event (Figure 2).

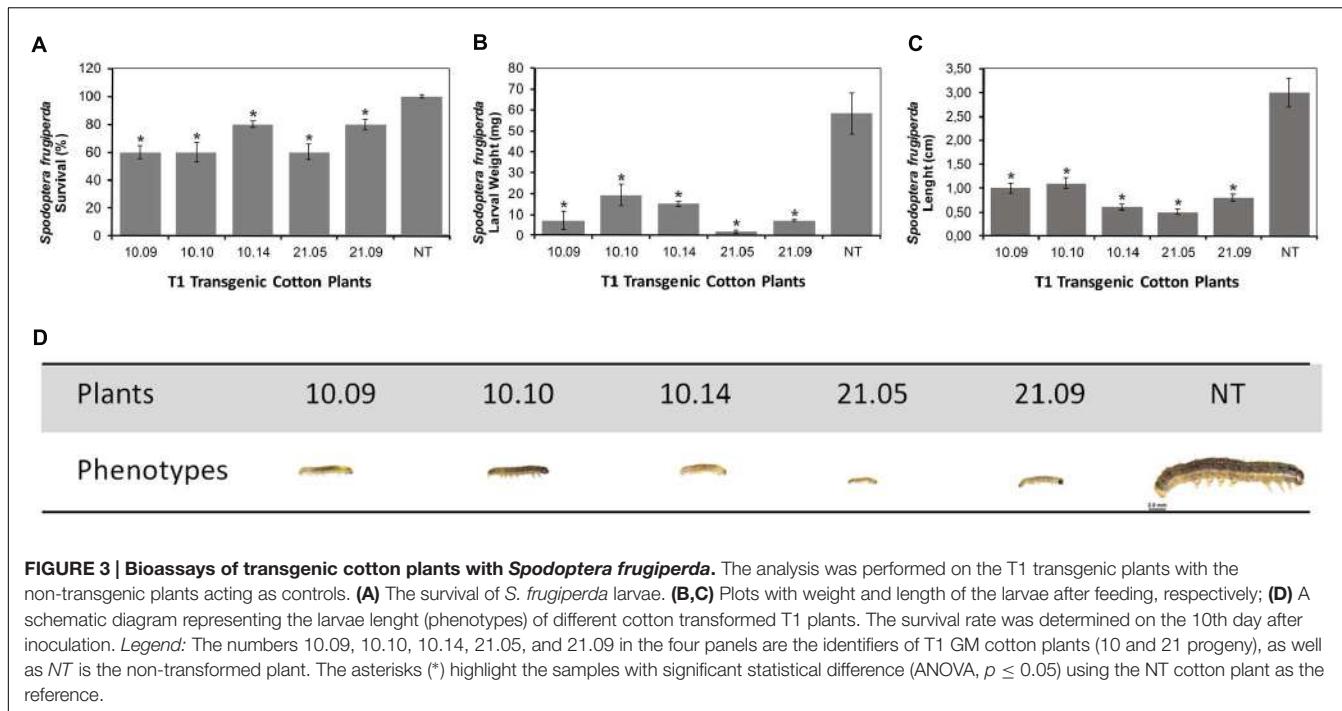
Insect Bioassays of Transgenic Plants

The Transgenic Cotton Plants Exhibited Toxicity to *Spodoptera frugiperda*

Five transgenic T1 plants (10.09, 10.10, 10.14, 21.05, and 21.09) were evaluated for their toxicity to cotton fall armyworm larvae. Initially the insects were fed with non-transgenic leaves for 2 days, and then they were transferred to transgenic leaves for 10 days. During the first 5 days, the experiment showed that the transgenic cotton plants were more resistant to *S. frugiperda*, compared to untransformed control, due to the fact that leaves have been slightly ingested by the larvae (Figure 3). Amongst the 6th and 10th days, nearly half of the leaves in the control plant had been consumed, while the transgenic leaves were



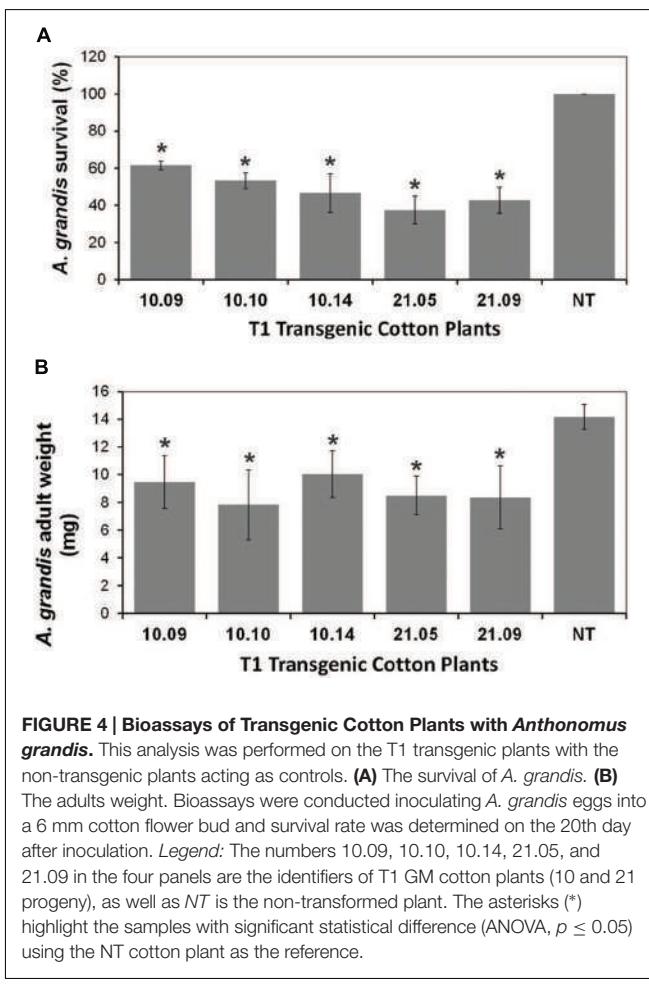
barely fed. The length from control larvae were significant larger than the ones that had fed on transgenic plants (Supplementary Figure S2). Besides the minor damage to the leaves, a significant



delay in the larvae growth fed on GM transformed plants were observed. Although, in terms of mortality, all 21 GM cotton plant progeny have shown greater toxicity to *S. frugiperda*, the five GM cotton plant progeny had significantly influenced the larvae development. All surviving larvae presented smaller lenght and were extremely weak, compared to larvae fed on non-transformed plants. The evaluated data showed that Cry1Ia12 toxin expressed in GM cotton plants was toxic to the cotton fall armyworms, as evidenced by larvae mortality rate up to 40%, after 10 days of experimental evaluation (Figure 3A). In the control insect group, all the larvae survived weighed approximately 60 mg (Figure 3B), while the weights of the surviving larvae fed on transgenic cotton plants ranged from 2 to 15 mg after 10 days of feeding, demonstrating be extremely smaller and weaker (Figures 3C,D), and obviously committing the next generation of the insect population.

The Transgenic Cotton Plants Exhibited Toxicity to *Anthonomus grandis*

The same T1 cotton plants expressing Cry1Ia12 used in *S. frugiperda* bioassay were also evaluate to their ability to confer resistance against to the cotton boll weevil. A total of ten floral buds were subjected to *A. grandis* bioassays. Once all the eggs have hatched is expected that the Cry1Ia12 protein expressed in the GM cotton plants do not block the hatching process. This statement is based on the fact that the toxin needs to be processed in the *A. grandis* midgut to become active (Schepf et al., 1998), which emphasizes the necessity that Cry proteins must be ingested by the insect to have activity. On the other hand, after 7 days, it was feasible to evaluate whether the larvae had fed, according the floral buds phenotypes, which became “fluffy,” in case of feeding. This feeding pattern



was evident in all non-transgenic plants. Compared with the untransformed, the transgenic plants expressing the Cry1Ia12 toxin showed substantially less damage to the floral bud after a week of feeding. The boll weevils completed their development and became adults in all non-transgenic plants after 20 days. Amongst transgenic plants, the number of adults that emerged was less than those emerged from control. Development delay in some groups was also observed (Supplementary Figure S3). The mortality of boll weevils in the experimental groups reached up to 60% after 20 days (**Figure 4A**), which was significantly higher than control group. All the larvae survived became adult insect on non-transgenic plants. The emerged insects that feed on floral buds from cotton line 10 progeny had a lower mortality rate than those which feed on the cotton line 21 progeny (**Figure 4B**).

DISCUSSION

The plant transformation primary goal is the production of fertile plants expressing a desirable foreign gene. To generate plants that express the desired traits, different techniques for inserting genes into plants have been developed since the early 1980s (De Block et al., 1984) and are currently widely used and commercially available (James, 2014). Cotton transformation methods normally use co-cultures with *A. tumefaciens* (Guo et al., 2007) and microprojectile bombardment (Aragão et al., 2005). It is well known that the transformation efficacy is affected by the plant material, genotype/variety, and type of explant (Wilkins et al., 2004; Rao et al., 2009; Anami et al., 2013; Chakravarthy et al., 2014; Juturu et al., 2015).

The pollen-tube pathway technique has been successfully used to transfer exogenous DNA into several plant species (Luo and Wu, 1989; Ni et al., 1998; Zhen et al., 1998; Yu et al., 1999). Herein, the establishment of a reliable and repeatable protocol for the pollen-tube pathway transformation technique was demonstrated, contributing to the generation of a GM Brazilian cotton variety, which expresses the Cry1Ia12 toxin that confers toxicity to the two important economic cotton insect pests, *A. grandis* and *S. frugiperda*. The pollen-tube pathway technique does not require tissue culture, which is the greatest advantage. In this context, the efficiency improvement observed with this technique relies on the seeds obtained from microinjected floral buds. In China, this transformation technique is usually applied on large scales to produce a vast number of seeds in the field (Huang and Wang, 2002). In contrast, Brazilian legislation is extremely restricted regarding to field experiments using GM plants. Therefore, such trials must be performed under greenhouse conditions, which decrease both the number of plants that can be microinjected and the seed yield. The pollen-tube pathway was efficient in transforming BRS Cedro cotton variety, reaching an efficiency of 0.01%, evaluated by the number of positive GM plants and viable seeds, as determined by PCR, Southern blot, ELISA and Western blot assays. This is important and justify the low number of cotton plants positively featured in this work, even starting from a large initial number of ovules.

Variations in gene expression in different transformation events have been reported in other studies (Deroles and Gardner, 1988; Robert et al., 1989) and could be due to variations in the transgene's integration into the target genome (Deroles and Gardner, 1988; Meyer, 1995a,b). Gene delivery strategies have also been explored to optimize the pollen-tube pathway technique. In cotton, the large size of the flowers allows for injection into an ovary.

According to Monsanto (2002), Cry1Ac protein content of Bollgard I cotton leaves was around $1.56 \mu\text{g g}^{-1}$ of leaf total soluble protein. Comparing the Bollgard I Cry1Ac expression levels with the cotton 10 and 21 progenies that best express Cry1Ia12 protein (10.14 and 21.05, respectively), it can be seen nearly the same toxin expression levels in both Cry1Ia12 cotton plants. Thus, this observation can be explained by several ways, highlighting: (i) intrinsic characteristics of each *cry* gene; (ii) the transgene insertion site in cotton genome, and (iii) gene promoter activity. Thus, in future studies is intended to use gene promoters that provide higher levels of expression, as well as presented by *uce* promoter, identified by Viana et al. (2011), which drive high expression in root and flower cotton tissues. Besides, several new gene promoters induced by biotic stress can be identified and characterized, especially after data analysis obtained from transcriptome of cotton flower buds infested with *A. grandis* larvae (Artico et al., 2014).

Since cotton was first transformed by two distinct groups in 1987 (Firoozabady et al., 1987; Umbeck et al., 1987), different traits have been introduced into cotton plants aiming either abiotic tolerance or biotic resistance (Juturu et al., 2015). The use of plant transformation to control insect pests started, when Perlak et al. (1990) developed transgenic cotton expressing the *B. thuringiensis* toxin Cry1Ac. After this achievement, GM cotton harboring *cry* genes to control insect pests have been available (James, 2014).

Once it was determined that Cry toxins are responsible for insect resistance in most GM plants, several studies were performed to evaluate the role of these toxins in response to insect stress. Tabashnik et al. (2002) showed that the Cry1Ac-resistant pink bollworm (*P. gossypiella*) had little or no ability to survive on second-generation transgenic cotton containing Cry2Ab alone or Cry1Ac plus Cry2Ab. Bioassays of several independent transgenic maize lines over-expressing the *cry1Ie* gene showed that these transgenic plants were highly toxic to the wild-type cotton bollworm (*Helicoverpa armigera*), producing mortality levels of 50% after 6 days of exposure (Zhang et al., 2013).

Even though the main class of genes used to obtain GM cotton resistant to insects, members of the *cry* family have limitations, mainly associated with molecular activities mechanisms. According to literature data, the active toxins bind with specific receptors on the brush border membrane of gut epithelial cells and is partially inserted into the membrane, generating pores. This results in colloid osmotic lysis of gut epithelial cells followed by the death of the insect (Hofmann and Lüthy, 1986; Schnepf et al., 1998). The most common resistance mechanism is a reduction of the toxin's ability to bind

to its specific midgut receptor(s). This may also confer cross-resistance to other toxins that share the same receptor (Ferré and Van Rie, 2002). In order to overcome this problem, various *cry* genes homologs have been characterized for insecticide function. According to specific studies, the CryII toxins group (where Cry1Ia12 is inserted) has wide host range and was initially characterized by their dual activity toward Lepidoptera and Coleoptera (Escudero et al., 2006). Among them, Martins et al. (2008) demonstrated in bioassays with heterologous Cry1Ia protein (expressed in baculovirus) in artificial diet that the recombinant protein had toxicity to *S. frugiperda* and *A. grandis* larvae. In parallel, Grossi-de-Sa et al. (2007) highlighted the *cry1Ia12* importance to *A. grandis* and *S. frugiperda* populations control in artificial diet. This feature has made it possible to test the susceptibility of *A. grandis* and *S. frugiperda* to a heterologous Cry1Ia-type toxin (Cry1Ia12 expressed in *Escherichia coli*) and demonstrated that the Cry1Ia12 toxin kills both insect larvae in concentration of 230 and 5 µg mL⁻¹ of artificial diet, respectively. The current study and those presented by Grossi-de-Sa are complementary. In both studies, *A. grandis* and *S. frugiperda* populations were controlled and they were differentiated by toxin administration (artificial diet and GM cotton plant). Thereby, assuming that Cry1Ia type protein sequences do not differ much from one another (Cry1Ia12 shows 99% of identity and similarity with other Cry1Ia toxins deposited in databases; Grossi-de-Sa et al., 2007), it is possible to suggest that the present study corroborates with others concerning the control of the cotton boll weevil and fall armyworm populations with Cry1Ia toxin variants administration.

Several studies showed a gradually increase in the number of insect populations resistant to Cry toxins. Downes et al. (2010) demonstrated that *Helicoverpa* species (Noctuidae) were resistant to Cry2Ab toxin in the second generation of *B. thuringiensis* cotton. Additionally, the emergence of *S. frugiperda* populations resistant to the *B. thuringiensis* toxin Cry1Fa expressed in corn was noted, forcing producers to use pesticides to reduce the damage caused by this insect pest (Monnerat et al., 2015). In order to retard the process of resistance, researchers postulate that transgenic plants should express high doses of the toxin or use more than one gene of interest in genetic transformation. Theoretically, a plant with two transgenes is significantly more effective in controlling insect pests than those expressing only one, since the insect that is resistant to a first toxin could likely be killed by a second (Roush, 1998; Zhao et al., 2005). Liu et al. (2005) demonstrated that *H. armigera* larvae in the first, second, and third instar could not survive if fed on transgenic cotton leaves expressing Cry1A + CpTI (Cowpea Trypsin Inhibitor) and Cry1Ac. In this present study, bioassays using *S. frugiperda* and *A. grandis* showed that cotton plants expressing Cry1Ia12 were toxic to both of these insect pests, but its insecticidal activity could be enhanced by associating with other molecules (Cry or non-Cry). Among them, we can point out: Cry8-type toxins (Nakasu et al., 2010; Oliveira et al., 2011; Navas et al., 2014), trypsin/chymotrypsin inhibitors (Franco et al., 2003; de Pg Gomes et al., 2005; Cruz et al., 2013), alpha-amylase inhibitors (Oliveira-Neto et al., 2003; Dias et al., 2005;

Bonavides et al., 2007) and *Streptomyces* cholesterol oxidase (Purcell et al., 1993). Moreover, it is worth emphasizing that gene knockdown by dsRNA technology has been widely used to silence important insect genes. A great example would be the *A. grandis* chitin synthase I (*AgCHI*) knockdown, that resulted in normal oviposition of unviable eggs and malformed alive larvae that were unable to develop in artificial diet (Firmino et al., 2013). Therefore, it would be also possible to associate the Cry1Ia12 toxin with dsRNA molecules in order to increase the control of *A. grandis* and *S. frugiperda* populations. Thus, Cry1Ia12 GM cotton plants can be used in breeding strategies to obtain GM cotton lines more effective in pest control, as well as presenting a reduction in emergence of resistant insects, especially *S. frugiperda*.

Considering, until now, the absence of cotton varieties with natural resistance to *A. grandis* infestation, as well as the great financial losses caused by this insect to cotton culture, we emphasize that the Cry1Ia12 GM cotton plants presented in this work are the first step in effective pesticide-free combat to this insect pest, even if the *A. grandis* mortality rate is still far from adequate. This observation is based mainly on the high cost of insecticides, besides the negative environmental impacts caused by these chemical agents. In Brazil, for example, the cost of insecticides to combat the boll weevil infestation in cotton crops ranged in 2015 between US\$ 100 and US\$ 300 per hectare (45% increase compared to last year; Brazilian Ministry of Agriculture, 2015). Thereby, a 60% mortality rate would be already significant, because would reach a large reduction in the cost of cotton production and consequently in insecticide management. Therefore, these GM cotton plants also present high potential *A. grandis* control and can be used in breeding programs to reduce the damage caused by this insect pest to cotton culture.

AUTHOR CONTRIBUTIONS

RdO carried out all experiments and data analysis. LdM and WL contributed to the bioassays design. FA contributed in achieving the ELISA experiments and help draft manuscript. OO-N helped establish the cotton transformation conditions. HM established the total protein extraction and Western-blot conditions. Mds, IL-T, and AB set the experimental conditions of PCR and Southern blot. MG conceived the study, planned the experiments, and helped draft the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00165>

REFERENCES

- Ali, A., Luttrell, R. G., Pitre, H. N., and Davis, F. M. (1989). Distribution of fall armyworm (Lepidoptera: Noctuidae) egg masses on cotton. *Environ. Entomol.* 18, 881–885. doi: 10.1093/ee/18.5.881
- Anami, S., Njuguna, E., Coussens, G., Aesaert, S., and Van Lijsebettens, M. (2013). Higher plant transformation: principles and molecular tools. *Int. J. Dev. Biol.* 57, 483–494. doi: 10.1387/ijdb.130232mv
- Aragão, F. J. L., Vianna, G. R., Carvalheira, S. B. R. C., and Rech, E. L. (2005). Germ line genetic transformation in cotton (*Gossypium hirsutum* L.) by selection of transgenic meristematic cells with a herbicide molecule. *Plant Sci.* 168, 1227–1233. doi: 10.1016/j.plantsci.2004.12.024
- Artico, S., Ribeiro-Alves, M., Oliveira-Neto, O. B., de Macedo, L. L. P., Silveira, S., Grossi-de-Sa, M. F., et al. (2014). Transcriptome analysis of *Gossypium hirsutum* flower buds infested by cotton boll weevil (*Anthonomus grandis*) larvae. *BMC Genomics* 15:854. doi: 10.1186/1471-2164-15-854
- Barros, E. M., Torres, J. B., Ruberson, J. R., and Oliveira, M. D. (2010). Development of *Spodoptera frugiperda* on different hosts and damage to reproductive structures in cotton. *Entomol. Exp. Appl.* 137, 237–245. doi: 10.1111/j.1570-7458.2010.01058.x
- Birch, R. G. (1997). Plant transformation: problems and strategies for practical application. *Annu. Rev. Plant Biol.* 48, 297–326. doi: 10.1146/annurev.aplant.48.1.297
- Bonavides, K. B., Pelegrini, P. B., Laumann, R. A., Grossi-de-Sa, M. F., Bloch, C. Jr., Melo, J. A. T., et al. (2007). Molecular identification of four different α -amylase inhibitors from Baru (*Dipteryx alata*) seeds with activity toward insect enzymes. *BMB Rep.* 40, 494–500.
- Bravo, A., Gomez, I., Porta, H., Garcia-Gomez, B. I., Rodriguez-Almazan, C., Pardo, L., et al. (2013). Evolution of *Bacillus thuringiensis* Cry toxins insecticidal activity. *Microb. Biotechnol.* 6, 17–26. doi: 10.1111/j.1751-7915.2012.00342.x
- Brazilian Ministry of Agriculture (2015). “Estatísticas”: Ministério da Agricultura, Pecuária e Abastecimento. Available at: http://www.agricultura.gov.br/vegetal/e_statisticas-2015
- Chakravarthy, V. S. K., Reddy, T. P., Reddy, V. D., and Rao, K. V. (2014). Current status of genetic engineering in cotton (*Gossypium hirsutum* L.): an assessment. *Crit. Rev. Biotechnol.* 34, 144–160. doi: 10.3109/07388551.2012.743502
- Chen, W. S., Chiu, C. C., Liu, H. Y., Lee, T. L., Cheng, J. T., Lin, C. C., et al. (1998). Gene transfer via pollen-tube pathway for anti-fusarium wilt in watermelon. *Biochem. Mol. Biol. Int.* 46, 1201–1209.
- Chilton, M. D. (2005). Adding diversity to plant transformation. *Nat. Biotechnol.* 23, 309–310. doi: 10.1038/Nbt0305-309
- Crickmore, N., Baum, J., Bravo, A., Lereclus, D., Narva, K., Sampson, K., et al. (2014). *Bacillus thuringiensis* Toxin Nomenclature [Online]. Available at: <http://www.bnomenclature.info/>
- Cruz, A. C. B., Massena, F. S., Miglioli, L., de Macedo, L. L. P., Monteiro, N. K. V., Oliveira, A. S., et al. (2013). Bioinsecticidal activity of a novel Kunitz trypsin inhibitor from Catanduva (*Piptadenia moniliformis*) seeds. *Plant Physiol. Biochem.* 70, 61–68. doi: 10.1016/j.plaphy.2013.04.023
- De Block, M., Herrera-Estrella, L., Van Montagu, M., Schell, J., and Zambryski, P. (1984). Expression of foreign genes in regenerated plants and in their progeny. *EMBO J.* 3, 1681–1689.
- de Pg Gomes, A., Dias, S. C., Bloch, C., Melo, F. R., Furtado, J. R., Monnerat, R. G., et al. (2005). Toxicity to cotton boll weevil *Anthonomus grandis* of a trypsin inhibitor from chickpea seeds. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 140, 313–319. doi: 10.1016/j.cbpc.2004.10.013
- Deroles, S. C., and Gardner, R. C. (1988). Expression and inheritance of kanamycin resistance in a large number of transgenic petunias generated by *Agrobacterium*-mediated transformation. *Plant Mol. Biol.* 11, 355–364. doi: 10.1007/Bf00027392
- Dias, S. C., Franco, O. L., Magalhaes, C. P., de Oliveira-Neto, O. B., Laumann, R. A., Figueira, E. L. Z., et al. (2005). Molecular cloning and expression of an α -amylase inhibitor from rye with potential for controlling insect pests. *Protein J.* 24, 113–123. doi: 10.1007/s10930-004-1518-4
- Downes, S., Mahon, R. J., Rossiter, L., Kauter, G., Leven, T., Fitt, G., et al. (2010). Adaptive management of pest resistance by *Helicoverpa* species (Noctuidae) in Australia to the Cry2Ab Bt toxin in Bollgard II® cotton. *Evol. Appl.* 3, 574–584. doi: 10.1111/j.1752-4571.2010.00146.x
- Engvall, E., and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochemistry* 8, 871–874. doi: 10.1016/0019-2791(71)90454-X
- Escudero, I. R., Estela, A., Porcar, M., Martinez, C., Oguiza, J. A., Escriche, B., et al. (2006). Molecular and insecticidal characterization of a Cry1I protein toxic to insects of the families Noctuidae, Tortricidae, Plutellidae, and Chrysomelidae. *Appl. Environ. Microbiol.* 72, 4796–4804. doi: 10.1128/Aem.02861-05
- Feitelson, J. S., Payne, J., and Kim, L. (1992). *Bacillus thuringiensis* – Insects and beyond. *Biotechnology* 10, 271–275. doi: 10.1038/Nbt0392-271
- Fernandes, M. G., Busoli, A. C., and Barbosa, J. C. (2002). Distribuição espacial de *Spodoptera frugiperda* (JE Smith, 1797) (Lepidoptera: Noctuidae) em algodoeiro. *Curr. Agric. Sci. Technol.* 8, 203–211.
- Ferré, J., and Van Rie, J. (2002). Biochemistry and genetics of insect resistance to *Bacillus thuringiensis*. *Annu. Rev. Entomol.* 47, 501–533. doi: 10.1146/annurev.ento.47.091201.145234
- Firmimo, A. A. P., de Assis Fonseca, F. C., de Macedo, L. L. P., Coelho, R. R., de Souza, J. D. A. Jr., Togawa, R. C., et al. (2013). Transcriptome analysis in cotton boll weevil (*Anthonomus grandis*) and RNA interference in insect pests. *PLoS ONE* 8:e85079. doi: 10.1371/journal.pone.0085079
- Firoozabady, E., DeBoer, D. L., Merlo, D. J., Halk, E. L., Amerson, L. N., Rashka, K. E., et al. (1987). Transformation of cotton (*Gossypium hirsutum* L.) by *Agrobacterium tumefaciens* and regeneration of transgenic plants. *Plant Mol. Biol.* 10, 105–116. doi: 10.1007/BF00016148
- Franco, O. L., dos Santos, R. C., Batista, J. A. N., Mendes, A. C. M., de Araujo, M. A. M., Monnerat, R. G., et al. (2003). Effects of black-eyed pea trypsin/chymotrypsin inhibitor on proteolytic activity and on development of *Anthonomus grandis*. *Phytochemistry* 63, 343–349. doi: 10.1016/S0031-9422(03)00108-0
- Gallo, D., Nakano, O., Silveira, N., and Carvalho, R. P. L. (2002). *Manual de Entomologia Agrícola*. Piracicaba: Agronômica Ceres.
- Greene, G. L., Leppla, N. C., and Dickerson, W. A. (1976). Velvetbean caterpillar: a rearing procedure and artificial medium. *J. Econ. Entomol.* 69, 487–488. doi: 10.1093/jee/69.4.487
- Grossi-de-Sa, M. F., de Magalhaes, M. Q., Silva, M. S., Silva, S. M. B., Dias, S. C., Nakasu, E. Y. T., et al. (2007). Susceptibility of *Anthonomus grandis* (cotton boll weevil) and *Spodoptera frugiperda* (fall armyworm) to a Cry1Ia-type toxin from a Brazilian *Bacillus thuringiensis* strain. *J. Biochem. Mol. Biol.* 40, 773–782. doi: 10.5483/BMBRep.2007.40.5.773
- Guimarães, L. M., Farias, D. F., Muchagata, R. C., de Magalhaes, M. Q., Campello, C. C., Rocha, T. L., et al. (2010). Short-term evaluation in growing rats of diet containing *Bacillus thuringiensis* Cry1Ia12 entomotoxin: nutritional responses and some safety aspects. *J. Biomed. Biotechnol.* 2010, 630267. doi: 10.1155/2010/630267
- Guo, X., Huang, C., Jin, S., Liang, S., Nie, Y., and Zhang, X. (2007). *Agrobacterium*-mediated transformation of Cry1C, Cry2A and Cry9C genes into *Gossypium hirsutum* and plant regeneration. *Biol. Plant.* 51, 242–248. doi: 10.1007/s10535-007-0048-2
- Hofmann, C., and Lüthy, P. (1986). Binding and activity of *Bacillus thuringiensis* delta-endotoxin to invertebrate cells. *Arch. Microbiol.* 146, 7–11. doi: 10.1007/BF00690150
- Hofte, H., and Whiteley, H. R. (1989). Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol. Rev.* 53, 242–255.
- Hu, Y., Georgiou, S. B., Kelleher, A. J., and Aroian, R. V. (2010). *Bacillus thuringiensis* Cry5B protein is highly efficacious as a single-dose therapy against an intestinal roundworm infection in mice. *PLoS Negl. Trop. Dis.* 4:e614. doi: 10.1371/journal.pntd.0000614
- Huang, J., and Wang, Q. (2002). Agricultural biotechnology development and policy in China. *AgBioForum* 5, 122–135.
- ISAAA (2015). ISAAA's GM Approval Database. Available at: <http://www.isaaa.org/gmapprovaldatabase/>
- James, C. (2014). Brief 49: Global Status of Commercialized Biotech/GM Crops: 2014. ISAAA Brief. Ithaca, NY: International Service for the Acquisition of Agri-biotech Applications, 290.
- Juturu, V. N., Mekala, G. K., and Kirti, P. B. (2015). Current status of tissue culture and genetic transformation research in cotton (*Gossypium* spp.). *Plant Cell Tissue Organ Cult.* 120, 813–839. doi: 10.1007/s11240-014-0640-z
- Khan, T., Reddy, V. S., and Leelavathi, S. (2010). High-frequency regeneration via somatic embryogenesis of an elite recalcitrant cotton genotype (*Gossypium*

- hirsutum* L.) and efficient *Agrobacterium*-mediated transformation. *Plant Cell Tissue Organ Cult.* 101, 323–330. doi: 10.1007/s11240-010-9691-y
- Kriticos, D. J., Ota, N., Hutchison, W. D., Beddow, J., Walsh, T., Tay, W. T., et al. (2015). The potential distribution of invading *Helicoverpa armigera* in North America: is it just a matter of time? *PLoS ONE* 10:e0119618. doi: 10.1371/journal.pone.0119618
- Kumar, M., Shukla, A. K., Singh, H., and Tuli, R. (2009). Development of insect resistant transgenic cotton lines expressing cry1EC gene from an insect bite and wound inducible promoter. *J. Biotechnol.* 140, 143–148. doi: 10.1016/j.biote.2009.01.005
- Larkin, P. (2004). *Somaclonal Variation: Origins and Causes*. New York, NY: Marcel Dekker.
- Leelavathi, S., Sunnichan, V. G., Kumria, R., Vijaykanth, G. P., Bhatnagar, R. K., and Reddy, V. S. (2004). A simple and rapid *Agrobacterium*-mediated transformation protocol for cotton (*Gossypium hirsutum* L.): embryogenic calli as a source to generate large numbers of transgenic plants. *Plant Cell Rep.* 22, 465–470. doi: 10.1007/s00299-003-0710-x
- Liu, D. P., Liao, L., Yuan, Y., and Liu, Y. Z. (1997). Gaining of soybean lines resistant to SMV by introducing exogenous DNA. *Soybean Sci.* 16, 277–281.
- Liu, X., Sun, C. I., and Zhang, Q. (2005). Effects of transgenic Cry1A+ CpTI cotton and Cry1Ac toxin on the parasitoid, Campoketis chlorideae (Hymenoptera: Ichneumonidae). *Insect Sci.* 12, 101–107. doi: 10.1111/j.1744-7917.2005.00012.x
- Luo, Z. X., and Wu, R. (1989). A simple method for the transformation of rice via the pollen tube pathway. *Plant Mol. Biol. Rep.* 7, 69–77. doi: 10.1007/BF02668692
- Mao, Y. B., Tao, X. Y., Xue, X. Y., Wang, L. J., and Chen, X. Y. (2011). Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms. *Transgenic Res.* 20, 665–673. doi: 10.1007/s11248-010-9450-1
- Martins, E. S., Aguiar, R. W. D. S., Martins, N. F., Melatti, V. M., Falcão, R., Gomes, A. C. M. M., et al. (2008). Recombinant Cry1Ia protein is highly toxic to cotton boll weevil (*Anthonomus grandis*, Boheman) and fall armyworm (*Spodoptera frugiperda*). *J. Appl. Microbiol.* 104, 1363–1371. doi: 10.1111/j.1365-2672.2007.03665.x
- Martins, E. S., Praça, L. B., Dumas, V. F., Silva-Werneck, J. O., Sone, E. H., Waga, I. C., et al. (2007). Characterization of *Bacillus thuringiensis* isolates toxic to cotton boll weevil (*Anthonomus grandis*). *Biol. Control* 40, 65–68. doi: 10.1016/j.bioc.2006.09.009
- McCabe, D. E., and Martinell, B. J. (1993). Transformation of elite cotton cultivars via particle bombardment of meristems. *Biotechnology* 11, 596–598. doi: 10.1038/Nbt0593-596
- Meyer, P. (1995a). Understanding and controlling transgene expression. *Trends Biotechnol.* 13, 332–337. doi: 10.1016/S0167-7799(00)88977-5
- Meyer, P. (1995b). Variation of transgene expression in plants. *Euphytica* 85, 359–366. doi: 10.1007/BF00023968
- Michiels, A., Van den Ende, W., Tucker, M., Van Riet, L., and Van Laere, A. (2003). Extraction of high-quality genomic DNA from latex-containing plants. *Anal. Biochem.* 315, 85–89. doi: 10.1016/S0003-2697(02)00665-6
- Miranda, J. E. (2006). *Distribuição Vertical de Lagartas de Spodoptera Frugiperda no Algodoeiro*. Campina Grande: Embrapa Algodão.
- Monnerat, R. (2000). *Criação Massal do Bicudo do Algodoeiro Anthonomus Grandis em Laboratório*. Brasília: Embrapa Recursos Genéticos e Biotecnologia.
- Monnerat, R., Martins, E. S., Macedo, C., Queiroz, P., Praça, L. B., Soares, C. M., et al. (2015). Evidence of field-evolved resistance of *Spodoptera frugiperda* to Bt corn expressing Cry1F in Brazil that is still sensitive to modified Bt toxins. *PLoS ONE* 10:e0119544. doi: 10.1371/journal.pone.0119544
- Monsanto (2002). *Monsanto Safety Summaries – Bollgard Cotton Event 531*. Available at: http://www.monsanto.com/products/documents/safety-summaries/bollgard_pss.pdf
- Nakasu, E. Y. T., Firmino, A. A. P., Dias, S. C., Rocha, T. L., Ramos, H. B., Oliveira, G. R., et al. (2010). Analysis of Cry8Ka5-binding proteins from *Anthonomus grandis* (Coleoptera: Curculionidae) midgut. *J. Invertebr. Pathol.* 104, 227–230. doi: 10.1016/j.jip.2010.01.012
- Navas, L. E., Berretta, M. F., Pérez, M. P., Amadio, A. F., Ortiz, E. M., Sauka, D. H., et al. (2014). Sequence and expression of two cry8 genes from *Bacillus thuringiensis* INTA Fr7-4, a native strain from Argentina. *J. Mol. Microbiol. Biotechnol.* 24, 241–248. doi: 10.1159/000365929
- Ni, W. C., Zhang, Z. L., Zhang, B. L., Xu, Y. J., and Guo, S. D. (1998). Development of transgenic insect-resistant cotton plants. *Sci. Agric. Sin.* 31, 8–13.
- Oerke, E. C. (2006). Crop losses to pests. *J. Agric. Sci.* 144, 31–43. doi: 10.1017/S0021859605005708
- Oh, T. J., Cullis, M. A., Kunert, K., Engelborghs, I., Swennen, R., and Cullis, C. A. (2007). Genomic changes associated with somaclonal variation in banana (*Musa* spp.). *Physiol. Plant.* 129, 766–774. doi: 10.1111/j.1399-3054.2007.00858.x
- Oliveira, G. R., Silva, M. C., Lucena, W. A., Nakasu, E. Y., Firmino, A. A., Beneventi, M. A., et al. (2011). Improving Cry8Ka toxin activity towards the cotton boll weevil (*Anthonomus grandis*). *BMC Biotechnol.* 11:85. doi: 10.1186/1472-6750-11-85
- Oliveira-Neto, O. B., Batista, J. A. N., Rigden, D. J., Franco, O. L., Falcão, R., Fragoso, R. R., et al. (2003). Molecular cloning of α -amylases from cotton boll weevil, *Anthonomus grandis* and structural relations to plant inhibitors: an approach to insect resistance. *J. Protein Chem.* 22, 77–87. doi: 10.1023/A:1023024012657
- Pan, Z. Z., Xu, L., Zhu, Y. J., Shi, H., Chen, Z., Chen, M. C., et al. (2014). Characterization of a new cry2Ab gene of *Bacillus thuringiensis* with high insecticidal activity against *Plutella xylostella* L. *World J. Microbiol. Biotechnol.* 30, 2655–2662. doi: 10.1007/s11274-014-1689-x
- Peppley, E. B., Allen, R., Song, P., and Shang, X. (2003). *Transformation of Onion Plants; Obtain Plant Cell, Transform with Vector, Recover Transformed Cells, Propagate Embryo*. Google Patents US6583335. Washington, DC: U.S. Patent and Trademark Office
- Perlak, F. J., Deaton, R. W., Armstrong, T. A., Fuchs, R. L., Sims, S. R., Greenplate, J. T., et al. (1990). Insect resistant cotton plants. *Biotechnology* 8, 939–943. doi: 10.1038/nbt1090-939
- Pitre, H. N., Mulrooney, J. E., and Hogg, D. B. (1983). Fall armyworm (Lepidoptera: Noctuidae) oviposition: crop preferences and egg distribution on plants. *J. Econ. Entomol.* 76, 463–466. doi: 10.1093/jee/76.3.463
- Purcell, J. P., Greenplate, J. T., Jennings, M. G., Ryerse, J. S., Pershing, J. C., Sims, S. R., et al. (1993). Cholesterol oxidase: a potent insecticidal protein active against boll weevil larvae. *Biochem. Biophys. Res. Commun.* 196, 1406–1413. doi: 10.1006/bbrc.1993.2409
- Rajasekaran, K. (2013). “Biostatic transformation of cotton embryogenic cell suspension cultures,” in *Transgenic Cotton: Methods and Protocols*, ed. B. Zhang (New York, NY: Springer), 59–70.
- Rajasekaran, K., Grula, J. W., Hudspeth, R. L., Pofelis, S., and Anderson, D. M. (1996). Herbicide-resistant Acala and Coker cottons transformed with a native gene encoding mutant forms of acetohydroxyacid synthase. *Mol. Breed.* 2, 307–319. doi: 10.1007/Bf00437909
- Rao, A. Q., Bakhsh, A., Kiani, S., Shahzad, K., Shahid, A. A., Husnain, T., et al. (2009). The myth of plant transformation. *Biotechnol. Adv.* 27, 753–763. doi: 10.1016/j.biotechadv.2009.04.028
- Rech, E. L., Vianna, G. R., and Aragao, F. J. L. (2008). High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nat. Protoc.* 3, 410–418. doi: 10.1038/Nprot.2008.9
- Robert, L. S., Thompson, R. D., and Flavell, R. B. (1989). Tissue-specific expression of a wheat high molecular-weight glutenin gene in transgenic tobacco. *Plant Cell* 1, 569–578. doi: 10.1105/tpc.1.6.569
- Roush, R. T. (1998). Two-toxin strategies for management of insecticidal transgenic crops: can pyramiding succeed where pesticide mixtures have not? *Philos. Trans. R. Soc. B Biol. Sci.* 353, 1777–1786. doi: 10.1098/rstb.1998.0330
- Sambrook, J., and Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual*. New York, NY: Cold Spring Harbor Laboratory Press.
- Santos, R. C., Marcellino, L. H., Monnerat, R. G., and Gander, E. S. (2003). Mechanical damage in cotton buds caused by the boll weevil. *Pesqui. Agropecu. Bras.* 38, 1351–1356. doi: 10.1590/S0100-204X2003001100015
- Schnepf, E., Crickmore, N., Van Rie, J., Lereclus, D., Baum, J., Feitelson, J., et al. (1998). *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol. Mol. Biol. Rev.* 62, 775–806.
- Soberón, M., Pardo, L., Muñoz-Garay, C., Sánchez, J., Gómez, I., Porta, H., et al. (2010). “Pore formation by Cry toxins,” in *Proteins: Membrane Binding and Pore Formation*, eds G. Anderluh and J. Lakey (Heidelberg: Bioscience and Springer Science + Business Media).
- Tabashnik, B. E., Dennehy, T. J., Sims, M. A., Larkin, K., Head, G. P., Moar, W. J., et al. (2002). Control of resistant pink bollworm (*Pectinophora gossypiella*) by transgenic cotton that produces *Bacillus thuringiensis* toxin Cry2Ab. *Appl. Environ. Microbiol.* 68, 3790–3794. doi: 10.1128/AEM.68.8.3790-3794.2002

- Umbeck, P., Johnson, G., Barton, K., and Swain, W. (1987). Genetically transformed cotton (*Gossypium hirsutum* L.) plants. *Nat. Biotechnol.* 5, 263–266. doi: 10.1038/nbt0387-263
- Vain, P. (2007). Thirty years of plant transformation technology development. *Plant Biotechnol. J.* 5, 221–229. doi: 10.1111/j.1467-7652.2006.00225.x
- Vajhala, C. S. K., Sadumpati, V. K., Nunna, H. R., Puligundla, S. K., Vu dem, D. R., and Khareedu, V. R. (2013). Development of transgenic cotton lines expressing *Allium sativum* agglutinin (ASAL) for enhanced resistance against major sap-sucking pests. *PLoS ONE* 8:e72542. doi: 10.1371/journal.pone.0072542
- Viana, A. A. B., Fragoso, R. R., Guimarães, L. M., Pontes, N., Oliveira-Neto, O. B., Artico, S., et al. (2011). Isolation and functional characterization of a cotton ubiquitination-related promoter and 5'UTR that drives high levels of expression in root and flower tissues. *BMC Biotechnol.* 11:115. doi: 10.1186/1472-6750-11-115
- Wilkins, T. A., Mishra, R., and Trolinder, N. L. (2004). Agrobacterium-mediated transformation and regeneration of cotton. *J. Food Agric. Environ.* 2, 179–187.
- Wu, J. H., Luo, X. L., Wang, Z., Tian, Y. C., Liang, A. H., and Sun, Y. (2008). Transgenic cotton expressing synthesized scorpion insect toxin aaHIT gene confers enhanced resistance to cotton bollworm (*Heliothis armigera*) larvae. *Biotechnol. Lett.* 30, 547–554. doi: 10.1007/s10529-007-9555-7
- Yang, A. F., Su, Q., An, L. J., Liu, J. F., Wu, W., and Qiu, Z. (2009). Detection of vector and selectable marker-free transgenic maize with a linear GFP cassette transformation via the pollen-tube pathway. *J. Biotechnol.* 139, 1–5. doi: 10.1016/j.jbiotec.2008.08.012
- Yu, H. X., Liu, J. J., Feng, Z. L., and Dong, J. D. (1999). Study on introduction of vermin-resistance gene (CpTI) into wheat through pollen-tube pathway method. *Shandong Agric. Sci.* 99, 5–8.
- Zhang, Y., Liu, Y., Ren, Y., Liu, Y., Liang, G., Song, F., et al. (2013). Overexpression of a novel Cry1Ia gene confers resistance to Cry1Ac-resistant cotton bollworm in transgenic lines of maize. *Plant Cell Tissue Organ Cult.* 115, 151–158. doi: 10.1007/s11240-013-0348-5
- Zhang, Y. S., Zhai, S., Yang, A., and Zhang, J. (2005). Maize transformation via pollen tube pathway and the inheritance of transgene in progeny. *High Technol. Lett.* 11, 202–206.
- Zhao, J. Z., Cao, J., Collins, H. L., Bates, S. L., Roush, R. T., Earle, E. D., et al. (2005). Concurrent use of transgenic plants expressing a single and two *Bacillus thuringiensis* genes speeds insect adaptation to pyramided plants. *Proc. Natl. Acad. Sci. U.S.A.* 102, 8426–8430. doi: 10.1073/pnas.0409324102
- Zhao, L. M., Liu, D. P., Sun, H., Yun, Y., and Huang, M. (1995). A sterile material of soybean gained by introducing exogenous DNA. *Soybean Sci.* 14, 83–87.
- Zhen, J. Z., Wu, Y. X., Wang, D. J., Zhang, J., Ma, Z. R., and Zhou, Z. Y. (1998). The exploration of the mechanism and genetic performance of the progenies gained from pollen-tube pathway transformation. *Chin. Sci. Bull.* 43, 561–566.
- Zhou, G., Weng, J., Zheng, Y., Huang, J., Qian, S., and Liu, G. (1983). Introduction of exogenous DNA into cotton embryos. *Methods Enzymol.* 101, 433–481. doi: 10.1016/0076-6879(83)01032-0

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Quantitative genetic analysis of agronomic and morphological traits in sorghum, *Sorghum bicolor*

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The productivity in sorghum is low, owing to various biotic and abiotic constraints. Combining insect resistance with desirable agronomic and morphological traits is important to increase sorghum productivity. Therefore, it is important to understand the variability for various agronomic traits, their heritabilities and nature of gene action to develop appropriate strategies for crop improvement. Therefore, a full diallel set of 10 parents and their 90 crosses including reciprocals were evaluated in replicated trials during the 2013–14 rainy and postrainy seasons. The crosses between the parents with early- and late-flowering flowered early, indicating dominance of earliness for anthesis in the test material used. Association between the shoot fly resistance, morphological, and agronomic traits suggested complex interactions between shoot fly resistance and morphological traits. Significance of the mean sum of squares for GCA (general combining ability) and SCA (specific combining ability) of all the studied traits suggested the importance of both additive and non-additive components in inheritance of these traits. The GCA/SCA, and the predictability ratios indicated predominance of additive gene effects for majority of the traits studied. High broad-sense and narrow-sense heritability estimates were observed for most of the morphological and agronomic traits. The significance of reciprocal combining ability effects for days to 50% flowering, plant height and 100 seed weight, suggested maternal effects for inheritance of these traits. Plant height and grain yield across seasons, days to 50% flowering, inflorescence exsertion, and panicle shape in the postrainy season showed greater specific combining ability variance, indicating the predominance of non-additive type of gene action/epistatic interactions in controlling the expression of these traits. Additive gene action in the rainy season, and dominance in the postrainy season for days to 50% flowering and plant height suggested G X E interactions for these traits.

Keywords: sorghum, combining ability, heritability, agronomic traits, morphological traits, GCA, SCA, grain yield

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is an important crop grown primarily in warm and dry climates with a wide range of adaptability to various agro-ecological conditions. It is the fifth most important food crop after wheat, rice, maize, and barley (FAO, 2004), and is widely grown in the semi-arid regions. It is the staple food for 600 million people living in the semi-arid regions.

India is the third largest sorghum producer after Nigeria and United States of America, with 6.25 million hectares of area under sorghum cultivation, and with a total production of 5.98 million tonnes (FAOSTAT, 2012).

Information on inheritance of agronomic and morphological traits is useful for improving genotypic performance across environments. In sorghum, both the additive and non-additive type of gene action governs the inheritance of morphological and agronomic traits (Nimbalkar and Bapat, 1992; Umakanth et al., 2002; Mohammed Maarouf, 2009) with considerable amount of G X E interaction (Jayanthi et al., 1996; Dhillon et al., 2006; Aruna et al., 2011a).

Most of the morphological traits in sorghum are associated with one or more economically important traits, and will be helpful in selecting the high yielding sorghum genotypes. Brown midrib increases the fodder quality, while the presence of awns acts as a mechanical barrier to bird damage (Porter et al., 1978; Kullaiswamy and Goud, 1983). Genotypes with tan-colored plants showed resistance to various fungal diseases while the genotypes with closed glumes are resistant to grain mold (Melake-Berhan et al., 1996; Murty, 2000).

Although, considerable progress has been made in identifying insect-resistant sorghums (Sharma, 1993; Sharma et al., 2003), but there is little progress in developing insect-resistant high yielding varieties for cultivation by the farmers. This is largely because of the lack of knowledge on inheritance of the agronomic and morphological characteristics associated with insect resistance and grain yield (Sharma et al., 2005; Riyazaddin et al., 2015). The combining ability analysis is useful to understand the nature of gene action, and has been used by the breeders to select the suitable parents for the crossing program. An understanding of the inheritance of morphological and agronomic traits will be helpful in combining the genes for insect resistance and desirable agronomic traits and grain characteristics to increase production and productivity of sorghum. Therefore, we developed a full diallel involving 10 parents to study the inheritance of morphological and agronomic traits. The combining ability studies will be helpful to identify genotypes which can be utilized in the hybridization.

MATERIALS AND METHODS

Experimental Material

Based on *per se* performance of sorghum genotypes in the field against shoot fly, *Atherigona soccata* and molecular diversity, 10 morphologically and genetically diverse sorghum genotypes (Annexure I in Supplementary Material) adapted to the rainy and postrainy seasons were selected and crossed in all possible combinations, which generated 45 direct crosses and 45 reciprocal crosses. These crosses along with the parents were evaluated in a randomized complete block design in three replications during the 2013–14 rainy and postrainy seasons at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad, Telangana, India (latitude 17.53°N, longitude 78.27°E, and altitude of 545 m).

Sowing of the test genotypes was carried out using a two cone planter. Each test plot consists of a row length of 2.0 m

and a row to row spacing of 75 cm. A distance of 10 cm was maintained in-between the plants within a row. Application of a basal dose of Ammonium phosphate to the field was carried out at 100 kg/ha. Each parent was sown in two rows, and a single row of F₁. Thinning of the test plots was carried out at 7 days after seedling emergence (DAE) and a plant population of 40 plants were retained in a test plot. At 30 DAE earthing up was carried out along with top dressing with urea at 100 kg/ha. During the postrainy season furrow irrigation was given to the experimental material. One set of the replicated test material was grown under protected conditions (application of carbofuran 3G granules in the leaf whorls at 7 days after seedling emergence, and cypermethrin spray after 5 days) to record the agronomic and morphological traits in the undamaged plants during the rainy and postrainy seasons.

Observations

Agronomic Characteristics

Data were recorded on days to 50% flowering, plant height, agronomic score, 100 seed weight, and grain yield. Days to 50% flowering was recorded when half of the panicles and nearly 50% of plants in the plot had attained the anthesis stage. Height of three plants was measured from the base of the plant to the tip of the panicle at physiological maturity in plants selected at random within a test plot. The agronomic desirability of the genotype was recorded at crop maturity on a 1–5 scale (1 = high productive potential, and 5 = poor productive potential). Data on 100 seed weight and grain yield/plot for parents, and grain yield/5 plants for F₁s were recorded after crop harvest.

Morphological Characteristics

Inflorescence exertion was scored on a 1–4 scale (1 = panicle fully exerted, and 4 = panicle recurved); panicle compactness on a 1–3 scale (1 = loose panicle, and 3 = compact panicle); panicle shape on a 1–4 scale (1 = erect panicle, and 4 = elliptic panicle); glume coverage on a 1–9 scale (1 = 25% grain covered with glumes, and 9 = glumes longer than the grain); awns on 1–2 scale (1 = absence of awns, 2 = presence of awns); grain luster on 1–2 scale (1 = non-lustrous grain, 2 = lustrous grain); and grain color on a 1–5 scale (1 = white colored grain and 5 = buff colored grain) (IBPGR and ICRISAT, 1993).

Statistical Analysis

Analysis of variance (ANOVA) was carried out using GenStat® 13th version (GenStat, 2010). F-test was used to test the significance differences between the genotypes, and least significance difference (LSD) for comparing the genotypic means at $P \leq 0.05$. Simple correlation coefficients were calculated to determine the association between the traits studied. Partitioning of the combining abilities (GCA and SCA) was done using the method 1 and model 1 of Griffing (1956), that provides the information on nature of parents, and the hybrid performance, using Windowstat (Indostat Services, 2004) software. The coefficient of variations at phenotypic and genotypic level variation was estimated using the formula adopted by Johnson et al. (1955) and predictability ratio using Baker (1978).

RESULTS

Agronomic Traits

Evaluation of 10 parents and 90 F₁s, including the reciprocals, showed significant differences for all the traits studied across seasons at $P \leq 0.01$. Days to 50% flowering was ranged from 61 to 81 days in the rainy season, 56–78 days in the postrainy season (**Table 1**). Almost all the crosses flowered early, with few exceptions. Crosses between the parents with early- and late-flowering were early-flowering, indicating dominance of earliness for anthesis in the test material used. The crosses CSV 15 X ICSV 25019, CSV 15 X PS 35805, ICSV 25019 X CSV 15, ICSV 25019 X PS 35805, ICSV 25019 X IS 2123, ICSV 25019 X Swarna, PS 35805 X CSV 15, PS 35805 X ICSV 25019, PS 35805 X IS 2146, PS 35805 X Swarna, Swarna X ICSV 25019, and Swarna X PS 35805 exhibited moderate plant height across seasons. Parents and the crosses with moderate plant height can be exploited in developing the commercial hybrids amenable for mechanical harvesting.

Ten crosses exhibited high 100 seed weight and grain yield with good agronomic desirability. Grain yield of the crosses CSV 15 X IS 2123, ICSV 25019 X Swarna, PS 35805 X Swarna, IS 2123 X CSV 15, IS 2123 X ICSV 25019, IS 2123 X Swarna, Swarna X ICSV 25019, and Swarna X IS 2123 was high in the rainy season.

Morphological and Grain Characteristics

All the panicle traits showed significant variability among the genotypes for all the characteristics studied, in both the rainy and postrainy seasons with significant variance ratio ($P \leq 0.01$) (**Table 2**). The mean scores for inflorescence exertion were 1.90 and 2.41; for panicle compactness 2.30 and 2.63; and for glume coverage 2.00 and 1.71, respectively, in the rainy and postrainy seasons.

Association of the Agronomic and Morphological Traits with Shoot Fly Resistance

Days to 50% flowering, inflorescence exertion, panicle compactness, glume coverage, and presence of awns were significantly and negatively correlated with shoot fly damage parameters across seasons, with few exceptions (**Table 3**). Plant height, 100 seed weight, and grain yield showed positive correlation with shoot fly damage across seasons.

Association between the Agronomic Traits

Agronomic score was positively correlated with days to 50% flowering and plant height, but negatively correlated with grain yield (**Table 4**). Days to 50% flowering were significantly and positively correlated with plant height, and negatively correlated with 100 seed weight and grain yield. Grain yield was positively correlated with plant height in the postrainy season, and 100 seed weight across seasons. Significant positive correlation was observed between plant height and 100 seed weight in the postrainy season.

Association between the Morphological Traits

Significant positive correlation was observed between inflorescence exertion and panicle compactness, and between awns and the panicle traits across seasons (**Table 5**). Panicle shape was positively correlated with inflorescence exertion, and panicle compactness in the postrainy season.

Combining Ability Analysis

Mean sum of squares for general combining ability of all the traits studied in the rainy season and postrainy seasons were significant at $P = 0.01$ (**Table 6**). Mean sum of squares due to SCA was significant for all the traits studied, during the rainy and postrainy seasons, except grain luster during rainy season and agronomic score and waxy bloom in the postrainy season indicating the role of both additive and non-additive nature of gene action in controlling most of the morphological and agronomic traits. The mean sum of squares due to reciprocal crosses was significant for days to 50% flowering and 100 seed weight across seasons, inflorescence exertion during the rainy season; and plant height, panicle compactness, and panicle shape during the postrainy season, suggesting the influence of cytoplasmic factors in the expression of these traits.

Estimates of General Combining Ability (gca), Specific Combining Ability (sca) and Reciprocal Effects

gca Effects of Agronomic Traits

gca effects of days to 50% flowering ranged from -2.87 (Phule Anuradha) to 3.36 (ICSV 700) in the rainy season, and from -3.65 (CSV 15) to 4.40 (ICSV 700) in the postrainy season (**Table 7**). Phule Anuradha (-2.87**), ICSV 25019 (-1.85**), IS 2146 (-0.77**) and Swarna (-1.37**) exhibited significant negative gca effects in the rainy season, and Phule Anuradha (-1.61**), CSV 15 (-3.65**), ICSV 25019 (-2.58**), PS 35805 (-2.58**), and Swarna (-2.60**) exhibited significant negative gca effects for days to 50% flowering in the postrainy season. ICSV 700 (3.36**, 4.40**, respectively, in the rainy and postrainy season), M 35-1 (0.50*, 2.30**), IS 2123 (1.33**, 1.70**), and IS 18551 (2.46**, 3.17**) across seasons and IS 2146 (1.44**) in the postrainy season showed significant positive gca effects for days to 50% flowering.

gca effects for plant height ranged from -44.49 (ICSV 25019) to 32.23 (ICSV 700) in the rainy season, -28.69 (ICSV 25019) to 20.59 (IS 18551) in the postrainy season. ICSV 25019 (-44.49** and -28.69**, respectively, in the rainy and postrainy seasons), PS 35805 (-42.96** and -27.69**) and Swarna (-16.10** and -3.36**) exhibited significant negative gca effects for plant height across seasons. ICSV 700, Phule Anuradha, M 35-1, CSV 15, IS 2123, IS 2146, and IS 18551 in the rainy season, and ICSV 700, Phule Anuradha, M 35-1, and IS 18551 in the postrainy season exhibited significant positive gca effects for plant height.

gca effects for 100 seed weight ranged from -0.31 (IS 18551) to 0.38 (Swarna) in the rainy and -0.43 (PS 35805) to 0.56 (Phule Anuradha) in the postrainy seasons. PS 35805 (-0.07**), IS 2123

TABLE 1 | Agronomic characteristics of sorghum genotypes (Parents and F₁'s) evaluated for resistance to sorghum shoot fly, *A. soccata* across seasons (ICRISAT, Patancheru, 2013–14).

Pedigree	Days to 50% flowering		Plant height (cm)		100 seed weight (g)		Grain yield (t/ha)		Agronomic score	
	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR
PARENTS										
ICSV 700	81	77	309	189	2.30	2.37	0.79	5.02	5.00	3.67
Phule Anuradha	62	63	259	179	2.90	4.23	1.22	6.51	4.67	4.33
M 35-1	75	72	306	187	2.40	3.50	0.66	6.87	5.00	3.67
CSV 15	71	61	254	180	2.50	3.03	1.54	5.23	3.00	3.00
ICSV 25019	65	64	131	109	2.30	2.10	1.90	3.01	2.00	3.00
PS 35805	69	65	121	102	2.20	2.37	1.70	3.12	2.00	3.00
IS 2123	73	72	283	176	2.13	2.50	0.77	5.64	5.00	4.33
IS 2146	68	73	279	181	1.80	2.33	0.88	5.56	5.00	4.67
IS 18551	78	78	313	203	1.70	2.23	0.51	4.23	5.00	3.33
Swarna	67	63	166	138	3.30	3.77	1.89	5.17	2.00	2.00
DIRECT CROSSES										
ICSV 700 X Phule Anuradha	65	75	314	236	2.73	3.77	3.58	15.18	4.33	2.67
ICSV 700 XM 35-1	65	70	321	218	2.63	4.00	3.73	12.51	3.67	3.33
ICSV 700 X CSV 15	75	71	327	209	2.37	3.30	4.73	14.18	2.33	4.00
ICSV 700 X ICSV 25019	67	68	308	199	2.80	3.63	4.80	12.72	3.00	3.00
ICSV 700 X PS 35805	67	67	309	204	2.47	3.07	6.25	12.13	3.33	3.67
ICSV 700 X IS 2123	68	69	303	219	2.50	3.40	4.12	13.73	4.67	4.33
ICSV 700 X IS 2146	69	71	318	208	2.53	3.53	2.57	5.49	4.00	3.67
ICSV 700 X IS 18551	67	70	339	231	2.40	3.30	5.31	11.87	3.67	3.33
ICSV 700 X Swarna	67	68	319	213	3.07	4.03	4.10	10.46	5.00	3.00
Phule Anuradha XM 35-1	64	66	273	202	2.60	4.37	2.93	11.99	4.00	3.33
Phule Anuradha X CSV 15	62	59	302	202	3.00	4.07	1.97	8.94	4.00	3.33
Phule Anuradha X ICSV 25019	61	60	280	183	2.87	3.97	2.58	10.69	3.67	2.67
Phule Anuradha X PS 35805	62	61	280	177	2.97	3.53	5.89	14.08	3.33	3.33
Phule Anuradha X IS 2123	63	64	280	188	2.60	3.83	3.40	12.40	4.33	4.00
Phule Anuradha X IS 2146	64	66	292	187	2.20	4.00	2.89	7.17	4.00	3.67
Phule Anuradha X IS 18551	65	65	310	208	2.27	3.47	4.68	12.70	3.33	4.00
Phule Anuradha X Swarna	63	62	297	199	3.43	4.33	4.76	9.44	3.33	3.33
M 35-1 X CSV 15	64	70	317	211	2.97	3.37	4.47	11.58	3.67	3.00
M 35-1 X ICSV 25019	63	63	304	203	2.77	3.83	3.34	13.94	3.33	3.33
M 35-1 X PS 35805	63	64	296	171	2.73	3.43	4.00	11.51	2.67	3.00
M 35-1 X IS 2123	66	70	296	198	2.57	3.50	3.84	14.34	4.67	4.33
M 35-1 X IS 2146	64	69	293	198	2.20	3.93	1.96	7.17	4.67	4.00
M 35-1 X IS 18551	67	70	322	211	2.37	3.17	4.69	14.47	3.67	3.00
M 35-1 X Swarna	69	67	314	208	2.37	3.13	4.49	11.56	3.33	3.33
CSV 15 X ICSV 25019	63	56	229	149	2.47	3.50	6.94	13.28	1.67	2.33
CSV 15 X PS 35805	65	57	250	158	2.50	3.33	7.16	12.62	2.00	2.67
CSV 15 X IS 2123	66	61	298	184	2.67	3.23	7.95	14.49	4.33	3.67
CSV 15 X IS 2146	64	63	290	197	2.77	3.30	2.88	7.34	3.33	3.67
CSV 15 X IS 18551	65	63	340	214	2.47	2.97	5.72	13.77	3.00	3.00
CSV 15 X Swarna	65	59	308	198	3.10	3.67	4.38	11.11	3.67	2.33
ICSV 25019 X PS 35805	66	60	127	104	1.97	2.43	4.33	7.57	2.00	3.00
ICSV 25019 X IS 2123	64	63	258	167	2.87	3.50	4.95	13.61	4.67	3.33
ICSV 25019 X IS 2146	63	64	273	173	2.80	3.70	2.41	6.99	4.33	3.67
ICSV 25019 X IS 18551	65	65	302	209	2.37	2.90	4.18	12.91	3.33	2.67
ICSV 25019 X Swarna	61	62	187	140	3.27	3.00	7.65	10.16	1.67	2.67

(Continued)

TABLE 1 | Continued

Pedigree	Days to 50% flowering		Plant height (cm)		100 seed weight (g)		Grain yield (t/ha)		Agronomic score	
	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR
PS 35805 X IS 2123	67	63	276	161	2.73	2.90	3.88	12.48	4.33	3.67
PS 35805 X IS 2146	63	61	261	180	2.53	3.20	2.14	7.20	3.67	3.33
PS 35805 X IS 18551	65	63	318	202	2.43	2.60	4.11	11.42	3.00	3.00
PS 35805 X Swarna	64	61	184	139	2.73	2.80	5.36	10.40	2.00	2.67
IS 2123 X IS 2146	68	71	292	196	1.97	3.10	2.01	6.51	4.67	4.33
IS 2123 X IS 18551	69	73	298	207	2.00	2.50	2.49	10.32	5.00	4.33
IS 2123 X Swarna	67	67	290	198	3.20	3.60	5.07	10.86	4.33	4.33
IS 2146 X IS 18551	67	72	290	211	2.10	3.03	2.23	8.33	4.67	4.67
IS 2146 X Swarna	63	62	297	210	2.63	3.87	3.13	5.40	4.33	3.67
IS 18551 X Swarna	68	65	327	222	2.90	3.50	5.06	12.10	3.67	3.00
RECIPROCAL CROSSES										
Phule Anuradha X ICSV 700	66	64	316	213	2.67	4.17	3.93	10.04	3.67	3.67
M 35-1 X ICSV 700	68	69	317	219	2.67	4.30	6.54	11.72	4.00	3.67
M 35-1 X Phule Anuradha	65	64	283	212	2.57	4.10	3.25	10.90	3.33	4.33
CSV 15 X ICSV 700	69	68	333	206	2.47	3.27	5.82	15.09	2.33	3.00
CSV 15 X Phule Anuradha	64	59	310	218	3.10	4.10	4.68	11.48	3.33	3.67
CSV 15 XM 35-1	63	64	302	212	2.90	4.13	4.14	14.25	3.67	3.67
ICSV 25019 X ICSV 700	68	67	311	198	2.57	3.47	5.02	12.99	3.00	3.00
ICSV 25019 X Phule Anuradha	61	59	282	187	3.30	4.13	5.97	11.13	3.00	2.67
ICSV 25019 XM 35-1	66	69	308	186	2.90	3.50	5.47	15.24	3.67	3.33
ICSV 25019 X CSV 15	64	59	230	158	2.47	3.00	7.46	11.77	2.00	2.67
PS 35805 X ICSV 700	71	66	309	203	2.53	3.07	6.29	12.33	2.67	3.33
PS 35805 X Phule Anuradha	63	68	288	211	2.93	3.27	4.20	17.28	3.33	3.33
PS 35805 XM 35-1	65	64	298	197	2.77	3.53	4.52	14.00	4.67	2.67
PS 35805 X CSV 15	65	57	238	152	2.40	2.90	6.74	10.79	2.83	3.00
PS 35805 X ICSV 25019	67	61	127	104	2.00	2.07	3.10	7.02	2.00	3.00
IS 2123 X ICSV 700	68	70	312	208	2.57	3.47	4.76	12.21	5.00	4.33
IS 2123 X Phule Anuradha	65	67	288	192	2.57	3.63	3.89	11.64	5.00	3.33
IS 2123 XM 35-1	69	68	312	197	2.37	3.50	2.90	13.93	4.33	4.33
IS 2123 X CSV 15	65	64	290	196	2.83	3.20	7.04	13.29	5.00	3.67
IS 2123 X ICSV 25019	67	64	269	161	2.77	3.20	5.09	13.91	4.33	3.67
IS 2123 X PS 35805	67	59	271	171	2.90	3.13	4.60	12.58	4.33	3.67
IS 2146 X ICSV 700	67	71	320	209	2.57	3.17	2.21	5.45	4.33	4.33
IS 2146 X Phule Anuradha	62	64	288	191	2.43	3.73	2.40	6.72	4.67	4.33
IS 2146 XM 35-1	66	68	293	199	2.20	3.70	2.76	7.08	5.00	4.67
IS 2146 X CSV 15	64	57	307	188	2.73	3.37	3.21	6.90	4.33	3.67
IS 2146 X ICSV 25019	64	64	270	166	2.37	3.43	1.96	6.93	4.50	4.67
IS 2146 X PS 35805	64	63	280	169	2.40	3.33	2.10	7.38	4.00	4.33
IS 2146 X IS 2123	68	69	300	199	1.90	3.30	2.32	6.62	4.67	4.67
IS 18551 X ICSV 700	71	71	343	211	2.13	3.20	4.82	10.72	4.00	3.33
IS 18551 X Phule Anuradha	65	65	308	209	2.30	3.43	4.84	14.73	3.67	3.67
IS 18551 XM 35-1	72	70	320	221	2.30	3.23	5.95	13.67	3.33	3.67
IS 18551 X CSV 15	67	64	336	228	2.47	2.97	6.16	12.98	3.33	3.00
IS 18551 X ICSV 25019	66	63	306	198	2.37	2.97	3.12	13.75	3.00	3.00
IS 18551 X PS 35805	69	64	308	207	2.23	2.77	4.28	13.02	3.00	3.00
IS 18551 X IS 2123	73	69	293	208	2.23	2.83	4.00	11.36	5.00	4.33
IS 18551 X IS 2146	68	71	320	197	1.87	2.93	2.35	5.85	5.00	4.33
Swarna X ICSV 700	68	66	318	226	2.90	4.20	5.82	8.62	3.33	3.00
Swarna X Phule Anuradha	61	58	313	204	3.30	4.60	4.73	9.44	3.67	3.33

(Continued)

TABLE 1 | Continued

Pedigree	Days to 50% flowering		Plant height (cm)		100 seed weight (g)		Grain yield (t/ha)		Agronomic score	
	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR
Swarna XM 35-1	64	62	316	193	3.13	4.47	4.36	10.39	3.33	3.00
Swarna X CSV 15	63	57	299	217	2.77	4.10	4.92	11.58	3.67	2.67
Swarna X ICSV 25019	61	59	187	142	2.83	2.90	7.38	9.46	2.00	2.67
Swarna X PS 35805	65	62	191	149	2.57	3.03	8.70	9.10	1.67	2.33
Swarna X IS 2123	64	63	294	177	2.90	3.73	4.94	10.79	4.33	4.00
Swarna X IS 2146	63	60	287	211	2.60	3.50	2.36	5.92	3.33	3.33
Swarna X IS 18551	65	67	330	230	2.83	3.40	5.27	11.69	3.67	3.00
Mean	66	65	286	191	2.60	3.38	4.10	10.43	3.69	3.46
SE ±	1.13	1.40	6.21	5.83	0.12	0.15	0.71	1.04	0.35	0.40
Vr (99, 198)	10.09**	10.90**	56.80**	22.91**	8.66**	12.87**	6.37**	9.65**	7.48**	2.41**
LSD (P 0.05)	3.14	3.90	17.33	16.26	0.34	0.42	1.97	2.90	0.97	1.12

**F probability significant at P 0.01; R, rainy season; PR, postrainy season.

(−0.06**), IS 2146 (−0.26**), and IS 18551 (−0.31**) in the rainy season, and ICSV 25019 (−0.22**), PS 35805 (−0.43**), IS 2123 (−0.16**), and IS 18551 (−0.40**) in the postrainy season exhibited significant negative *gca* effects for 100 seed weight. Whereas, the genotypes Phule Anuradha (0.20**), CSV 15 (0.09**), and Swarna (0.38**) in the rainy season, and ICSV 700 (0.07*), Phule Anuradha (0.56**), M 35-1 (0.33**), and Swarna (0.29**) in the postrainy season showed positive *gca* effects for 100 seed weight.

gca effects of sorghum grain yield ranged from −1.79 (IS 2146) to 0.90 (CSV 15) in the rainy season, and −3.86 (IS 2146) to 1.27 (M 35-1) in the postrainy season. The *gca* effects of Phule Anuradha (−0.43**), M 35-1 (−0.34*), and IS 2146 (−1.79**) in the rainy season, and IS 2146 (−3.86**) and Swarna (−0.99**) in the postrainy season exhibited significant negative *gca* effects for grain yield. The genotypes CSV 15 (0.90**), ICSV 25019 (0.40**), PS 35805 (0.48**), and Swarna (0.74**) in the rainy season and ICSV 700 (0.44*), Phule Anuradha (0.52*), M 35-1 (1.27**), CSV 15 (0.86**), IS 2123 (0.89**), and IS 18551 (0.77**) in the postrainy season showed significant positive *gca* effects for grain yield. *gca* effects of agronomic score ranged from −0.75 (PS 35805) to 0.96 (IS 2123) in the rainy season, and −0.48 (Swarna) to 0.66 (IS 2146) in the postrainy season.

***gca* Effects of Morphological Traits**

The *gca* effects of inflorescence exertion ranged from −0.63 (Swarna) to 0.57 (IS 2123) in the rainy season, and −1.06 (Swarna) to 1.51 (IS 2123) in the postrainy season (Table 7). *gca* effects of panicle compactness ranged from −0.25 (Swarna) to 0.62 (IS 2123) in the rainy season, and −0.50 (CSV 15) to 0.37 (IS 2123 and IS 2146) in the postrainy season. Six genotypes exhibited significant and negative *gca* effects and two genotypes exhibited positive and significant *gca* effects for panicle compactness in the rainy season. Three genotypes exhibited significant negative *gca* effects, while five genotypes exhibited significant positive *gca* effects for panicle compactness in the postrainy season.

gca effects of the panicle shape ranged from −0.94 (Swarna) to 0.58 (IS 2146) in the postrainy season. The general combining ability of glume cover ranged from −0.65 to 2.52 in the rainy season, and −0.51 to 2.89 in the postrainy season. All the genotypes exhibited significant negative *gca* effects except IS 18551 (2.52** and 2.89**) with significant positive *gca* effects for glume coverage across seasons. *gca* effects of awns ranged from −0.36 to 0.25 in the rainy season, and −0.35 to 0.25 in the postrainy season. CSV 15, ICSV 25019, PS 35805, and Swarna exhibited significant negative *gca* effects, while ICSV 700, Phule Anuradha, M 35-1, IS 2123, IS 2146, and IS 18551 exhibited significant positive *gca* effects for presence of awns across seasons.

Specific Combining Ability (*sca*) Effects

***sca* effects of agronomic traits**

The *sca* effects for days to 50% flowering ranged from −3.66 to 3.19 and −3.20 to 3.61 during the rainy and postrainy season, respectively. For plant height the *sca* effects ranged from −71.09 to 45.68 and −30.09 to 22.75, for 100 seed weight from −0.56 to 0.37 and −0.50 to 0.38, for grain yield from −1.24 to 2.65 and −3.24 to 4.70, respectively, in the rainy and postrainy seasons. For agronomic score from −1.02 to 0.91 in the rainy season (Table 8). Ten hybrids in the rainy season and nine hybrids in the postrainy season exhibited significant negative *sca* effects for days to 50% flowering. ICSV 700 X CSV 15 across seasons, and Phule Anuradha X PS 35805, M 35-1 X CSV 15 in the postrainy season showed significant positive *sca* effects for days to 50% flowering.

Significant negative *sca* effects for plant height were observed for 14 hybrids in the rainy season, and 10 hybrids in the postrainy season. Fifteen hybrids across seasons, Phule Anuradha X Swarna, ICSV 25019 X IS 2123, ICSV 25019 X IS 2146, PS 35805 X IS 2123, IS 2123 X Swarna in the rainy season, and M 35-1 X CSV 15 in the postrainy season exhibited significant positive *sca* effects for plant height.

Significant negative *sca* effects for grain yield were observed in Phule Anuradha X CSV 15, ICSV 25019 X PS 35805 across seasons, CSV 15 X Swarna in the rainy season, and ICSV 700

TABLE 2 | Panicle and grain characteristics of sorghum genotypes (Parents and F₁'s) evaluated for resistance to sorghum shoot fly, *A. soccata* across seasons (ICRISAT, Patancheru, 2013–14).

Pedigree	Inflorescence exertion		Panicle compactness		Panicle shape	Glume coverage		Awns		Grain luster
	2013 R	2013 PR	2013 R	2013 PR	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 PR
PARENTS										
ICSV 700	2.00	2.00	2.00	3.00	4.00	5.00	5.00	2.00	2.00	2.00
Phule Anuradha	2.33	3.00	2.00	3.00	4.00	3.00	1.00	2.00	2.00	2.00
M 35-1	2.00	2.00	2.00	3.00	4.00	3.00	1.00	2.00	2.00	2.00
CSV 15	2.00	1.00	2.00	2.00	2.00	1.00	1.00	1.00	1.00	2.00
ICSV 25019	2.67	2.00	3.00	3.00	4.00	1.00	1.00	1.00	1.00	2.00
PS 35805	3.00	2.00	3.00	3.00	4.00	1.00	1.00	1.00	1.00	2.00
IS 2123	2.33	4.00	3.00	3.00	3.00	3.00	1.00	2.00	2.00	2.00
IS 2146	2.00	4.00	3.00	3.00	3.00	3.00	1.00	2.00	2.00	2.00
IS 18551	2.00	2.00	2.00	3.00	4.00	9.00	9.00	2.00	2.00	1.00
Swarna	1.00	1.00	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
DIRECT CROSSES										
ICSV 700 x Phule Anuradha	2.00	2.67	2.00	3.00	4.00	1.67	1.00	2.00	2.00	2.00
ICSV 700 x M 35-1	2.00	2.33	2.00	3.00	4.00	1.67	1.00	2.00	2.00	2.00
ICSV 700 x CSV 15	1.67	1.33	2.00	2.00	1.33	1.00	1.00	1.00	1.00	2.00
ICSV 700 x ICSV 25019	2.00	1.67	2.00	2.33	2.00	1.00	1.00	1.00	1.00	2.00
ICSV 700 x PS 35805	2.00	1.00	2.00	3.00	4.00	1.67	1.00	1.00	1.00	2.00
ICSV 700 x IS 2123	2.00	4.00	3.00	3.00	3.00	1.67	1.00	2.00	2.00	2.00
ICSV 700 x IS 2146	2.00	3.00	2.67	3.00	3.33	2.33	1.00	2.00	2.00	2.00
ICSV 700 x IS 18551	1.33	2.33	2.00	3.00	4.00	5.67	4.33	2.00	2.00	1.67
ICSV 700 x Swarna	2.00	1.00	2.00	2.00	1.00	1.67	1.67	1.00	1.00	2.00
Phule Anuradha x M 35-1	2.00	3.00	2.00	2.67	3.00	1.00	1.00	2.00	2.00	2.00
Phule Anuradha x CSV 15	1.67	1.67	2.00	2.00	1.33	1.00	1.00	1.00	1.00	2.00
Phule Anuradha x ICSV 25019	2.00	2.33	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
Phule Anuradha x PS 35805	2.00	3.00	2.33	2.33	2.00	1.00	1.00	1.00	1.00	2.00
Phule Anuradha x IS 2123	2.67	4.00	3.00	3.00	3.00	1.67	1.00	2.00	2.00	2.00
Phule Anuradha x IS 2146	2.67	4.00	2.67	3.00	3.00	1.67	1.00	2.00	2.00	2.00
Phule Anuradha x IS 18551	2.00	2.33	2.00	3.00	4.00	3.00	5.00	2.00	2.00	2.00
Phule Anuradha x Swarna	1.67	1.33	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
M 35-1 x CSV 15	1.00	1.00	2.00	2.33	2.67	1.00	1.67	1.00	1.00	2.00
M 35-1 x ICSV 25019	1.00	1.00	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
M 35-1 x PS 35805	1.00	1.67	2.33	3.00	4.00	2.33	1.00	1.00	1.00	2.00
M 35-1 x IS 2123	2.00	4.00	2.67	3.00	3.00	1.67	1.00	2.00	2.00	2.00
M 35-1 x IS 2146	2.33	4.00	2.67	3.00	3.00	1.00	1.00	2.00	2.00	2.00
M 35-1 x IS 18551	1.67	2.33	2.00	3.00	4.00	5.00	4.33	2.00	2.00	2.00
M 35-1 x Swarna	2.00	1.33	2.00	3.00	4.00	3.00	1.00	1.00	1.00	2.00
CSV 15 x ICSV 25019	2.33	3.00	2.00	2.00	1.33	1.00	1.00	1.00	1.00	2.00
CSV 15 x PS 35805	2.33	1.67	2.00	2.00	1.33	1.00	1.00	1.00	1.00	2.00
CSV 15 x IS 2123	2.00	4.00	3.00	3.00	3.33	1.67	1.00	1.00	1.00	2.00
CSV 15 x IS 2146	2.00	2.33	2.67	3.00	3.67	1.00	1.00	1.00	1.00	2.00
CSV 15 x IS 18551	1.33	1.33	2.00	2.00	1.00	4.33	3.67	1.33	1.00	2.00
CSV 15 x Swarna	1.00	1.00	2.00	1.00	2.00	1.67	1.00	1.00	1.00	2.00
ICSV 25019 x PS 35805	3.00	3.00	2.33	3.00	4.00	1.00	1.00	1.00	1.00	2.00
ICSV 25019 x IS 2123	3.00	4.00	3.00	3.00	3.00	1.00	1.00	1.00	1.00	2.00
ICSV 25019 x IS 2146	2.33	4.00	2.67	3.00	3.67	1.00	1.00	1.00	1.00	2.00
ICSV 25019 x IS 18551	1.00	1.00	2.00	2.67	3.00	3.00	3.00	1.00	1.00	2.00
ICSV 25019 x Swarna	1.00	1.33	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
PS 35805 x IS 2123	2.33	4.00	3.00	3.00	3.00	2.33	1.00	1.00	1.00	2.00

(Continued)

TABLE 2 | Continued

Pedigree	Inflorescence exertion		Panicle compactness		Panicle shape	Glume coverage		Awns		Grain luster
	2013 R	2013 PR	2013 R	2013 PR	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 PR
PS 35805 x IS 2146	2.33	3.33	3.00	3.00	3.67	1.00	1.00	1.00	1.00	2.00
PS 35805 x IS 18551	1.33	1.67	2.00	2.00	1.00	3.67	3.67	1.00	1.00	1.67
PS 35805 x Swarna	1.00	1.00	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
IS 2123 x IS 2146	3.00	4.00	3.00	3.00	3.00	1.67	1.00	2.00	2.00	2.00
IS 2123 x IS 18551	2.67	4.00	3.00	3.00	3.00	4.33	4.33	2.00	2.00	2.00
IS 2123 x Swarna	2.00	4.00	2.67	3.00	3.67	1.00	1.00	1.00	1.00	2.00
IS 2146 x IS 18551	2.33	3.00	3.00	3.00	3.00	2.33	3.67	2.00	2.00	2.00
IS 2146 x Swarna	1.00	2.00	2.00	3.00	4.00	1.67	1.00	1.00	1.00	2.00
IS 18551 x Swarna	1.00	1.00	2.00	2.00	1.00	4.33	4.33	1.00	1.00	2.00
RECIPROCAL CROSSES										
Phule Anuradha x ICSV 700	2.00	3.00	2.00	3.00	4.00	1.67	1.00	2.00	2.00	2.00
M 35-1 x ICSV 700	1.67	2.67	2.00	3.00	4.00	1.67	1.00	2.00	2.00	2.00
M 35-1 x Phule Anuradha	2.33	2.00	2.00	2.33	2.00	1.00	1.00	2.00	1.67	1.67
CSV 15 x ICSV 700	1.67	1.33	2.00	2.33	2.33	1.67	1.00	1.00	1.00	2.00
CSV 15 x Phule Anuradha	1.33	1.33	2.00	2.00	1.67	1.00	1.00	1.00	1.00	2.00
CSV 15 x M 35-1	1.33	1.33	2.00	2.00	1.33	1.00	1.67	1.00	1.00	2.00
ICSV 25019 x ICSV 700	1.00	1.67	2.00	2.67	3.00	1.67	1.00	1.00	1.00	2.00
ICSV 25019 x Phule Anuradha	1.33	1.33	2.00	2.33	2.00	1.67	1.00	1.00	1.00	2.00
ICSV 25019 x M 35-1	1.33	1.67	2.00	2.67	3.00	1.67	1.00	1.00	1.00	2.00
ICSV 25019 x CSV 15	2.00	2.67	2.00	2.00	1.67	1.00	1.00	1.00	1.00	2.00
PS 35805 x ICSV 700	1.67	1.33	2.00	3.00	4.00	1.00	1.00	1.00	1.00	2.00
PS 35805 x Phule Anuradha	1.67	2.00	2.00	3.00	4.00	1.00	1.00	1.00	1.00	2.00
PS 35805 x M 35-1	1.00	1.33	2.00	3.00	4.00	1.67	1.00	1.00	1.00	2.00
PS 35805 x CSV 15	2.33	2.33	2.00	2.00	1.67	1.00	1.00	1.00	1.00	2.00
PS 35805 x ICSV 25019	3.00	3.00	2.00	3.00	4.00	1.00	1.00	1.00	1.00	2.00
IS 2123 x ICSV 700	2.67	4.00	3.00	3.00	3.00	2.33	1.00	2.00	2.00	2.00
IS 2123 x Phule Anuradha	2.67	4.00	3.00	3.00	3.00	1.67	1.00	2.00	2.00	2.00
IS 2123 x M 35-1	3.00	4.00	3.00	3.00	3.00	1.67	1.00	2.00	2.00	2.00
IS 2123 x CSV 15	2.67	4.00	2.67	3.00	3.00	1.67	1.00	1.00	1.00	2.00
IS 2123 x ICSV 25019	2.00	3.67	3.00	3.00	3.00	1.67	1.00	1.00	1.00	2.00
IS 2123 x PS 35805	2.67	3.67	3.00	3.00	3.00	1.00	1.00	1.00	1.00	2.00
IS 2146 x ICSV 700	2.33	3.67	2.67	3.00	3.33	1.67	1.00	2.00	2.00	2.00
IS 2146 x Phule Anuradha	2.67	4.00	3.00	3.00	3.00	1.67	1.00	2.00	1.67	2.00
IS 2146 x M 35-1	2.67	4.00	3.00	3.00	3.00	1.00	1.00	2.00	2.00	2.00
IS 2146 x CSV 15	1.00	3.33	2.33	3.00	4.00	1.00	1.67	1.00	1.00	2.00
IS 2146 x ICSV 25019	2.33	3.67	3.00	3.00	3.67	1.00	1.00	1.00	1.00	2.00
IS 2146 x PS 35805	2.67	3.67	3.00	3.00	3.33	1.00	1.00	1.00	1.00	2.00
IS 2146 x IS 2123	3.00	4.00	3.00	3.00	3.00	1.67	1.00	2.00	2.00	2.00
IS 18551 x ICSV 700	2.00	2.67	2.00	3.00	4.00	5.67	3.67	2.00	2.00	2.00
IS 18551 x Phule Anuradha	2.33	2.67	2.00	3.00	4.00	4.33	5.00	2.00	2.00	2.00
IS 18551 x M 35-1	2.00	2.33	2.00	3.00	4.00	5.00	5.00	2.00	2.00	2.00
IS 18551 x CSV 15	1.00	1.00	2.00	2.00	1.33	2.33	4.33	1.00	1.00	1.67
IS 18551 x ICSV 25019	1.33	1.00	2.00	2.00	1.00	3.67	3.00	1.00	1.00	2.00
IS 18551 x PS 35805	1.67	1.33	2.00	2.33	2.00	3.67	3.67	1.00	1.00	2.00
IS 18551 x IS 2123	3.00	4.00	3.00	3.00	3.00	4.33	4.33	2.00	2.00	2.00
IS 18551 x IS 2146	3.00	2.33	3.00	3.00	3.00	3.67	3.67	2.00	2.00	2.00
Swarna x ICSV 700	1.33	1.00	2.00	2.00	1.00	1.67	1.00	1.00	1.00	2.00
Swarna x Phule Anuradha	1.00	1.00	2.00	2.00	1.33	1.67	1.00	1.00	1.00	2.00
Swarna x M 35-1	1.33	1.00	2.00	2.33	1.00	1.00	1.00	1.00	1.00	2.00
Swarna x CSV 15	1.00	1.00	2.00	1.00	2.00	1.00	1.00	1.00	1.00	2.00

(Continued)

TABLE 2 | Continued

Pedigree	Inflorescence exertion		Panicle compactness		Panicle shape	Glume coverage		Awns		Grain luster
	2013 R	2013 PR	2013 R	2013 PR	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 PR
Swarna x ICSV 25019	1.00	1.00	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
Swarna x PS 35805	1.00	1.00	2.00	2.00	1.00	1.00	1.00	1.00	1.00	2.00
Swarna x IS 2123	1.67	3.00	3.00	3.00	3.33	1.00	1.00	1.00	1.00	2.00
Swarna x IS 2146	1.67	1.00	2.00	3.00	4.00	1.67	1.00	1.00	1.00	2.00
Swarna x IS 18551	1.00	1.00	2.00	2.00	1.00	4.33	5.00	1.00	1.00	2.00
Mean	1.90	2.41	2.30	2.63	2.75	2.00	1.71	1.40	1.35	1.98
SE ±	0.25	0.29	0.12	0.12	0.35	0.43	0.33	0.03	0.05	0.07
Vr	6.49**	15.17**	13.65**	18.62**	9.81**	10.81**	19.91**	208.27**	102.88**	3.18**
LSD (P 0.05)	0.69	0.81	0.33	0.32	0.99	1.20	0.92	0.09	0.13	0.19

*, **Correlation coefficient significant at P 0.05 and P 0.01, respectively; R, rainy season; PR, postrainy season.

TABLE 3 | Association of agronomic and panicle traits with expression of resistance to sorghum shoot fly, *Atherigona soccata* (ICRISAT, Patancheru, 2013–14).

Traits	Plants with shoot fly eggs (%)		Number of shoot fly eggs/plant	Shoot fly deadhearts (%)		ORS
	2013 R	2013 PR		2013 R	2013 PR	
Days to 50% flowering	-0.20* (-0.41**)		0.13 (-0.07)		-0.31** (-0.47**)	0.09 (-0.24**)
Plant height	0.13 (-0.01)		0.15 (-0.06)		0.35** (0.07)	-0.07 (-0.02)
100 seed weight	0.21* (0.28**)		-0.03 (-0.04)		0.39** (0.32**)	0.13 (0.15)
Grain yield	0.16* (0.03)		-0.01 (-0.17*)		0.24** (0.05)	0.05 (0.22*)
Agronomic score	-0.03 (-0.43**)		0.09 (-0.17*)		0.02 (-0.41**)	-0.10 (-0.38**)
Inflorescence exertion	-0.19* (-0.37**)		-0.06 (-0.14)		-0.37** (-0.41**)	-0.18* (-0.31**)
Panicle compactness	-0.13 (-0.55**)		-0.01 (-0.18*)		-0.30** (-0.60**)	-0.28** (-0.45**)
Panicle shape	(-0.48**)		(-0.10)		(-0.49**)	(-0.38**)
Glume coverage	-0.17* (-0.16*)		0.15 (0.08)		-0.18* (-0.44**)	-0.01 (-0.13)
Awns	-0.12 (-0.41**)		0.01 (-0.07)		-0.18* (-0.44**)	0.10 (-0.17*)

*, **Correlation coefficients significant at P 0.05 and P 0.01, respectively; ORS, overall resistance score; The values inside the parentheses are for postrainy season, whereas the values outside the parentheses are for rainy season.

TABLE 4 | Association between the agronomic traits in the postrainy season adapted sorghums (ICRISAT, Patancheru, 2013–14).

Traits	Agronomic score	Days to 50% flowering	Plant height	100 seed weight
Days to 50% flowering	0.24** (0.45**)	1.00		
Plant height	0.52** (0.24**)	0.22* (0.41**)	1.00	
100 seed weight	-0.17 (0.01)	-0.51** (-0.23**)	0.04 (0.43**)	1.00
Grain yield	-0.47** (-0.21*)	-0.26** (-0.08)	0.00 (0.36**)	0.36** (0.21*)

*, **Correlation coefficients significant at P 0.05 and P 0.01, respectively; The values inside the parentheses are for postrainy season, whereas the values outside the parentheses are for rainy season.

X IS 2146 in the postrainy season. Four hybrids across seasons, seven in the rainy season and eight hybrids in the postrainy season, exhibited significant and positive *sca* effects for grain yield. ICSV 700 X CSV 15, Phule Anuradha XM 35-1, Phule Anuradha X IS 18551, M 35-1 X IS 18551, CSV 15 X ICSV

TABLE 5 | Association between the panicle traits in the postrainy season sorghums (ICRISAT, Patancheru, 2013–14).

Traits	Inflorescence exertion	Panicle compactness	Panicle shape	Glume coverage	Awns
Panicle compactness	0.66** (0.66**)	1.00			
Panicle shape	(0.46**)	(0.86**)	1.00		
Glume coverage	-0.03 (-0.14)	-0.12 (0.05)	(0.07)	1.00	
Awns	0.45** (0.53**)	0.30** (0.52**)	(0.44**)	0.44** (0.29**)	1.00
Grain luster	(0.10)	(0.01)	(-0.03)	(-0.55**)	(-0.13)

*, **Correlation coefficient significant at P 0.05 and P 0.01, respectively; The values inside the parentheses are for postrainy season, whereas the values outside the parentheses are for rainy season.

25019, ICSV 25019 X Swarna, PS 35805 X Swarna, IS 2123 X IS 2146 exhibited significant negative *sca* effects while ICSV 700 X Swarna, M 35-1 X PS 35805, CSV 15 X IS 2123, CSV 15 X Swarna, ICSV 25019 X IS 2123, ICSV 25019 X IS 2146 exhibited

TABLE 6 | Analysis of variance (ANOVA) showing mean sum of squares of general, specific and reciprocal combining abilities of F₁(10 X 10) diallel across seasons (ICRISAT, Patancheru, 2013–14).

Source	GCA		SCA		Reciprocal		Error	
	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR
Days to 50% flowering	75.23**	168.15**	10.31**	8.40**	2.74**	4.92**	1.27	1.96
Plant height (cm)	14639.30**	5747.19**	1856.50**	494.31**	40.27	69.04**	38.62	33.99
100 seed weight (g)	0.81**	2.07**	0.10**	0.18**	0.02*	0.05**	0.02	0.02
Grain yield (t/ha)	11.80**	44.52**	3.94**	12.95**	0.70	1.08	0.50	1.08
Agronomic score	6.83**	3.06**	0.44**	0.15	0.17	0.11	0.12	0.16
Inflorescence exertion	2.14**	10.80**	0.34**	0.58**	0.11**	0.11	0.06	0.09
Panicle compactness	1.68**	1.74**	0.08**	0.17**	0.01	0.03**	0.01	0.01
Panicle shape	—	4.88**	—	1.40**	—	0.32**	—	0.13
Glume coverage	17.63**	21.08**	0.70**	0.49**	0.21	0.03	0.19	0.11
Awns	1.91**	1.85**	0.13**	0.13**	0.00	0.00	0.00	0.00
Grain luster	0.02**	0.04**	0.02	0.02**	0.00	0.01	0.00	0.00

*, **F probability significant at P 0.05 and P 0.01, respectively; GCA, general combining ability; SCA, specific combining ability; R, rainy season; PR, postrainy season.

TABLE 7 | Estimates of general combining ability of agronomic and panicle traits of parents (10 X 10 diallel) across seasons (ICRISAT, Patancheru, 2013–14).

Traits	ICSV 700	Phule Anuradha	M 35-1	CSV 15	ICSV 25019	PS 35805	IS 2123	IS 2146	IS 18551	Swarna
Days to 50% flowering	3.36** (4.40**)	-2.87** (-1.61**)	0.50* (2.30**)	-0.35 (-3.65**)	-1.85** (-2.58**)	-0.44 (-2.58**)	1.33** (1.70**)	-0.77** (1.44**)	2.46** (3.17**)	-1.37** (-2.60**)
Plant height (cm)	32.23** (19.42**)	5.59** (7.92**)	19.43** (10.53**)	5.19** (1.87)	-44.49** (-28.69**)	-42.96** (-27.69**)	3.87** (-2.13)	5.96** (1.53)	31.29** (20.59**)	-16.10** (-3.36**)
100 seed weight (g)	-0.02 (0.07*)	0.20** (0.56**)	0.01 (0.33**)	0.09** (0.01)	0.04 (-0.22**)	-0.07** (-0.43**)	-0.06* (-0.16**)	-0.26** (-0.05)	-0.31** (-0.40**)	0.38** (0.29**)
Grain yield (t/ha)	0.23 (0.44*)	-0.43** (0.52*)	-0.34* (1.27**)	0.90** (0.86**)	0.40** (0.07)	0.48** (0.03)	-0.14 (0.89*)	-1.79** (-3.86**)	-0.06 (0.77**)	0.74** (-0.99**)
Agronomic score	0.13 (0.04)	0.18* (0.11)	0.26** (0.11)	-0.46** (-0.31**)	-0.73** (-0.39**)	-0.75** (-0.31**)	0.96** (0.59*)	0.69** (0.66*)	0.18* (-0.01)	-0.47** (-0.48**)
Inflorescence exertion	-0.05 (-0.18**)	0.12* (0.17**)	-0.13* (-0.16*)	-0.23** (-0.53**)	0.00 (-0.16*)	0.13* (-0.21**)	0.57** (1.51**)	0.35** (0.96*)	-0.12* (-0.34**)	-0.63** (-1.06**)
Panicle compactness	-0.17** (0.13**)	-0.13** (0.00)	-0.15** (0.13**)	-0.20** (-0.50**)	-0.03 (-0.10*)	0.02 (0.05*)	0.62** (0.37*)	0.43** (0.37*)	-0.13** (0.02)	-0.25** (-0.47**)
Panicle shape	(0.41**)	(0.01)	(0.35**)	(-0.70**)	(-0.24**)	(0.20**)	(0.31**)	(0.58**)	(0.01)	(-0.94**)
Glume coverage	0.35** (0.03)	-0.28** (-0.31**)	-0.05 (-0.27**)	-0.65** (-0.31**)	-0.65** (-0.51**)	-0.55** (-0.44**)	-0.01 (-0.37**)	-0.31** (-0.41**)	2.52** (2.89**)	-0.38** (-0.31**)
Awns	0.24** (0.25**)	0.24** (0.21**)	0.24** (0.23**)	-0.35** (-0.35**)	-0.36** (-0.35**)	-0.36** (-0.35**)	0.24** (0.25**)	0.24** (0.23**)	0.25** (0.25**)	-0.36** (-0.35**)
Grain luster	(0.01)	(0.01)	(0.01)	(0.01)	(0.02)	(0.01)	(0.02)	(0.02)	(-0.13**)	(0.02)

*, **t-test significant at 0.05 and 0.01 probability levels; The values outside the parentheses are for rainy season and inside the parentheses are for postrainy season.

significant positive *sca* effects for agronomic score in the rainy season.

sca Effects of Morphological Traits

The *sca* effects of the inflorescence exertion ranged from -0.92 to 0.95 and -0.91 to 1.11, for panicle compactness from -0.52 to 0.37 and -0.67 to 0.50, for glume coverage -1.22 to 0.78 and -1.09 to 0.71, for awns from -0.25 to 0.36 and 0.25 to 0.35, respectively, in the rainy and postrainy seasons. For panicle shape it ranged from -1.46 to 1.60 and grain luster

from -0.16 to -0.01 in the postrainy season (Table 9). The genotypes with significant *gca* and/or *sca* for morphological traits can be utilized in developing sorghum cultivars for use by the farmers.

Reciprocal Combining Ability Effects of Agronomic and Morphological Traits

M 35-1 X ICSV 700, ICSV 25019 X M 35-1, IS 18551 X ICSV 700, IS 18551 X M 35-1, IS 18551 X PS 35805, IS 18551 X IS 2123 in the rainy season, PS 35805 X Phule Anuradha in the postrainy

TABLE 8 | Estimates of specific combining ability effects of agronomic traits of F₁ crosses (10 X 10 diallel) of sorghum across seasons (ICRISAT, Patancheru, 2013–14).

Pedigree	Days to 50% flowering		Plant height (cm)		100 seed weight (g)		Grain yield (t/ha)		Agronomic score
	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 R
ICSV 700 X Phule Anuradha	-0.80	1.30	-8.32*	6.19	-0.06	-0.05	-0.11	1.22	0.00
ICSV 700 XM 35-1	-3.66**	-2.62**	-18.26**	-2.53	0.08	0.37**	1.16*	-0.03	-0.25
ICSV 700 X CSV 15	3.19**	3.16**	7.10	-4.98	-0.23**	-0.18	0.05	2.90**	-1.02**
ICSV 700 X ICSV 25019	-0.15	0.10	36.21**	16.69**	0.09	0.32**	0.21	1.91**	-0.09
ICSV 700 X PS 35805	-0.06	-0.74	34.11**	21.24**	0.01	0.04	1.51**	1.33	-0.07
ICSV 700 X IS 2123	-2.83**	-2.02*	-13.80**	5.13	0.04	0.14	0.29	1.21	0.05
ICSV 700 X IS 2146	-0.90	0.25	-4.79	-3.53	0.25**	-0.06	-0.13	-1.55*	-0.34
ICSV 700 X IS 18551	-2.80**	-1.99*	-7.89	-9.81*	0.02	0.20*	0.83	-0.36	-0.16
ICSV 700 X Swarna	-0.46	-0.05	16.72**	12.47**	0.05	0.38**	-0.07	-0.34	0.82**
Phule Anuradha XM 35-1	0.57	-0.77	-32.19**	-2.14	-0.21**	-0.04	-0.24	-0.77	-0.46*
Phule Anuradha X CSV 15	0.09	-0.99	9.85*	9.30*	0.18*	0.13	-1.21*	-1.60*	0.26
Phule Anuradha X ICSV 25019	-0.41	-1.72	34.53**	14.86**	0.27**	0.32**	0.22	-0.11	0.20
Phule Anuradha X PS 35805	-0.16	3.61**	35.76**	22.75**	0.24**	-0.12	0.92*	4.70**	0.21
Phule Anuradha X IS 2123	-0.60	0.16	-11.09**	-6.70	-0.14	-0.06	0.12	0.19	-0.16
Phule Anuradha X IS 2146	0.50	0.10	-7.02	-11.48**	-0.21*	-0.04	0.79	-0.15	-0.22
Phule Anuradha X IS 18551	-1.06	-1.80	-13.49**	-11.09**	-0.19*	-0.10	1.18*	1.99**	-0.55*
Phule Anuradha X Swarna	-0.06	-1.20	29.75**	6.19	0.21*	0.23*	0.36	-0.52	0.10
M 35-1 X CSV 15	-2.95**	2.76**	-0.64	8.35*	0.25**	0.03	-0.31	0.35	0.18
M 35-1 X ICSV 25019	-0.45	0.70	45.68**	21.69**	0.21*	0.18	0.28	2.82**	0.28
M 35-1 X PS 35805	-2.20**	-0.97	35.01**	10.13**	0.23**	0.20*	0.04	1.03	0.46*
M 35-1 X IS 2123	-0.63	-0.09	-4.89	-2.09	-0.06	-0.05	-0.23	1.55*	-0.41
M 35-1 X IS 2146	-0.70	-0.15	-17.54**	-4.65	-0.13	0.15	0.41	-0.72	0.20
M 35-1 X IS 18551	0.74	-0.39	-15.10**	-5.92	0.05	-0.11	1.64**	1.60*	-0.63**
M 35-1 X Swarna	1.07	-0.29	26.18**	2.47	-0.22**	-0.20*	-0.04	0.27	-0.15
CSV 15 X ICSV 25019	-0.60	-1.19	-16.73**	-10.76**	-0.24**	0.08	1.83**	1.16	-0.66**
CSV 15 X PS 35805	-0.35	-1.69	-3.80	-10.09**	-0.15	0.15	1.50**	0.38	-0.06
CSV 15 X IS 2123	-1.61*	-0.64	-0.65	-0.64	0.14	-0.02	2.65**	1.71*	0.48*
CSV 15 X IS 2146	-1.01	-2.70**	1.70	-2.09	0.34**	-0.01	-0.13	-0.32	-0.08
CSV 15 X IS 18551	-1.91*	-1.27	15.80**	7.75*	0.10	-0.02	1.02*	1.31	-0.24
CSV 15 X Swarna	-0.41	-0.84	28.76**	17.80**	-0.12	0.21*	-1.06*	1.04	0.91**
ICSV 25019 X PS 35805	2.49**	0.75	-71.09**	-30.09**	-0.56**	-0.49**	-1.24**	-3.24**	-0.21
ICSV 25019 X IS 2123	-0.11	-0.70	18.73**	3.80	0.26**	0.34**	0.69	2.37**	0.58*
ICSV 25019 X IS 2146	-0.01	-0.44	24.70**	5.69	0.23**	0.45**	-0.51	0.31	0.77**
ICSV 25019 X IS 18551	-1.08	-1.84*	31.60**	20.52**	0.06	0.17	-0.75	2.05**	0.03
ICSV 25019 X Swarna	-1.91*	0.43	-38.22**	-17.76**	0.06	-0.50**	2.29**	0.29	-0.66**
PS 35805 X IS 2123	-0.36	-3.20**	26.92**	5.02	0.37**	0.22*	-0.17	1.19	0.43
PS 35805 X IS 2146	-1.60*	-1.94*	22.06**	9.69*	0.22**	0.36**	-0.65	0.69	0.20
PS 35805 X IS 18551	-1.16	-2.34*	38.97**	20.63**	0.13	0.13	-0.31	0.99	-0.12
PS 35805 X Swarna	0.00	1.26	-38.93**	-15.98**	-0.24**	-0.33**	1.73**	0.29	-0.64**
IS 2123 X IS 2146	1.30	1.78	0.80	6.91	-0.33**	0.02	0.02	-0.90	-0.67**
IS 2123 X IS 18551	0.74	1.21	-25.08**	-2.15	-0.10	-0.16	-0.65	-1.25	0.17
IS 2123 X Swarna	-0.43	0.48	18.95**	1.80	0.15	0.15	0.32	0.50	0.15
IS 2146 X IS 18551	0.00	1.65	-17.72**	-9.14*	-0.03	0.05	0.07	-0.26	0.28
IS 2146 X Swarna	-1.00	-3.09**	16.32**	21.47**	-0.08	0.06	-0.27	0.07	-0.07
IS 18551 X Swarna	-0.73	0.35	27.64**	17.97**	0.22**	0.18	0.40	1.68*	0.27

*, **t-test significant at P 0.05 and P 0.01 respectively; R, rainy season; PR, postrainy season.

season, and PS 35805 X Phule Anuradha across seasons, exhibited significant negative reciprocal effects for days to 50% flowering (**Table 10**). CSV 15 X ICSV 700 and Swarna X IS 18551 in the

rainy season, six hybrids in the postrainy season, and Swarna X M 35-1 and Swarna X IS 2123 across seasons exhibited significant positive reciprocal effects for days to 50% flowering. The crosses

TABLE 9 | Estimates of specific combining ability effects of panicle traits of F₁ crosses (10 X 10 diallel) of sorghum, across seasons (ICRISAT, Patancheru, 2013–14).

Pedigree	Inflorescence exertion		Panicle compactness		Panicle shape	Glume coverage		Awns		Grain luster
	2013 R	2013 PR	2013 R	2013 PR	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 PR
ICSV 700 X Phule Anuradha	0.02	0.43*	-0.03	0.23**	0.82**	-0.42	-0.43*	0.16**	0.19**	0.01
ICSV 700 XM 35-1	0.10	0.43*	-0.02	0.10	0.49*	-0.65*	-0.46*	0.16**	0.17**	0.01
ICSV 700 X CSV 15	0.03	-0.37	0.03	-0.10	-0.63**	-0.39	-0.43*	-0.25**	-0.25**	0.01
ICSV 700 X ICSV 25019	-0.37*	-0.41*	-0.13	-0.17*	-0.43	-0.39	-0.23	-0.24**	-0.25**	-0.01
ICSV 700 X PS 35805	-0.17	-0.86**	-0.18*	0.18*	0.64**	-0.49	-0.29	-0.24**	-0.25**	0.01
ICSV 700 X IS 2123	-0.10	0.26	0.22**	-0.13	-0.48*	-0.35	-0.36	0.16**	0.15**	-0.01
ICSV 700 X IS 2146	-0.05	0.14	0.07	-0.13	-0.41	-0.05	-0.33	0.16**	0.17**	-0.01
ICSV 700 X IS 18551	-0.08	0.61**	-0.03	0.22**	0.82**	0.78**	-0.63**	0.15**	0.15**	-0.02
ICSV 700 X Swarna	0.43**	-0.17	0.08	-0.30**	-1.23**	-0.32	-0.09	-0.24**	-0.25**	-0.01
Phule Anuradha XM 35-1	0.27	0.08	-0.05	-0.27**	-0.61**	-0.687*	-0.13	0.16**	0.04	-0.16**
Phule Anuradha X CSV 15	-0.30	-0.56**	0.00	-0.13	-0.56*	-0.09	-0.09	-0.25**	-0.21**	0.01
Phule Anuradha X ICSV 25019	-0.37*	-0.59**	-0.17*	-0.37**	-1.03**	0.25	0.11	-0.24**	-0.21**	-0.01
Phule Anuradha X PS 35805	-0.33*	0.13	-0.05	-0.02	0.04	-0.19	0.04	-0.24**	-0.21**	0.01
Phule Anuradha X IS 2123	0.07	-0.09	0.18*	0.00	-0.08	-0.05	-0.03	0.16**	0.19**	-0.01
Phule Anuradha X IS 2146	0.28	0.46*	0.20*	0.00	-0.35	0.25	0.01	0.16**	0.04	-0.01
Phule Anuradha X IS 18551	0.25	0.26	-0.07	0.35**	1.22**	-0.59*	0.71**	0.15**	0.19**	0.14**
Phule Anuradha X Swarna	-0.07	-0.36	0.05	-0.17*	-0.66**	-0.02	-0.09	-0.24**	-0.21**	-0.01
M 35-1 X CSV 15	-0.38*	-0.56**	0.02	-0.10	-0.40	-0.32	0.54*	-0.25**	-0.23**	0.01
M 35-1 X ICSV 25019	-0.62**	-0.76**	-0.15	-0.33**	-0.86**	0.01	0.07	-0.24**	-0.23**	-0.01
M 35-1 X PS 35805	-0.92**	-0.54**	-0.03	0.18*	0.70**	0.58*	0.01	-0.24**	-0.23**	0.01
M 35-1 X IS 2123	0.15	0.24	0.03	-0.13	-0.41	-0.29	-0.06	0.16**	0.17**	-0.01
M 35-1 X IS 2146	0.37*	0.79**	0.22**	-0.13	-0.68**	-0.65*	-0.03	0.16**	0.19**	-0.01
M 35-1 X IS 18551	0.17	0.43*	-0.05	0.22**	0.89**	0.51	0.34	0.15**	0.17**	0.14**
M 35-1 X Swarna	0.52**	-0.02	0.07	0.37**	0.34	0.41	-0.13	-0.24**	-0.23**	-0.01
CSV 15 X ICSV 25019	0.48**	1.11**	-0.10	-0.03	-0.31	0.28	0.11	0.35**	0.35**	-0.01
CSV 15 X PS 35805	0.52**	0.33	-0.15	-0.18*	-0.75**	0.18	0.04	0.35**	0.35**	0.01
CSV 15 X IS 2123	0.08	0.61**	0.08	0.50**	0.80**	0.31	-0.03	-0.25**	-0.25**	-0.01
CSV 15 X IS 2146	-0.53**	-0.01	-0.07	0.50**	1.20**	-0.05	0.34	-0.25**	-0.23**	-0.01
CSV 15 X IS 18551	-0.40*	-0.37	0.00	-0.15*	-0.89**	-0.55	-0.29	-0.10*	-0.25**	-0.02
CSV 15 X Swarna	-0.05	0.18	0.12	-0.67**	0.89**	0.35	-0.09	0.35**	0.35**	-0.01
ICSV 25019 X PS 35805	0.95**	0.96**	-0.15	0.42**	1.29**	0.18	0.24	0.36**	0.35**	-0.01
ICSV 25019 X IS 2123	0.02	0.08	0.08	0.10	0.17	-0.02	0.17	-0.24**	-0.25**	-0.02
ICSV 25019 X IS 2146	0.07	0.63**	0.10	0.10	0.57*	-0.05	0.21	-0.24**	-0.23**	-0.02
ICSV 25019 X IS 18551	-0.63**	-0.91**	-0.17*	-0.22**	-0.53*	-0.55	-1.09**	-0.25**	-0.25**	0.13**
ICSV 25019 X Swarna	-0.28	-0.02	-0.05	-0.07	-0.58*	0.01	0.11	0.36**	0.35**	-0.02
PS 35805 X IS 2123	-0.12	0.13	0.03	-0.05	-0.26	0.21	0.11	-0.24**	-0.25**	-0.01
PS 35805 X IS 2146	0.10	0.34	0.22**	-0.05	-0.03	-0.15	0.14	-0.24**	-0.23**	-0.01
PS 35805 X IS 18551	-0.43**	-0.36	-0.22**	-0.53**	-1.46**	-0.32	-0.49*	-0.25**	-0.25**	-0.02
PS 35805 X Swarna	-0.47*	-0.14	-0.10	-0.22**	-1.01**	-0.09	0.04	0.36**	0.35**	-0.01
IS 2123 X IS 2146	0.17	-0.87**	-0.38**	-0.37**	-0.65**	-0.02	0.07	0.16**	0.17**	-0.02
IS 2123 X IS 18551	0.47**	0.43*	0.18*	-0.02	-0.08	-0.19	0.11	0.15**	0.15**	0.13**
IS 2123 X Swarna	-0.02	0.64**	0.13	0.47**	1.37**	-0.62*	-0.03	-0.24**	-0.25**	-0.02
IS 2146 X IS 18551	0.52**	-0.36	0.37**	-0.02	-0.35	-1.22**	-0.53*	0.15**	0.17**	0.13**
IS 2146 X Swarna	-0.30	-0.81**	-0.52**	0.47**	1.60**	0.35	0.01	-0.24**	-0.23**	-0.02
IS 18551 X Swarna	-0.17	-0.01	0.05	-0.18*	-0.83**	0.18	0.37	-0.25**	-0.25**	0.13**

*, **Significant at P 0.05 and P 0.01 probability levels; R, rainy season; PR, postrainy season.

CSV 15 X Phule Anuradha, PS 35805 X Phule Anuradha, PS 35805 X ICSV 700, and Swarna X CSV 15 exhibited significant negative and the crosses Phule Anuradha X ICSV 700, ICSV

25019 XM 35-1, IS 18551 X ICSV 700, and Swarna X IS 2123 showed significant positive reciprocal effects for plant height in the postrainy season.

TABLE 10 | Estimates of reciprocal combining ability effects of reciprocal crosses (10 X 10 diallel) of sorghum across seasons (ICRISAT, Patancheru, 2013–14).

Pedigree	Days to 50% flowering		Plant height (cm)		100 seed weight (g)		Inflorescence exertion		Panicle compactness		Panicle shape	
	2013 R	2013 PR	2013 PR	2013 R	2013 PR	2013 R	2013 PR	2013 PR	2013 PR	2013 PR	2013 PR	2013 PR
Phule Anuradha X ICSV 700	-0.50	5.33**	11.11**	0.03	-0.20*	—	—	—	—	—	—	—
M 35-1 X ICSV 700	-1.70*	0.33	-0.56	-0.02	-0.15	0.17	—	—	—	—	—	—
M 35-1 X Phule Anuradha	-0.33	0.83	-5.00	0.02	0.13	-0.17	0.17*	0.17*	0.50*	—	—	—
CSV 15 X ICSV 700	3.00**	1.50	1.67	-0.05	0.02	—	-0.17*	-0.17*	-0.50*	—	—	—
CSV 15 X Phule Anuradha	-1.00	0.33	-7.78*	-0.05	-0.02	0.17	—	—	-0.17	—	—	—
CSV 15 XM 35-1	0.67	3.00**	-0.56	0.03	-0.38**	-0.17	0.17*	0.17*	0.67**	—	—	—
ICSV 25019 X ICSV 700	-0.83	0.50	0.56	0.12	0.08	0.50**	-0.17*	-0.17*	-0.50*	—	—	—
ICSV 25019 X Phule Anuradha	-0.33	0.33	-1.67	-0.20**	-0.08	0.30*	-0.17*	-0.17*	-0.50*	—	—	—
ICSV 25019 XM 35-1	-1.70*	-3.00**	8.89*	-0.07	0.17	-0.17	-0.33**	-0.33**	-1.00**	—	—	—
ICSV 25019 X CSV 15	-0.67	-1.50	-4.44	—	0.25*	0.17	—	—	-0.17	—	—	—
PS 35805 X ICSV 700	-2.00**	0.67	0.56	-0.03	—	0.17	—	—	—	—	—	—
PS 35805 X Phule Anuradha	-0.67	-3.67**	-17.22**	0.02	0.13	0.17	-0.33**	-0.33**	-1.00**	—	—	—
PS 35805 XM 35-1	-0.67	-0.33	-12.78**	-0.02	-0.05	—	—	—	—	—	—	—
PS 35805 X CSV 15	0.33	—	2.78	0.05	0.22*	—	—	—	—	—	-0.17	—
PS 35805 X ICSV 25019	-0.33	-0.50	—	-0.02	0.18	—	—	—	—	—	—	—
IS 2123 X ICSV 700	—	-0.67	5.56	-0.03	-0.03	-0.30*	—	—	—	—	—	—
IS 2123 X Phule Anuradha	-0.67	-1.17	-2.22	0.02	0.10	—	—	—	—	—	—	—
IS 2123 XM 35-1	-1.33	0.83	0.56	0.10	—	-0.50**	—	—	—	—	—	—
IS 2123 X CSV 15	0.50	-1.33	-5.56	-0.08	0.02	-0.30*	—	—	0.17	—	—	—
IS 2123 X ICSV 25019	-1.17	-0.67	2.78	0.05	0.15	0.50**	—	—	—	—	—	—
IS 2123 X PS 35805	—	1.83*	-5.00	-0.08	-0.12	-0.17	—	—	—	—	—	—
IS 2146 X ICSV 700	1.17	—	-0.56	-0.02	0.18	-0.17	—	—	—	—	—	—
IS 2146 X Phule Anuradha	1.00	0.83	-2.22	-0.12	0.13	—	—	—	—	—	—	—
IS 2146 XM 35-1	-1.17	0.50	-0.56	—	0.12	-0.17	—	—	—	—	—	—
IS 2146 X CSV 15	—	3.00**	4.45	0.02	-0.03	0.50**	—	—	-0.17	—	—	—
IS 2146 X ICSV 25019	-0.17	—	3.89	0.20**	0.13	—	—	—	—	—	—	—
IS 2146 X PS 35805	-0.67	-1.17	5.56	0.07	-0.07	-0.17	—	—	0.17	—	—	—
IS 2146 X IS 2123	—	0.83	-1.67	0.03	-0.10	—	—	—	—	—	—	—
IS 18551 X ICSV 700	-1.80*	-0.50	10.00*	0.13	0.05	-0.30*	—	—	—	—	—	—
IS 18551 X Phule Anuradha	—	—	-0.56	-0.02	0.02	-0.17	—	—	—	—	—	—
IS 18551 XM 35-1	-2.50**	—	-5.00	0.03	-0.03	-0.17	—	—	—	—	—	—
IS 18551 X CSV 15	-1.00	-0.17	-6.67	—	—	0.17	—	—	-0.17	—	—	—
IS 18551 X ICSV 25019	-0.33	1.00	5.56	—	-0.03	-0.17	0.33**	0.33**	1.00**	—	—	—
IS 18551 X PS 35805	-1.70*	-0.17	-2.22	0.10	-0.08	-0.17	-0.17*	-0.17*	-0.50*	—	—	—
IS 18551 X IS 2123	-2.00**	2.00*	-0.56	-0.12	-0.17	-0.17	—	—	—	—	—	—
IS 18551 X IS 2146	-0.50	0.50	7.22	0.12	0.05	-0.30*	—	—	—	—	—	—
Swarna X ICSV 700	-0.33	0.67	-6.11	0.08	-0.08	0.30*	—	—	—	—	—	—
Swarna X Phule Anuradha	0.83	1.83*	-2.78	0.07	-0.13	0.30*	—	—	-0.17	—	—	—
Swarna XM 35-1	2.30**	2.33*	7.22	-0.40**	-0.67**	0.30*	0.33**	0.33**	1.50**	—	—	—
Swarna X CSV 15	0.67	0.83	-9.45*	0.20*	-0.22*	—	—	—	—	—	—	—
Swarna X ICSV 25019	-0.33	1.17	-1.11	0.20**	0.05	—	—	—	—	—	—	—
Swarna X PS 35805	-0.33	-0.33	-5.00	0.08	-0.12	—	—	—	—	—	—	—
Swarna X IS 2123	1.70*	2.17*	10.56**	0.15	-0.07	0.17	—	—	0.17	—	—	—
Swarna X IS 2146	0.33	1.00	-0.55	0.02	0.18	-0.30*	—	—	—	—	—	—
Swarna X IS 18551	1.80*	-1.17	-3.89	0.03	0.05	—	—	—	—	—	—	—

*; **t-test significant at 0.05 and 0.01 probability levels; ORS, overall resistance score; R, rainy season; PR, postrainy season.

ICSV 25019 X Phule Anuradha in the rainy season and Phule Anuradha X ICSV 700, CSV 15 XM 35-1, Swarna X CSV 15 in the postrainy season, and Swarna X M 35-1 across seasons, exhibited

significant negative reciprocal effects for 100 seed weight. IS 2146 X ICSV 25019, Swarna X CSV 15, Swarna X ICSV 25019 in the rainy season and ICSV 25019 X CSV 15, PS 35805 X CSV 15

in the postrainy season exhibited significant positive reciprocal effects for 100 seed weight.

Combining Ability Estimates and Genetic Parameters

Variance due to GCA (σ^2_g) was higher than the variance due to SCA (σ^2_s) for glume cover across seasons (Table 11); agronomic score and waxy bloom in the rainy season and days to 50% flowering and inflorescence exertion in the postrainy season also showed high *gca* variance, indicating the predominance of additive gene action in controlling the expression of these traits. Plant height and grain yield exhibited higher σ^2_s than the σ^2_g across seasons; days to 50% flowering and inflorescence exertion in the rainy season, and panicle shape in the postrainy season showed high σ^2_s than the variance due to *gca*, indicating the predominance of non-additive type of gene action in controlling the expression of these traits. The other traits that had similar σ^2_g and σ^2_s exhibited both additive and non-additive type of gene action.

Glume cover showed greater additive (σ^2_a) than the dominance variance (σ^2_d) across seasons. Agronomic score, waxy bloom, and panicle compactness in the rainy season, and days to 50% flowering, plant height, 100 seed weight, and inflorescence exertion in the postrainy season showed higher additive variance than the dominance variance. Overall resistance score, grain yield, and plant color exhibited higher dominance variance than the additive variance across seasons. Days to 50% flowering, plant height, and inflorescence exertion in the rainy season and panicle shape in the postrainy season possessed higher dominance variance than the additive variance.

Glume cover and awns exhibited high GCA/SCA ratios across seasons. Agronomic score, waxy bloom, and panicle compactness in the rainy season, and days to 50% flowering, plant height, 100 seed weight, and inflorescence exertion in the postrainy season exhibited high GCA/SCA ratios, indicating the additive type of gene action controlling the expression of these traits. Panicle compactness, glume cover, and awns showed high predictability ratios across seasons. The predictability ratios for agronomic score, and waxy bloom in the rainy season, and days to 50% flowering, plant height, 100 seed weight, and inflorescence exertion in the postrainy season were quite high. Heritability estimates of the traits studied ranged from 0.10 to 0.71 (narrow-sense heritability), and 0.85 to 1.00 (broad-sense heritability) in the rainy season, and 0.17 to 0.82 (narrow-sense heritability), and 0.67 to 0.99 (broad-sense heritability) in the postrainy season. Almost all the traits exhibited moderate to high heritability values, except grain yield and grain luster across the seasons. Panicle shape in the postrainy season exhibited low (≤ 0.30) narrow-sense heritability.

DISCUSSION

Significance of *F*-values for all the traits studied indicated the presence of high variability in the parents used for developing the full diallel. Plant height, 100 seed weight and grain yield were associated with susceptibility to shoot fly. Days to 50% flowering,

agronomic score, inflorescence exertion, panicle compactness, panicle shape, glume coverage, and awns were associated with shoot fly resistance.

Association between the shoot fly resistance, morphological, and agronomic traits suggested complex interactions between shoot fly and plant traits. Significance of the GCA and SCA mean sum of squares for all the traits across seasons suggested that both the additive and non-additive nature of gene action for agronomic and panicle characteristics. The significance of reciprocal combining ability effects for days to 50% flowering, plant height, and 100 seed weight, suggesting possible role of cytoplasmic factors in inheritance of these traits. These interactions should be taken into consideration while developing strategies for improving sorghums for shoot fly resistance and high grain yield.

Genotypes with negative GCA effects for days to 50% flowering can be utilized to develop the hybrids with early flowering. To develop hybrids with moderate height, care should be taken to select the parents with moderate plant height. Additive gene action in the rainy season and dominance in the postrainy season for days to 50% flowering and plant height suggested G X E interactions for these traits. This contrasting gene expression in the rainy and postrainy seasons for days to 50% flowering and plant height suggested the season specific breeding of these traits for sorghum improvement. Meng et al. (1998), Rafiq et al. (2002), and Mohammed Maarouf (2009) reported additive gene action for days to 50% flowering, while Ereno (1998) reported additive gene action for plant height. Higher magnitude of SCA variance was reported by Manickam and Vijendra Das (1994) and Umakanth et al. (2002) for both the plant traits. High GCA/SCA and predictability ratios for 100 seed weight in the postrainy season indicated the predominance of additive gene action, whereas both additive and non-additive gene action was observed in the rainy season. Grain yield exhibited higher SCA variance suggesting the predominance of dominance (non-additive) type of gene action (Wilson et al., 1978; Singhania, 1980; Amsalu, 1987; Hovny et al., 2000; Umakanth et al., 2002; Girma et al., 2010). However, the importance of both the additive and non-additive gene action was observed for 100 seed weight by Toure et al. (1996).

Knowledge of the genetic architecture of grain yield, and morphological and agronomic traits will be useful for formulating a meaningful breeding strategy for developing improved genotypes. Genetic diversification of sorghum for key traits is important for sustaining the yield gains and mapping QTL underlying agronomically important traits is a key step in understanding their genetic control and for using the tightly linked markers for marker-assisted breeding for crop improvement (Srinivas et al., 2009; Ashok Kumar et al., 2011; Nagaraja Reddy et al., 2013, 2014). Many studies were conducted in identifying the QTL regions of different traits associated with insect resistance as well as the morphological and agronomic traits (Satish et al., 2009; Srinivas et al., 2009; Aruna et al., 2011b; Nagaraja Reddy et al., 2013, 2014). Based on the present inheritance studies of the agronomic and morphological traits and as well as the QTL information available one can effectively plan suitable breeding strategies for sorghum improvement.

TABLE 11 | Estimates of various genetic parameters for different agronomic and panicle traits of sorghum across seasons (ICRISAT, Patancheru, 2013–14).

Traits	Days to 50% flowering	Plant height (cm)	100 seed weight (g)	Grain yield (t/ha)	Agronomic score	Inflorescence exertion	Panicle compactness	Panicle shape	Glume coverage	Awns	Grain lustre
$\sigma^2 g$	3.70 (8.31)	730.03 (285.66)	0.04 (0.10)	0.57 (2.17)	0.34 (0.15)	0.10 (0.54)	0.08 (0.09)	(0.24)	0.87 0.87	0.10 0.10	—
$\sigma^2 s$	9.05 (6.44)	1817.89 (460.32)	0.08 (0.16)	3.44 (11.87)	0.32	0.27 (0.49)	0.07 (0.16)	(1.27)	0.51 (0.38)	0.12 (0.12)	0.02 (0.01)
$\sigma^2 r$	0.74 (1.48)	(17.53)	(0.02)	—	—	0.03 (0.09)	(0.01)	(0.1)	— (0.11)	— (0.19)	—
$\sigma^2 e$	1.27 (1.96)	38.62 (33.99)	0.02 (0.02)	0.50 (1.08)	0.12 (0.16)	0.06 (0.09)	0.01 (0.01)	(0.13)	0.19 (0.11)	— (0.19)	—
$\sigma^2 a$	7.40 (16.62)	1460.07 (571.32)	0.08 (0.20)	1.13 (4.34)	0.67 (0.29)	0.21 (1.07)	0.17 (0.17)	(0.48)	1.74 (2.10)	0.19 (0.19)	—
$\sigma^2 d$	9.05 (6.44)	1817.89 (460.32)	0.08 (0.16)	3.44 (11.87)	0.32	0.27 (0.49)	0.07 (0.16)	(1.27)	0.51 (0.38)	0.12 (0.12)	0.02 (0.01)
$\sigma^2 p$	18.45 (26.50)	3317.39 (1083.15)	0.18 (0.40)	5.17 (17.29)	1.14 (0.41)	0.57 (1.66)	0.25 (0.35)	(1.97)	2.46 (2.55)	0.32 (0.31)	0.02 (0.02)
h_{ns}^2	0.40 (0.63)	0.44 (0.53)	0.45 (0.51)	0.22 (0.25)	0.59 (0.71)	0.37 (0.65)	0.68 (0.50)	(0.24)	0.71 (0.82)	0.60 (0.60)	0.10 (0.17)
h_b^2	0.89 (0.87)	0.99 (0.95)	0.90 (0.91)	0.88 (0.94)	0.87 (0.67)	0.85 (0.94)	0.95 (0.94)	(0.89)	0.92 (0.97)	1.00 (0.99)	1.00 (0.78)
GCA/SCA ratio	0.41 (1.29)	0.40 (0.62)	0.49 (0.64)	0.16 (0.18)	1.04	0.38 (1.09)	1.26 (0.55)	(0.19)	1.70 (2.73)	0.77 (0.75)	0.06 (0.14)
Predictability ratio	0.45 (0.72)	0.45 (0.55)	0.5 (0.56)	0.25 (0.27)	0.68	0.43 (0.69)	0.72 (0.53)	(0.27)	0.77 (0.85)	0.61 (0.6)	0.10 (0.22)

$\sigma^2 g$, gca variance; $\sigma^2 s$, sca variance; $\sigma^2 r$, reciprocal variance; $\sigma^2 e$, environmental/error variance; $\sigma^2 a$, additive variance; $\sigma^2 d$, dominance variance; $\sigma^2 p$, phenotypic variance; h_{ns}^2 , narrow-sense heritability; h_b^2 , broad-sense heritability; GCA, general combining ability; SCA, specific combining ability; The values in the parentheses are for postrainy season and off the parentheses are for rainy season.

Most of the hybrids studied exhibited higher grain yield than the parents even if one of the parent was high yielding, suggesting that one of the parent should possess high grain yield ability for developing high yielding hybrids. This is very critical in sorghum improvement considering the fact that hybrids are preferred over the varieties worldover, barring Africa. Most of the commercial hybrids show 30–40% yield superiority over the best varieties in a given ecology. The panicle trait such as inflorescence exertion exhibited predominance of additive gene action in the postrainy season, and dominance gene action in the rainy season, while glume cover and presence of awns showed predominance of additive gene action.

The genotypes CSV 15, ICSV 25019, PS 35805, and Swarna exhibited negative gca effects for almost all the traits, but positive gca effects for grain yield. Hence, these genotypes can be effectively used in breeding the high yielding sorghums. The crosses involving the genotype IS 2146 either as male or female parent showed a decrease in grain yield being a poor combiner coupled with low *per se* mean yield. Phule Anuradha and M 35-1 showed positive gca effects for 100 seed weight but negative gca effects for grain yield in the rainy season, and positive effects in the postrainy season, suggesting that these genotypes can be effectively utilized for breeding high yielding shoot fly resistant sorghums for the postrainy season. Both M 35-1 and Phule Anuradha are highly adapted to postrainy environments, and are very popular with farmers. ICSV 25019, PS 35805, IS 2123, and IS 18551 exhibited negative gca effects for 100 seed weight, but showed positive gca effects for grain yield. Hence, these genotypes can be utilized for breeding high yielding sorghums

with shoot fly resistance. Though the genotypes CSV 15 and Swarna showed positive gca effects for 100 seed weight and grain yield, but these may not be good parents in a shoot fly resistance breeding program. ICSV 700, IS 2123, and IS 18551 showed positive gca effects for most of the traits and these can be utilized for improving shoot fly resistance. Higher narrow- and broad-sense heritability estimates suggested high heritability of these traits across environments, and greater role of additive gene action, suggesting that selection is effective for improving these traits. This information can be used for developing high yielding cultivars with insect resistance for sustainable crop production.

CONCLUSIONS

Genotypic response varies across seasons, and hence, it is important to identify genotypes with desirable agronomic traits, insect resistance, and high grain yield for different seasons and locations. The genotypes ICSV 700, Phule Anuradha, M 35-1, ICSV 25019, PS 35805, IS 2123, and IS 18551 exhibiting moderate to high shoot fly resistance and desirable agronomic traits, can be effectively used in sorghum improvement. Both additive and non-additive type of gene action governs the morphological (inflorescence exertion, panicle compactness, and panicle shape) and agronomic (days to 50% flowering, plant height and 100 seed weight) traits and hence it is important to exploit heterosis breeding for improving agronomic and morphological traits and grain yield in sorghum. The significance of reciprocal effects for some of the traits (days to 50% flowering, plant height, and 100 seed weight) suggested that apart from the direct genetic

effects, the cytoplasmic factors also played an important role in inheritance of these traits. An understanding of the association between shoot fly resistance and morphological and agronomic traits will be useful to improve the strategies to develop shoot fly-resistant cultivars with desirable plant types, and season specific adaptation for sustainable crop production.

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REFERENCES

- Amsalu, A. (1987). *Heterosis and Combining Ability Studies in Grain Sorghum [Sorghum bicolor (L.) Moench]*. M.Sc. thesis, Mahatma Phule Agricultural University, Maharashtra.
- Aruna, C., Bhagwat, V. R., Madhusudhana, R., Vittal, S., Hussain, T., Ghorade, R. B., et al. (2011b). Identification and validation of genomic regions that affect shoot fly resistance in sorghum [Sorghum bicolor (L.) Moench]. *Theor. Appl. Genet.* 122, 1617–1630. doi: 10.1007/s00122-011-1559-y
- Aruna, C., Bhagwat, V. R., Vittal, S., Hussain, T., Ghorade, R. B., Khandalkar, H. G., et al. (2011a). Genotype X Environment interactions for shoot fly resistance in sorghum [Sorghum bicolor (L.) Moench]: response of recombinant inbred lines. *Crop Prot.* 30, 623–630. doi: 10.1016/j.cropro.2011.02.007
- Ashok Kumar, A., Reddy, B. V. S., Sharma, H. C., Hash, C. T., Srinivasa Rao, P., Ramaiah, B., et al. (2011). Recent advances in sorghum genetic enhancement research at ICRISAT. *Am. J. Plant Sci.* 2, 589–600. doi: 10.4236/ajps.2011.24070
- Baker, R. J. (1978). Issues in diallel analysis. *Crop Sci.* 18, 533–536. doi: 10.2135/cropsci1978.0011183X001800040001x
- Melake-Berhan, A., Butler, L. G., Ejeta, G., and Menkir, A. (1996). Grain mold resistance and polyphenol accumulation in sorghum. *J. Agr. Food Chem.* 44, 2428–2434. doi: 10.1021/jf950580x
- Dhillon, M. K., Sharma, H. C., Folkertsma, R. T., and Chandra, S. (2006). Genetic divergence and molecular characterisation of sorghum hybrids and their parents for reaction to *Atherigona soccata* (Rondani). *Euphytica* 149, 199–210. doi: 10.1007/s10681-005-9067-2
- Ereno, D. (1998). *Study of Combining Ability in Lowland Grain Sorghum [Sorghum bicolor (L.) Moench]*. M.Sc. thesis, Alemaya University, Ethiopia.
- FAO (2004). *Production Yearbook*. Rome: FAO.
- FAOSTAT (2012). *Crops Primary Equivalent*. Available online at: <http://faostat.fao.org>. (Retrieved 15th March, 2015).
- GenStat (2010). *Introduction to GenStat for Windows Genstat, 13th Edn*. Harpenden: Lawes Agricultural Trust, Rothamsted Experimental Station.
- Girma, M., Amsalu, A., and Ketema, B. (2010). Combining ability for yield and its components in ethiopian sorghum [Sorghum bicolor (L.) Moench] landraces. *East Afr. J. Sci.* 4, 34–40. doi: 10.4314/eajsci.v4i1.7152
- Griffing, B. (1956). Concept of general and specific combining ability in relation to diallel crossing systems. *Aust. J. Biol. Sci.* 9, 463–493.
- Hovny, M. R. A., El-Naggouly, O. O., and Hassaballa, E. A. (2000). Combining ability and heterosis in grain sorghum [Sorghum bicolor (L.) Moench]. *Assiut J. Agr. Sci.* 31, 1–16.
- IBPGR and ICRISAT (1993). *Descriptors for Sorghum [Sorghum bicolor (L.) Moench]*. International Board for Plant Genetic Resources. Rome: International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru.
- Indostat Services (2004). *Windostat*. Hyderabad: Indostat Services.
- Jayanthi, P. D. K., Reddy, B. V. S., Reddy, D. D. R., Gour, T. B., and Nwanze, K. F. (1996). “Genetics of shoot fly resistance in sorghum hybrids of cytoplasmic male sterile lines,” in *Abstracts of Poster Sessions: 2nd International Crop Science Congress: Crop Productivity and Sustainability—Shaping the Future* (New Delhi: National Academy of Agricultural Sciences, Indian Council of Agricultural Research), 152.
- Johnson, H. W., Robinson, H. F., and Comstock, R. E. (1955). Estimates of genetic and environmental variability in soybean. *Agron. J.* 47, 314–318. doi: 10.2134/agronj1955.00021962004700070009x
- Kullaiswamy, B. Y., and Goud, J. V. (1983). New genes for awning in sorghum [Sorghum bicolor (L.) Moench]. *Madras Agr. J.* 70, 355–359.
- Manickam, S., and Vijendra Das, L. D. (1994). Line X tester analysis in forage sorghum. *Int. Sorghum Millet Newslett.* 35, 79–80.
- Meng, C. G., An, X. M., Zhang, F. Y., Zheng, J. B., Wang, L. X., and Li, P. L. (1998). Analysis of combining ability of newly developed sorghum male sterile lines. *Acta Agr. Boreali-Sinica* 13, 81–85.
- Mohammed Maarouf, I. (2009). Line X tester analysis across locations and years in Sudanese x exotic lines of forage sorghum. *J. Plant Breed. Crop Sci.* 1, 311–319. Available online at: http://www.academicjournals.org/article/article1379424511_Mohammed.pdf
- Murty, D. S. (2000). “Breeding for grain mold resistance in sorghum: opportunities and limitations,” in *Technical and Institutional Options for Sorghum Grain Mold Management: Proceedings of an International Consultation, 18–19 May 2000*, eds A. Chandrashekhar, R. Bandyopadhyay and A. J. Hall (Patancheru: International Crops Research Institute for the Semi-Arid Tropics (ICRISAT)), 225–227.
- Nagaraja Reddy, R., Madhusudhana, R., Murali Mohan, S., Chakravarthi, D. V. N., Mehtre, S. P., Seetharama, N., et al. (2013). Mapping QTL for grain yield and other agronomic traits in post-rainy sorghum [Sorghum bicolor (L.) Moench]. *Theor. Appl. Genet.* 126, 1921–1939. doi: 10.1007/s00122-013-2107-8
- Nagaraja Reddy, R., Madhusudhana, R., Murali Mohan, S., Seetharama, N., and Jagannatha Vishnu, P. (2014). Detection and validation of stay-green QTL in post-rainy sorghum involving widely adapted cultivar, M35-1 and a popular stay-green genotype B35. *BMC Genomics* 15:909. doi: 10.1186/1471-2164-15-909
- Nimbalkar, V. S., and Bapat, D. R. (1992). Inheritance of shoot fly resistance in sorghum. *J. Maharashtra Agric. Univ.* 17, 93–96.
- Porter, K. S., Axtell, J. D., Lechtenberg, V. L., and Colenbrander, V. F. (1978). Phenotype, fiber composition, and *in vitro* dry matter disappearance of chemically induced brown midrib (bmr) mutants of sorghum. *Crop Sci.* 18, 205–208. doi: 10.2135/cropsci1978.0011183X001800020002x
- Rafiq, S. M., Thete, R. Y., Madhusudhana, R., and Umakanth, A. U. (2002). Combining ability studies for grain yield and its components in postrainy season sorghum grown in medium-deep and shallow soils. *Int. Sorghum Millets Newslett.* 43, 33–37. Available online at: http://oar.icrisat.org/1106/1/RA_00378.pdf
- Riyazaddin, M. D., Kavi Kishor, P. B., Ashok Kumar, A., Belum Reddy, V. S., Rajendra, S. M., and Sharma, H. C. (2015). Mechanisms and diversity of resistance to sorghum shoot fly, *Atherigona soccata*. *Plant Breed.* 134, 423–436. doi: 10.1111/pbr.12276
- Satish, K., Srinivas, G., Madhusudhana, R., Padmaja, P. G., Nagaraja Reddy, R., Murali Mohan, S., et al. (2009). Identification of quantitative trait loci for resistance to shoot fly in sorghum [Sorghum bicolor (L.) Moench]. *Theor. Appl. Genet.* 119, 1425–1439. doi: 10.1007/s00122-009-1145-8
- Sharma, H. C. (1993). Host-Plant Resistance to insects in sorghum and its role in integrated pest management. *Crop Prot.* 12, 11–34. doi: 10.1016/0261-2194(93)90015-B

- Sharma, H. C., Reddy, B. V. S., Dhillon, M. K., Venkateswaran, K., Singh, B. U., Pampapathy, G., et al. (2005). Host plant resistance to insects in sorghum: present status and need for future research. *Int. Sorghum Millets Newslett.* 46, 36–43. Available online at: http://oar.icrisat.org/1215/1/ISMN-46_36-43_2005.pdf
- Sharma, H. C., Taneja, S. L., Kameswara Rao, N., and Prasada Rao, K. E. (2003). *Evaluation of Sorghum Germplasm for Resistance to Insect Pests. Information Bulletin no. 63.* Patancheru: International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).
- Singhania, D. L. (1980). Heterosis and combining ability studies in grain sorghum. *Indian J. Genet.* 40, 463–471.
- Srinivas, G., Satish, K., Madhusudhana, R., Nagaraja Reddy, R., Murali Mohan, S., and Seetharama, N. (2009). Identification of quantitative trait loci for agronomically important traits and their association with genic-microsatellite markers in sorghum. *Theor. Appl. Genet.* 118, 1439–1454. doi: 10.1007/s00122-009-0993-6
- Toure, A., Miller, F. R., and Rosenow, D. T. (1996). Heterosis and combining ability for grain yield and yield components in guinea sorghums. *J. Afr. Crop Sci.* 4, 383–391.
- Umakanth, A. V., Madhusudhana, K., Madhavi Latha, P., Hema, K., and Kaul, S. (2002). Genetic architecture of yield and its contributing characters in postrainy season sorghum. *Int. Sorghum Millets Newslett.* 43, 37–40. Available online at: http://oar.icrisat.org/1106/1/RA_00378.pdf
- Wilson, N. D., Weibel, D. E., and McNew, R. W. (1978). Diallel analyses of grain yield, percent protein, and protein yield in grain sorghum. *Crop Sci.* 18, 491–495.

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Plant Genetic Background Increasing the Efficiency and Durability of Major Resistance Genes to Root-knot Nematodes Can Be Resolved into a Few Resistance QTLs

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With the banning of most chemical nematicides, the control of root-knot nematodes (RKNs) in vegetable crops is now based essentially on the deployment of single, major resistance genes (*R*-genes). However, these genes are rare and their efficacy is threatened by the capacity of RKNs to adapt. In pepper, several dominant *R*-genes are effective against RKNs, and their efficacy and durability have been shown to be greater in a partially resistant genetic background. However, the genetic determinants of this partial resistance were unknown. Here, a quantitative trait loci (QTL) analysis was performed on the F_{2:3} population from the cross between Yolo Wonder, an accession considered partially resistant or resistant, depending on the RKN species, and Doux Long des Landes, a susceptible cultivar. A genetic linkage map was constructed from 130 F₂ individuals, and the 130 F₃ families were tested for resistance to the three main RKN species, *Meloidogyne incognita*, *M. arenaria*, and *M. javanica*. For the first time in the pepper-RKN pathosystem, four major QTLs were identified and mapped to two clusters. The cluster on chromosome P1 includes three tightly linked QTLs with specific effects against individual RKN species. The fourth QTL, providing specific resistance to *M. javanica*, mapped to pepper chromosome P9, which is known to carry multiple NBS-LRR repeats, together with major *R*-genes for resistance to nematodes and other pathogens. The newly discovered cluster on chromosome P1 has a broad spectrum of action with major additive effects on resistance. These data highlight the role of host QTLs involved in plant-RKN interactions and provide innovative potential for the breeding of new pepper cultivars or rootstocks combining quantitative resistance and major *R*-genes, to increase both the efficacy and durability of RKN control by resistance genes.

Keywords: *Capsicum annuum*, *Meloidogyne* spp., quantitative resistance, major resistance, resistance durability

INTRODUCTION

Root-knot nematodes (RKNs), *Meloidogyne* spp., are major plant pathogens worldwide. These extremely polyphagous endoparasites can infest more than 5,500 plant species, including many field and greenhouse crops (Goodey et al., 1965). Since the banning of most chemical nematicides, due to environmental and public health issues, resistant cultivars have become an increasing important weapon against these pests. This method efficiently controls RKN populations, and is economically sustainable, innocuous to health and environment-friendly. RKN resistance is generally mediated by single, major resistance genes (*R*-genes), which can easily be introgressed into cultivars through back-crossing and phenotypic or marker-assisted selection (MAS). For this reason, major *R*-genes are widely used in the breeding of RKN-resistant cultivars and/or rootstocks. However, their efficacy is threatened by the capacity of RKNs to adapt. Indeed, *R*-genes apply a selective pressure on nematode populations, increasing the risk of virulent nematode populations emerging (Castagnone-Sereno, 2006), and this greatly limits their use. Several management strategies have been developed, to prevent the breakdown of resistance by pathogens. Most of these approaches are based on spatiotemporal management of the deployment of *R*-genes: (i) alternation of different *R*-genes in the crop rotation, (ii) use of mixtures of cultivars with different *R*-genes, or (iii) pyramiding, the introduction of several *R*-genes into the same cultivar (Kiyosawa, 1982; Mundt, 2002; Pink, 2002). The use of such strategies requires several *R*-genes to be available, with no emergence of cross-virulent pathogens. Recent experimental studies have shown that the pyramiding of *R*-genes is the best method for promoting effective, durable RKN resistance in pepper (Djian-Caporalino et al., 2014).

Several recent studies, on very different pathosystems, have shown that the genetic background of the plant greatly influences *R*-gene efficiency, potentially slowing the adaptation of pathogen populations to *R*-gene-carrying cultivars (Palloix et al., 2009; Brun et al., 2010; Fournet et al., 2013; Barbary et al., 2014). In some pathosystems, this greater durability has been shown to result from quantitative trait loci (QTLs), which slow the selection of variants virulent against the *R*-gene and decrease the size of the pathogen population (e.g., Quenouille et al., 2014). Plant genetic background is rarely considered in breeding programs for RKN resistance, despite its contribution to *R*-gene efficiency and durability. Indeed, breeding for resistance with QTLs is more complex and costly than the use of *R*-genes. In particular, the introgression of QTLs into elite cultivars must not impair other agronomically important crop traits, such as yield, quality criteria and adaptation, or other physiological characteristics.

In pepper (*Capsicum annuum* L.), several dominant *R*-genes, the *Me* genes and the *N* gene, have been characterized in detail (Hare, 1956; Hendy et al., 1985; Djian-Caporalino et al., 1999; Thies and Fery, 2000). These genes map to a genetic cluster on pepper chromosome P9 (Djian-Caporalino et al., 2007; Fazari et al., 2012). Three of these genes, *Me3*, *Me1*, and *N*, are routinely used in breeding programs. These genes are effective against a wide range of RKN species, including *M. arenaria*,

M. incognita, and *M. javanica*, the most common species in temperate and tropical areas. They differ in their mode of action: *Me3* and *Me1* are stable at high temperature (Djian-Caporalino et al., 1999), whereas the efficacy of *N* is temperature-dependent (Thies and Fery, 1998). In addition, *Me3* and *Me1* differ in the spatiotemporal location of the resistance response triggered by a nematode attack. *Me3* triggers an early hypersensitive response in the root epidermis at the nematode penetration site, whereas *Me1* triggers a later response in the root vascular cylinder (Bleve-Zacheo et al., 1998; Pegard et al., 2005). It was long thought that there was a relationship between the mode of action of these *R*-genes and their durability, as the emergence of virulent populations has been reported only for *N* and *Me3* (Castagnone-Sereno et al., 1996; Thies, 2011). However, the risk of *Me1*-virulent RKN populations emerging and of the development of multi-virulent populations might increase with the extensive deployment of these resistance genes in agriculture. The efficacy of these genes has been shown to be higher when they are introgressed into a partially resistant background and lower if they are introgressed into a highly susceptible background (Barbary et al., 2014). These same genetic backgrounds were also previously shown to affect the durability of field resistance (Djian-Caporalino et al., 2014). However, no quantitative resistance loci that could be combined with major genes to increase the efficacy and durability of genetic RKN control have yet been identified in the pepper germplasm.

We report here a QTL analysis dissecting the genetic backgrounds previously shown to modulate the efficacy and durability of resistance. An F_{2:3} progeny derived from a cross between a partially resistant (Yolo Wonder, YW) and a highly susceptible (Doux Long des Landes, DLL) pepper inbred line was tested for quantitative resistance to the three main RKN species (*M. incognita*, *M. arenaria*, and *M. javanica*). Four new major QTLs were mapped to two separate clusters. The first, containing one QTL, colocalized with the cluster of *Me* genes on pepper chromosome P9. The second included three QTLs against the three *Meloidogyne* species located on pepper chromosome P1. This new cluster could potentially be used for innovative breeding strategies to increase *R*-gene efficacy and durability for the control of RKNs.

MATERIALS AND METHODS

Plant Material

A population of 130 F_{2:3} families derived from a cross between YW and DLL was used. These pepper cultivars were selected from the INRA pepper germplasm collection at Avignon, France (CRB-Leg, INRA-GAFL), on the basis of their different levels of resistance to nematode species. DLL is highly susceptible to the three main RKN species: *M. arenaria*, *M. javanica*, and *M. incognita*. YW is partially resistant (i.e., low-level symptoms) to *M. incognita* (Figure 1), totally resistant to *M. javanica* and has variable levels of resistance to *M. arenaria* (i.e., totally or partially resistant), depending on the RKN isolate considered (Djian-Caporalino et al., 1999). A single F₁ hybrid plant was self-pollinated

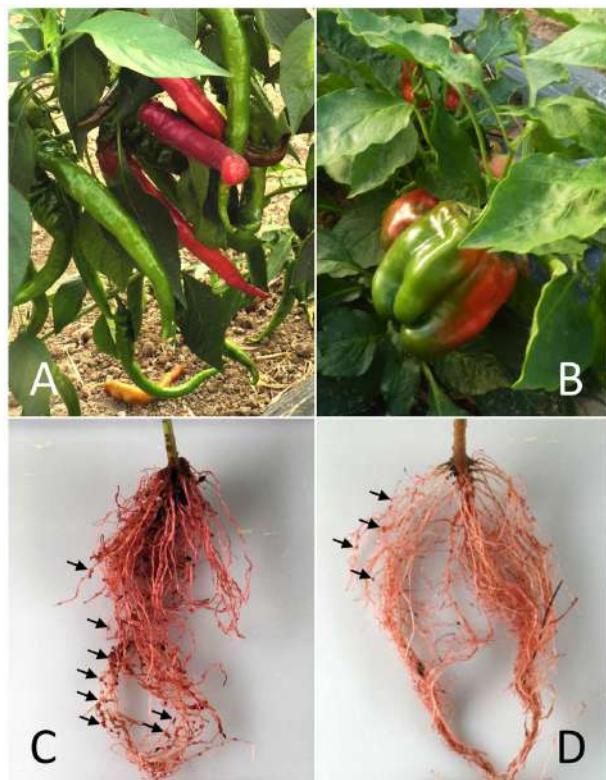


FIGURE 1 | The pepper/*Meloidogyne incognita* pathosystem used in this study. The susceptible and resistant pepper cultivars Doux Long des Landes (DLL) (A) and Yolo Wonder (YW) (B) and their respective root systems (C,D) 6 weeks after inoculation with the nematodes. Egg masses (EMs; arrows) were stained with cold eosin (red).

to generate 130 F₂ plants, which were used to construct the genetic map. The 130 F₂ plants were self-pollinated to generate 130 F₃ families, which were used to assess disease resistance.

Nematode Isolates

Three RKN species were used for resistance tests in controlled conditions. The first, *M. incognita* (Morelos isolate), causes disease on DLL, which is susceptible, whereas YW is partially resistant. The other two species used were *M. arenaria* (Marmande isolate) and *M. javanica* (Avignon isolate). DLL is susceptible and YW is highly resistant to these two species (Djian-Caporalino et al., 1999). These nematodes were obtained from the INRA *Meloidogyne* collection maintained at Institut Sophia Agrobiotech in Sophia Antipolis, France. These three *Meloidogyne* species have a mitotic parthenogenetic mode of reproduction. We therefore considered all the second-stage juveniles (J2s) hatching from a single egg mass to constitute a clonal line. Before multiplication, these isolates were specifically identified on the basis of isoesterase electrophoresis (Dalmasso and Berge, 1978) or sequence characterized amplified region (SCAR) PCR (Zijlstra et al., 2000).

DNA Extraction, Genotyping of Molecular Markers, and Linkage Map

Genomic DNA was isolated from the young leaves of both parents, the F₁ and the individuals of the mapping population, as described by Fulton et al. (1995). After RNase treatment, the concentration and purity of DNA were assessed with a NanoDrop 2000 spectrophotometer (Thermo Scientific), and the final DNA concentration was adjusted to 20 ng/μL for PCR.

The F₂ mapping population was genotyped with 58 markers previously used in other populations: one B94 SCAR marker (Djian-Caporalino et al., 2007), 13 simple sequence repeat (SSR) markers previously mapped in pepper (Alimi et al., 2013), 44 single-nucleotide polymorphism (SNP) markers from Nicolaï et al. (2012). In addition, 272 new SNP markers were provided by Syngenta Seeds. We used these markers to construct a genetic linkage map with Mapmaker software version 3.0b (Lander et al., 1987), using a LOD score threshold of 3.0 and a maximum recombination fraction of 0.3. Distances between markers were calculated with the Kosambi mapping function. For each linkage group (LG), marker order was checked with the 'ripple' command and markers were retained only if the LOD score value was greater than 2.0. The LGs were assigned to pepper chromosomes on the basis of the positions of SSR and SNP markers common to the genetic maps for pepper published by Alimi et al. (2013) and Quenouille et al. (2014).

Experimental Procedures for Evaluating Nematode Resistance

Resistance was assessed on the F₃ progenies. For each RKN isolate, 16 F₃ seeds per F_{2:3} family were sown individually in 9 cm plastic pots containing steam-sterilized sandy soil covered with a 1 cm layer of loam. F₃ plants were split evenly (i.e., eight plants per F₃) between two growth chambers maintained at 24°C (±2°C) with a 12-h light/12-h dark cycle and a relative humidity of 60–70%. Parental lines, the F₁ progeny and two resistant controls (HD149 and HD330) were included in the experimental design. The 130 F_{2:3} families and controls were randomly arranged within each growth chamber. Six to seven-week-old plants (4–6 true leaves) were each inoculated with 500 freshly hatched J2s suspended in water, for experiments with *M. arenaria* and *M. javanica*, and with 1,000 J2s suspended in water for experiments with *M. incognita*. This difference in inoculum was based on the behavior of YW with respect to the species used (resistant or partially resistant). It has been shown that a higher inoculum density is required to reveal the differences between the partially resistant parent (YW) and the highly susceptible parent (DLL). J2s were obtained in a mist chamber, from previously inoculated susceptible tomato roots (cultivar Saint Pierre). Six to seven weeks after inoculation (i.e., a period long enough for completion of the nematode life cycle), plants were harvested, carefully washed individually with tap water and frozen at -20°C until scoring. Before analysis, the roots were thawed and stained by incubation for 10 min in a cold aqueous solution of eosin (0.1 g/l water), for the specific staining of egg masses (EMs). The roots were rinsed and examined under a magnifying glass. The number of EMs was counted for each plant

and the median number of EMs per plant for each F_3 family (and for the control genotype) was determined, for estimation of the phenotypic value of each F_2 .

Statistical Analyses

R software¹ was used for descriptive statistics. Analyses of variance (ANOVA) were carried out for each phenotypic trait (i.e., for resistance to each RKN species), to estimate genotypic/environmental effects. For each phenotypic trait, narrow-sense heritability (h^2) was estimated with the formula $h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_E^2/n)$, where σ_G^2 corresponds to the genotypic variance and σ_E^2 to the environment variance (including block, interaction, and error effects) and n is the number of replicates per $F_{2:3}$ family (two growth rooms). Additive and dominance effects were calculated as described by Stuber et al. (1987). The normality of phenotype distributions was assessed with Shapiro-Wilks tests ($\alpha = 0.05$).

Quantitative trait loci analyses were performed with the R/qt1 package of R software (Broman et al., 2003). QTLs were detected by regression analysis, SIM, CIM, and non-parametric interval mapping (model = “np” in the R/qt1 package) for the non-Gaussian phenotype distributions, although the residues were normal, making it possible to carry out a regression analysis to estimate additive and R^2 values. All the methods yielded similar results (the same QTLs at the same positions; data not shown), although the QTL peaks were slightly less sharp with the non-parametric procedure. A permutation test was performed with 1,000 replicates to determine the genome-wide LOD threshold empirically at the 5% probability level, for each phenotypic trait individually. The LOD threshold was estimated at 3.6 for the three traits. For each QTL, the confidence interval (CI) was defined as a 2-LOD drop-off around the maximum LOD score. R^2 coefficients were calculated with the ‘fitql’ function of R/qt1.

RESULTS

Linkage Map

Four of the 330 markers tested on the F_2 mapping population remained unlinked. The genetic linkage map was therefore constructed with 326 markers: 13 SSRs, 312 SNPs, and 1 SCAR. Markers displaying significant deviation from the expected Mendelian ratio of 1:2:1 were retained (indicated by asterisks in Supplementary Data 1). The map comprised 12 LGs, corresponding to the 12 pepper chromosomes (P1–P12) and covering an overall length of 1436 cM (Supplementary Data 1).

Segregation of Resistance to the Three RKN Species

The frequency distributions of resistance to *M. incognita*, *M. arenaria*, and *M. javanica* in the $F_{2:3}$ families are shown in Figure 2. For *M. incognita*, the effects of both genotype and block were significant ($p\text{-value} = 0.000653$ and $p\text{-value} < 2.00^{e-16}$, respectively) as revealed by ANOVA. The regression of F_3 values

from block 1 over block 2 was significant ($R_{\text{Pearson}} = 0.32$, $p = 0.00027$) with higher values in block 1. Individual EM data were therefore adjusted by multiplying the data from block 2 by the regression coefficient (value: 1.4). This linear adjustment removed the block effect and the data were pooled for further analyses. The phenotypes of the control pepper lines were consistent with the expected results (Table 1): YW presented a lower infestation rate than DLL (mean of 250 and 330 EMs/plant, respectively). The F_1 progeny was skewed toward DLL (319 EMs/plant), with a d/a ratio of -0.72, indicating additive to partly dominant inheritance in favor of susceptibility. A Shapiro-Wilk test showed that the values for the $F_{2:3}$ families were normally distributed ($W = 0.99$, $p\text{-value} = 0.2029$; Figure 2A). The h^2 value was 0.48.

For the experiment with *M. arenaria*, ANOVA revealed a significant effect of genotype ($p\text{-value} = 3.26^{e-15}$), but no significant block effect ($p\text{-value} = 0.0805$). YW and DLL were resistant and susceptible (mean values of 5 and 69 EMs/plant, respectively), as expected (Table 1). The F_1 phenotype was skewed toward YW (14 EMs/plant), with a d/a ratio of 0.72, indicating that resistance was additive to partly dominant in favor of resistance. The values for the $F_{2:3}$ families were not normally distributed, as confirmed by a Shapiro-Wilk test ($W = 0.92$, $p\text{-value} = 8.63^{e-07}$; Figure 2B). The distribution was skewed toward resistance. Neither logarithmic nor square-root transformation resulted in normality (data not shown). Resistance to *M. arenaria* was highly heritable, as h^2 was 0.76.

For the experiment with *M. javanica*, ANOVA showed a significant effect of genotype ($p\text{-value} < 2.00^{e-16}$) but no significant block effect ($p > 0.183$). YW was highly resistant and DLL was susceptible, as expected (means of 1 and 131 EMs/plant, respectively; Table 1). The F_1 displayed an intermediate phenotype, with skewing toward YW (mean of 17 EMs/plant), with a d/a ratio equal to 0.75, indicating that resistance was mostly dominant, but slightly additive. The Shapiro-Wilk test indicated that the data for the $F_{2:3}$ families were not normally distributed ($W = 0.66$, $p\text{-value} = 2.80^{e-15}$; Figure 2C). Neither logarithmic nor square-root transformation yielded normality. Heritability was high for resistance to *M. javanica* ($h^2 = 0.87$).

Mapping QTLs for Resistance to the Three Meloidogyne Isolates

Simple interval mapping (SIM), composite interval mapping (CIM) and non-parametric (“np” or Kruskal-Wallis) analysis were performed to identify QTLs for resistance. However, as CIM and non-parametric analyses did not improve QTL detection, only the results for SIM are shown. Only one QTL for resistance to *M. incognita* was detected on pepper chromosome P1, with a LOD_{\max} at 179.2 cM and an R^2 value of 40.9, corresponding to 85% of the heritability ($h^2 = 0.48$; Figure 3; Table 2). This QTL was named *Minc-P1*. The d/a ratio of -0.38 at the closest marker (SP1790) indicates a mostly additive effect of this QTL, with a partial dominance effect for susceptibility.

¹<http://www.r-project.org/>

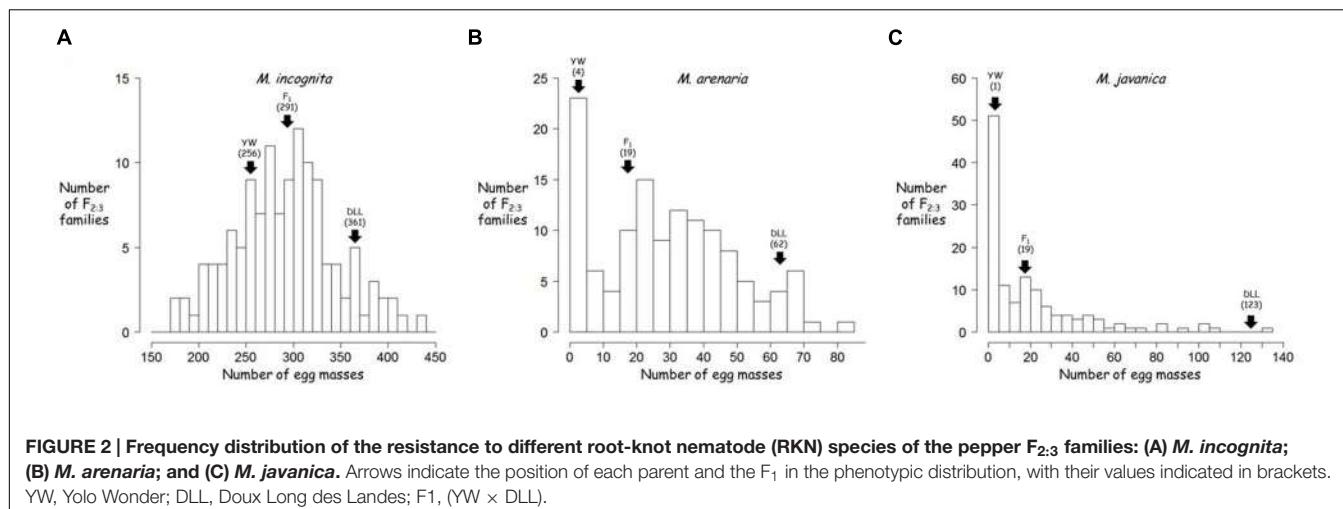


TABLE 1 | Summary of root-knot nematode (RKN) reproduction capacity in the parental lines, F1 and F_{2:3} progeny (130 F_{2:3} families) from the pepper cross (YW × DLL), for different RKN species.

RKN species	Parents		F ₁	F _{2:3} families				
	YW	DLL		Maximum	Minimum	Mean	SD	<i>h</i> ²
<i>Meloidogyne incognita</i>	250	330	319	519	172	332	67	0.48
<i>Meloidogyne arenaria</i>	5	69	14	82	0	25	21	0.76
<i>Meloidogyne javanica</i>	1	131	17	132	0	17	26	0.87

Reproduction capacities were evaluated as the median number of egg masses (EMs) on 16 plants for each genotype. YW, Yolo Wonder (resistant or partially resistant genotype); DLL, Doux Long des Landes (susceptible genotype); F₁, hybrid F₁; SD, standard deviation; *h*², broad-sense heritability ($h^2 = \sigma_G^2 / (\sigma_G^2 + \sigma_E^2/n)$).

Similarly, only one QTL for resistance to *M. arenaria* was detected and mapped to chromosome P1 (*Mare-P1*) with a LOD_{max} at 177.0 cM, close to *Minc-P1*. This QTL, with an *R*² value of 73.8 (97% of the *h*² value) and a *d/a* ratio of 0.28, acts as a major additive QTL, with a weak dominance effect in favor of resistance.

Two QTLs for resistance to *M. javanica* were detected. The first, *Mjav-P1*, was located on chromosome P1 with a LOD_{max} at 178.0 cM, an *R*² of 31.9 and a *d/a* ratio of 0.29, indicating a mostly additive effect. The second QTL, *Mjav-P9*, was detected on the distal part of chromosome P9 (closest marker SP381) with an *R*² of 52.4 and a *d/a* of 0.73, indicating a mostly dominant effect in favor of resistance. Together, *Mjav-P1* and *Mjav-P9* explained 61.2% of the phenotypic variance, corresponding to 71% of the *h*² value.

All the resistance-conferring alleles at these four QTLs originated from the partially resistant parent YW.

Looking for Recombinant Individuals Within the P1 QTL Cluster

F₂ individuals with recombinant genotypes for the QTL cluster on chromosome P1 were surveyed, focusing on their genotypes for the markers and the phenotypes of their F₃ progenies (homogeneous resistant, homogeneous susceptible, or segregating). Four F₂ individuals were found to be recombinant for the alleles at the markers within the P1 QTL cluster (recombination between SP1790 and SP1798).

Two F_{2:3} progenies for these individuals clearly corresponded to phenotypic recombinants in terms of their resistance to *M. arenaria* and *M. incognita*, as attested by the phenotypes of the F₃ progenies (Table 3). Resistance to *M. javanica* was less informative, probably due to the major effect of the second locus *Mjav-P9*.

DISCUSSION

The pepper genetic map constructed in this study with 130 F₂ plants from the cross between YW and DLL comprised 12 LGs, consistent with the known number of chromosomes in pepper, and it covered a total length of 1436 cM, consistent with previous maps for pepper (Lefebvre et al., 2002; Paran et al., 2004; Wu et al., 2009). However, as only a few of the previously used markers proved to be polymorphic between the parental lines YW and DLL, new SNPs had to be developed to complete the map. The sequences supporting the SNPs targeting the QTLs are provided in Supplementary Data 2. This new mapping population was developed because it was shown in a previous study that *R*-genes are more effective and durable against RKN attacks when introgressed into the YW genetic background than when introgressed into the DLL genetic background (Barbary et al., 2014; Djian-Caporalino et al., 2014). This difference is thought to result from the partial resistance alleles carried by YW, which seem to protect the *R*-genes. This new map was

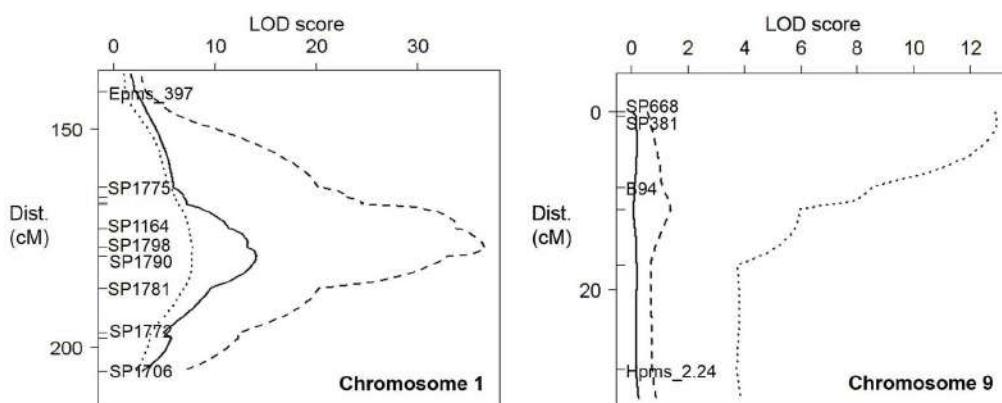


FIGURE 3 | Quantitative trait loci (QTLs) against *M. incognita* (solid line), *M. arenaria* (dashed line), and *M. javanica* (dotted line) on pepper chromosome 1 (Left) and chromosome 9 (Right). On the left of each linkage group (LG), distances in centimorgans, flanking markers, and the marker closest to the resistance factors are indicated.

constructed to identify these resistance factors and associated molecular markers, which should constitute valuable resources for further MAS.

The 130 F_{2:3} [YW × DLL] families were tested against the three main RKN species, *M. incognita*, *M. arenaria*, and *M. javanica*. The QTL analyses identified four new major QTLs affecting reproductive capacity, located in two separate clusters. No minor-effect QTL was detected, and the phenotypic variance explained by the major QTLs for each resistance trait closely fitted the *h*² values (71–97%), indicating that almost all the genetic variance was explained by these major QTLs. Three of these QTLs were grouped on pepper chromosome P1, with overlapping CIs, in a 30 cM region. The YW alleles at these QTLs each confer

resistance to a single species of RKN: *M. incognita*, *M. arenaria*, or *M. javanica*. These are the first QTLs conferring resistance to RKNs to have been detected in pepper, but QTLs conferring resistance to *Meloidogyne* spp., have already been mapped in soybean (Li et al., 2001), cotton (Shen et al., 2006), and cowpea (Muchero et al., 2009). This is also the first report of nematode resistance factors mapping to a genomic location other than chromosome P9 in pepper (i.e., on chromosome P1). All the RKN *R*-genes previously identified in pepper mapped to a cluster on P9 (Djian-Caporalino et al., 2007; Fazari et al., 2012). It is unclear whether *Minc-P1*, *Mare-P1*, and *Mjav-P1* on P1 are all part of a single gene with a broad spectrum of action, or whether they belong to separate genes forming a new cluster, as observed on

TABLE 2 | Quantitative trait loci (QTLs) for resistance to the different RKN species in the pepper F_{2:3} progeny.

RKN species	QTL	Chr. ^a	Location (cM)	Closest marker	LOD	CI ^b	Additive effect (a)	Dominance effect (d)	d/a ratio	R ²
<i>M. incognita</i>	<i>Minc-P1</i>	1	179.2	SP1790	14.1	173.4–184.0	-55.7	21.1	-0.38	40.9
<i>M. arenaria</i>	<i>Mare-P1</i>	1	177.0	SP1798	36.6	173.4–179.0	-24.5	-6.8	0.28	73.8
<i>M. javanica</i>	<i>Mjav-P1</i>	1	178.0	SP1798	7.7	163.9–190.5	-14.2	-4.1	0.29	31.9
	<i>Mjav-P9</i>	9	1.0	SP381	12.9	0.0–6.4	-19.1	-13.9	0.73	52.4

^aChromosome. ^bCI: Confidence interval, defined as a LOD-2 drop-off around the maximum LOD score. Negative values for additive (a) or dominance (d) effects indicate that the allele from the resistant parent decreases the phenotype value.

TABLE 3 | Recombinant genotypes within the cluster of QTLs on chromosome P1 containing *Minc-P1* and *Mare-P1*.

F _{2:3} family	Alleles (YW versus DLL) at the markers				Phenotype* (EMs)	
	SP1164	SP1798	SP1790	SP1781	<i>M. arenaria</i>	<i>M. incognita</i>
17	YW/YW	YW/YW	YW/DLL	YW/DLL	R (3)	He (310)
22	YW/DLL	YW/DLL	DLL/DLL	m.d.	He (13)	S (478)
29	DLL/DLL	DLL/DLL	YW/DLL	YW/DLL	S (48)	S (398)
66	m.d.	YW/DLL	DLL/DLL	DLL/DLL	S (37)	He (306)

Alleles at the marker: YW, Yolo Wonder (resistant allele); DLL, Doux Long des Landes (susceptible allele); m.d., missing data. *Observed segregation in the F₃ family: R, all F₃ plants resistant, S, all F₃ plants susceptible, He, segregation of susceptible, and resistant plants within the F₃ family. The value in brackets is the median number of EMs per plant for each F_{2:3} family.

P9 for *Me3*, *Me1*, and *N*, which have different spectra of action against RKN species (Hendy et al., 1985; Thies and Fery, 2000). However, our results provide two lines of evidence in support of these QTLs belonging to different genes within a cluster. Firstly, for the *Mare-P1* and *Mjav-P1* QTLs, the resistant YW allele displayed partial dominance, whereas partial dominance of the susceptible allele from DLL was observed for *Minc-P1*, suggesting different modes of action. Secondly, *F₂* individuals displaying genetic recombination between the markers at the peak values of the *Minc-P1* and *Mare-P1* loci were detected and the phenotypes of the corresponding *F₃* families confirmed recombination in the *F₂* plant, with a homozygous resistant or susceptible genotype at one QTL and a heterozygous genotype at the other QTL (Table 3). Despite their very tight linkage, these QTLs thus probably belong to different genes conferring different specificities against RKN populations. These findings provide further evidence that broad-spectrum quantitative resistance can result from the combination of race-specific resistance factors.

An additional major QTL, *Mjav-P9*, with a major dominant effect for resistance against *M. javanica*, was mapped to pepper chromosome P9, close to the B94 marker. The detection of a new resistance factor at this site was not unexpected, because the P9 genomic region also carries the *Me* and *N* genes (Fazari et al., 2012), together with the *R*-gene *Bs2*, which confers resistance to the bacterial pathogen *Xanthomonas campestris* pv. *Vesicatoria* (Mazourek et al., 2009), and QTLs for resistance to potyvirus PVY (Wang et al., 2008) and to the oomycete *Phytophthora capsici* (Thabuis et al., 2004). The genomic sequence of this P9 chromosomal region also has a high density of NBS-LRR genes from the *Bs2* subclass (82 genes), highlighting the “explosive expansion of the pepper genome” relative to those of other Solanaceae species (Kim et al., 2014). This expansion, which probably involved tandem duplications, resulted in diversification and a clustering of the *R*-genes on chromosome P9, and the *Mjav-P9* QTL, which has a mostly dominant effect, probably belongs to this cluster.

Broad-spectrum *R*-genes are often preferentially used in breeding programs, but previous studies have shown that the use of *R*-genes in an inappropriate genetic background may decrease their efficacy, in turn affecting their durability. The strategy of combining an *R*-gene with a partially resistant genetic background (i.e., a background carrying relevant QTLs) to increase its durability has been evaluated and validated in other pathosystems (Palloix et al., 2009; Brun et al., 2010; Fournet et al., 2013). Quenouille et al. (2012) suggested that this effect was due mostly to the additional resistance conferred by QTLs from the genetic background, decreasing the size of the pathogen population and, thus, the risk of emergence and of the further selection of virulent variants. For interactions between pepper and RKNs, YW proved to be a better genetic background than DLL for strengthening the efficacy of *Me1* or *Me3* (Barbary et al., 2014) and reducing the frequency of *Me3* resistance breakdown (Djian-Caporalino et al., 2014). However, the genetic determinants of this partial resistance had never been characterized. The new QTLs identified in this study (i.e., *Minc-P1*, *Mare-P1*, *Mjav-P1*, and *Mjav-P9*) are, thus, good candidates for pyramiding with *Me1*, *Me3*, or *N*, providing new

opportunities for combining major and partial resistance factors together in pepper cultivars.

In terms of plant breeding, the location of the newly identified resistance factors on pepper chromosome P1 should facilitate their introgression by MAS, alongside current *R*-genes. Indeed, all the resistance genes effective against RKNs mapped to date are closely linked on pepper chromosome P9 (Fazari et al., 2012). However, they are in repulsion phases, with the different genes carried by different pepper accessions. *Minc-P1*, *Mare-P1*, and *Mjav-P1* are independent of this cluster and all are carried by the same accession (YW). This should make it easier to generate homozygous plant genotypes harboring resistance factors from both the P9 and P1 clusters. Breeders could also make use of *Minc-P1*, *Mare-P1*, and *Mjav-P1*, which do not act as fully dominant *R*-genes and may act through different resistance mechanisms. He et al. (2014) reported two QTLs affecting two different processes in RKN attack on cotton plants: root galling and egg production. These QTLs provided strong resistance to RKNs when combined in the same genotype. We are currently investigating this aspect in our pathosystem, by performing histological time-course studies to explore the spatiotemporal induction of plant responses conferred by these new resistance factors in pepper. In particular, the kinetics of J2 invasion in the roots, and the timing and location of cell necrosis (if any) will be investigated. On the basis of our preliminary observations, we hypothesize that the newly identified QTLs will induce defense reactions different from the classical HR triggered by the major *R*-genes *Me1* and *Me3*. We therefore anticipate that the successful combination of these qualitative and quantitative resistance factors into elite cultivars will provide new opportunities for enhanced and durable RKN resistance in pepper.

AUTHOR CONTRIBUTIONS

AB, PC-S, CD-C, AP, and BC conceived the project, contribute to technical work, to data treatment and article writing, AF and NM organized and performed technical work.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00632>

REFERENCES

- Alimi, N. A., Bink, M., Dieleman, J. A., Magan, J. J., Wubs, A. M., Palloix, A., et al. (2013). Multi-trait and multi-environment QTL analyses of yield and a set of physiological traits in pepper. *Theor. Appl. Genet.* 126, 2597–2625. doi: 10.1007/s00122-013-2160-3
- Barbary, A., Palloix, A., Fazari, A., Marteu, N., Castagnone-Sereno, P., and Djian-Caporalino, C. (2014). The plant genetic background affects the efficiency of the pepper major nematode resistance genes Me1 and Me3. *Theor. Appl. Genet.* 127, 499–507. doi: 10.1007/s00122-013-2235-1
- Bleve-Zacheo, T., Bongiovanni, M., Melillo, M. T., and Castagnone-Sereno, P. (1998). The pepper resistance genes Me1 and Me3 induce differential penetration rates and temporal sequences of root cell ultrastructural changes upon nematode infection. *Plant Sci.* 133, 79–90. doi: 10.1016/S0168-9452(98)00021-1
- Broman, K., Wu, W. H., Sen, S., and Churchill, G. A. (2003). R/qtl: QTL mapping in experimental crosses. *Bioinformatics* 19, 889–890. doi: 10.1093/bioinformatics/btg112
- Brun, H., Chevre, A. M., Fitt, B. D., Powers, S., Besnard, A. L., Ermel, M., et al. (2010). Quantitative resistance increases the durability of qualitative resistance to *Leptosphaeria maculans* in *Brassica napus*. *New Phytol.* 185, 285–299. doi: 10.1111/j.1469-8137.2009.03049.x
- Castagnone-Sereno, P. (2006). Genetic variability and adaptive evolution in parthenogenetic root-knot nematodes. *Heredity (Edinb.)* 96, 282–289. doi: 10.1038/sj.hdy.6800794
- Castagnone-Sereno, P., Bongiovanni, M., Palloix, A., and Dalmasso, A. (1996). Selection for *Meloidogyne incognita* virulence against resistance genes from tomato and pepper and specificity of the virulence/resistance determinants. *Eur. J. Plant Pathol.* 102, 585–590. doi: 10.1007/BF01877026
- Dalmasso, A., and Berge, J. B. (1978). Molecular polymorphism and phylogenetic relationship in some *Meloidogyne* spp.: application to the taxonomy of *Meloidogyne*. *J. Nematol.* 10, 323–332.
- Djian-Caporalino, C., Fazari, A., Arguel, M. J., Vernie, T., Van de Castele, C., Faure, I., et al. (2007). Root-knot nematode (*Meloidogyne* spp.) Me resistance genes in pepper (*Capsicum annuum* L.) are clustered on the P9 chromosome. *Theor. Appl. Genet.* 114, 473–486. doi: 10.1007/s00122-006-0447-3
- Djian-Caporalino, C., Palloix, A., Fazari, A., Marteu, N., Barbary, A., Abad, P., et al. (2014). Pyramiding, alternating or mixing: comparative performances of deployment strategies of nematode resistance genes to promote plant resistance efficiency and durability. *BMC Plant Biol.* 14:53. doi: 10.1186/1471-2229-14-53
- Djian-Caporalino, C., Pijarowski, L., Januel, A., Lefebvre, V., Daubèze, A. M., Palloix, A., et al. (1999). Spectrum of resistance to root-knot nematodes and inheritance of heat-stable resistance in pepper (*Capsicum annuum* L.). *Theor. Appl. Genet.* 99, 496–502. doi: 10.1007/s001220051262
- Fazari, A., Palloix, A., Wang, L. H., Hua, M. Y., Sage-Palloix, A. M., Zhang, B. X., et al. (2012). The root-knot nematode resistance N-gene co-localizes in the Me-genes cluster on the pepper (*Capsicum annuum* L.) P9 chromosome. *Plant Breed.* 131, 665–673. doi: 10.1111/j.1439-0523.2012.01994.x
- Fournet, S., Kerlan, M. C., Renault, L., Dantec, J. P., Rouaux, C., and Montarry, J. (2013). Selection of nematodes by resistant plants has implications for local adaptation and cross-virulence. *Plant Pathol.* 62, 184–193. doi: 10.1111/j.1365-3059.2012.02617.x
- Fulton, T. M., Chunwongse, J., and Tanksley, S. D. (1995). Microprep protocol for extraction of DNA from tomato and other herbaceous plants. *Plant Mol. Biol. Rep.* 13, 207–209. doi: 10.1007/BF02670897
- Goodey, T., Goodey, J. B., Franklin, M. T., and Hooper, D. J. (1965). *The Nematode Parasites of Plants Catalogued Under Their Hosts*, 3rd Edn. Farnham Royal: Commonwealth Agricultural Bureaux, 214.
- Hare, W. W. (1956). Resistance in pepper to *Meloidogyne incognita* acrata. *Phytopathology* 46, 98–104.
- He, Y., Kumar, P., Shen, X., Davis, R. F., Van Becelaere, G., May, O. L., et al. (2014). Re-evaluation of the inheritance for root-knot nematode resistance in the upland cotton germplasm line M-120 RNR revealed two epistatic QTLs conferring resistance. *Theor. Appl. Genet.* 127, 1343–1351. doi: 10.1007/s00122-014-2302-2
- Hendy, H., Pochard, E., and Dalmasso, A. (1985). Transmission héréditaire de la résistance aux *Meloidogyne* portée par deux lignées de *Capsicum annuum*: études de descendances d'homozygotes issues d'androgénèse. *Agronomie* 5, 93–100. doi: 10.1051/agro:19850201
- Kim, S., Park, M., Yeom, S. I., Kim, Y. M., Lee, J. M., and Lee, H. A. (2014). Genome sequence of the hot pepper provides insights into the evolution of pungency in *Capsicum* species. *Nat. Genet.* 46, 270–278. doi: 10.1038/ng.2877
- Kiyosawa, S. (1982). Genetics and epidemiological modeling of breakdown of plant disease resistance. *Annu. Rev. Phytopathol.* 20, 93–117. doi: 10.1146/annurev.py.20.090182.000052
- Lander, E. S., Green, P., Abrahamson, J., Barlow, A., Daly, M. J., Lincoln, S. E., et al. (1987). MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. *Genomics* 1, 174–181. doi: 10.1016/0888-7543(87)90010-3
- Lefebvre, V., Pflieger, S., Thabuis, A., Caranta, C., Blattes, A., Chauvet, J. C., et al. (2002). Towards the saturation of the pepper linkage map by alignment of three intraspecific maps including known-function genes. *Genome* 45, 839–854. doi: 10.1139/g02-053
- Li, Z., Jakkula, L., Hussey, R. S., Tamulonis, J. P., and Boerma, H. R. (2001). SSR mapping and confirmation of the QTL from PI96354 conditioning soybean resistance to southern root-knot nematode. *Theor. Appl. Genet.* 103, 1167–1173. doi: 10.1007/s001220100672
- Mazourek, M., Cirulli, E. T., Collier, S. M., Landry, L. G., Kang, B. C., Quirin, E. A., et al. (2009). The fractionated orthology of Bs2 and Rx/Gpa2 supports shared synteny of disease resistance in the Solanaceae. *Genetics* 182, 1351–1364. doi: 10.1534/genetics.109.101022
- Muchero, W., Matthews, W. C., Diop, N. N., Bhat, P. R., Wanamaker, S., Ehlers, J. D., et al. (2009). QTL mapping of root-knot nematode resistance in cowpea (*Vigna unguiculata*) using EST derived SNP markers. *J. Nematol.* 41, 361–362.
- Mundt, C. C. (2002). Use of multiline cultivars and cultivar mixtures for disease management. *Annu. Rev. Phytopathol.* 40, 381–410. doi: 10.1146/annurev.phyto.40.011402.113723
- Nicolai, M., Pisani, C., Bouchet, J. P., Vuylsteke, M., and Palloix, A. (2012). Discovery of a large set of SNP and SSR genetic markers by high-throughput sequencing of pepper (*Capsicum annuum*). *Genet. Mol. Res.* 11, 2295–2300. doi: 10.4238/2012.August.13.3
- Palloix, A., Ayme, V., and Moury, B. (2009). Durability of plant major resistance genes to pathogens depends on the genetic background, experimental evidence and consequences for breeding strategies. *New Phytol.* 183, 190–199. doi: 10.1111/j.1469-8137.2009.02827.x
- Paran, I., Rouppe Van Der Voort, J., Lefebvre, V., Jahn, M., Landry, L., Van Schriek, M., et al. (2004). An integrated genetic linkage map of pepper (*Capsicum* spp.). *Mol. Breed.* 13, 251–261.
- Pegard, A., Brizzard, G., Fazari, A., Soucaze, O., Abad, P., and Djian-Caporalino, C. (2005). Histological characterization of resistance to different root-knot nematode species related to phenolics accumulation in *Capsicum annuum*. *Phytopathology* 95, 158–165. doi: 10.1094/PHYTO-95-0158
- Pink, D. (2002). Strategies using genes for non-durable disease resistance. *Euphytica* 124, 227–236. doi: 10.1023/A:1015638718242
- Quenouille, J., Montarry, J., Palloix, A., and Moury, B. (2012). Farther, slower, stronger: how the plant genetic background protects a major resistance gene from breakdown. *Mol. Plant Pathol.* 14, 109–118. doi: 10.1111/j.1364-3703.2012.00834.x
- Quenouille, J., Paulhiac, E., Moury, B., and Palloix, A. (2014). Quantitative trait loci from the host genetic background modulate the durability of a resistance gene: a rational basis for sustainable resistance breeding in plants. *Heredity (Edinb.)* 112, 579–587. doi: 10.1038/hdy.2013.138
- Shen, X. L., Van Becelaere, G., Kumar, P., Davis, R. F., May, O. L., and Chee, P. (2006). QTL mapping for resistance to root-knot nematodes in the M-120 RNR upland cotton line (*Gossypium hirsutum* L.) of the Auburn 623 RNR source. *Theor. Appl. Genet.* 113, 1539–1549. doi: 10.1007/s00122-006-0401-4
- Stuber, C. W., Edwards, M. D., and Wendel, J. F. (1987). Molecular marker-facilitated investigations of quantitative trait loci in maize. II. Factors influencing yield and its component traits. *Crop Sci.* 27, 639–648. doi: 10.2135/cropsci1987.0011183X002700040006x
- Thabuis, A., Lefebvre, V., Bernard, G., Daubèze, A. M., Phaly, T., Pochard, E., et al. (2004). Phenotypic and molecular evaluation of a recurrent selection program for a polygenic resistance to *Phytophthora capsici* in pepper. *Theor. Appl. Genet.* 109, 342–351. doi: 10.1007/s00122-004-1633-9

- Thies, J. A. (2011). Virulence of *Meloidogyne incognita* to expression of N gene in pepper. *J. Nematol.* 43, 90–94.
- Thies, J. A., and Fery, R. L. (1998). Modified expression of the N gene for southern root-knot nematode resistance in pepper at high soil temperatures. *J. Am. Soc. Hortic. Sci.* 123, 1012–1015.
- Thies, J. A., and Fery, R. L. (2000). Characterization of resistance conferred by the N gene to *Meloidogyne arenaria* Races 1 and 2, *M. hapla*, and *M. javanica* in two sets of isogenic lines of *Capsicum annuum* L. *J. Am. Soc. Hortic. Sci.* 125, 71–75.
- Wang, L. H., Zhang, B., Caranta, C., Mao, S., and Palloix, A. (2008). Molecular markers assisted selection for three QTLs resistant to PVY in pepper (*Capsicum annuum* L.). *Acta Hortic. Sin.* 35, 53–58.
- Wu, F., Eannetta, N. T., Xu, Y., Durrett, R., Mazourek, M., Jahn, M. M., et al. (2009). A COSII genetic map of the pepper genome provides a detailed picture of synteny with tomato and new insights into recent chromosome evolution in the genus *Capsicum*. *Theor. Appl. Genet.* 118, 1279–1293. doi: 10.1007/s00122-009-0980-y
- Zijlstra, C., Donkers-Venne, D., and Fargette, M. (2000). Identification of *Meloidogyne incognita*, *M. javanica* and *M. arenaria* using sequence characterised amplified region (SCAR) based PCR assays. *Nematology* 2, 847–853. doi: 10.1163/156854100750112798

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Genome-Wide Identification and Analysis of the SBP-Box Family Genes under *Phytophthora capsici* Stress in Pepper (*Capsicum annuum* L.)

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SQUAMOSA promoter binding protein (SBP)-box genes encode plant-specific transcription factors that are extensively involved in many physiological and biochemical processes, including growth, development, and signal transduction. However, pepper (*Capsicum annuum* L.) SBP-box family genes have not been well characterized. We investigated SBP-box family genes in the pepper genome and characterized these genes across both compatible and incompatible strain of *Phytophthora capsici*, and also under different hormone treatments. The results indicated that total 15 members were identified and distributed on seven chromosomes of pepper. Phylogenetic analysis showed that SBP-box genes of pepper can be classified into six groups. In addition, duplication analysis within pepper genome, as well as between pepper and *Arabidopsis* genomes demonstrated that there are four pairs of homology of SBP-box genes in the pepper genome and 10 pairs between pepper and *Arabidopsis* genomes. Tissue-specific expression analysis of the CaSBP genes demonstrated their diverse spatiotemporal expression patterns. The expression profiles were similarly analyzed following exposure to *P. capsici* inoculation and hormone treatments. It was shown that nine of the CaSBP genes (CaSBP01, 02, 03, 04, 05, 06, 11, 12, and 13) exhibited a dramatic up-regulation after compatible HX-9 strain (*P. capsici*) inoculation, while CaSBP09 and CaSBP15 were down-regulated. In case of PC strain (*P. capsici*) infection six of the CaSBP genes (CaSBP02, 05, 06, 11, 12, and 13) were arose while CaSBP14 was down regulated. Furthermore, Salicylic acid, Methyl jasmonate and their biosynthesis inhibitors treatment indicated that some of the CaSBP genes are potentially involved in these hormone regulation pathways. This genome-wide identification, as well as characterization of evolutionary relationships and expression profiles of the pepper CaSBP genes, will help to improve pepper stress tolerance in the future.

Keywords: *Capsicum annuum* L., SBP-box family genes, Phylogenetic analysis, *Phytophthora capsici*, hormone treatments

INTRODUCTION

Transcription factors (TFs) are DNA-binding proteins that regulate gene expression at the level of mRNA transcription. They are capable of activating or repressing the transcription of multiple target genes (Yang et al., 2008). In plants, TFs play essential roles in the regulation of many developmental processes (Li et al., 2013). SQUAMOSA promoter binding protein (SBP)-box genes encode a TFs that contain a highly conserved DNA-binding domain termed the SBP domain (Klein et al., 1996; Cardon et al., 1999). This domain comprises approximately 76 amino acid residues that are involved in both DNA binding and nuclear localization, including two zinc-binding sites (Yamasaki et al., 2004). The *AmSBP1* and *AmSBP2* genes of *Antirrhinum majus* were the first SBP-box genes to be discovered based on their ability to interact with the promoter sequence of the floral meristem identity gene SQUAMOSA (Klein et al., 1996). Additional SBP-box genes were later identified, isolated, and characterized in many plants, including *Arabidopsis thaliana* (Cardon et al., 1999), silver birch (Lannenpaa et al., 2004), *Salvia miltiorrhiza* (Zhang et al., 2014), rice (Xie et al., 2006), maize (Chuck et al., 2010), tomato (Salinas et al., 2012), grape (Hou et al., 2013b), and *Gossypium hirsutum* (Zhang et al., 2015).

SQUAMOSA promoter binding protein genes have been found to play a role in the gene regulatory network of the flower formation pathway, and many studies have revealed that these genes are closely related to flower development (Klein et al., 1996; Cardon et al., 1997; Shikata et al., 2009). Moreover, recent studies showed that SBP-box genes are involved in signal transduction and responses to abiotic and biotic stress in many species. For instance, *AtSPL14* has been found to be involved in determining sensitivity to the programmed cell death-inducing fungal toxin fumonisin B1 (Stone et al., 2005). *AtSPL2* (*At5g43270*), which is modified in transgenic *Arabidopsis* overexpressing the JASMONATE CARBOXYL METHYLTRANSFERASE gene (*AtJMT*) response to jasmonic acid mediated resistance pathway (Jung et al., 2007). *VpSBP5* likely participates in regulating resistance to *Erysiphe necator* by activating the SA-induced systemic acquired resistance pathway and MeJA-induced wound signaling pathway in grapes (Hou et al., 2013b). However, little is currently known about the SBP-box genes in pepper, especially regarding resistance to *Phytophthora* blight.

Pepper (*Capsicum annuum* L.) is one of the most important vegetable crops worldwide. The *Phytophthora* blight in pepper is caused by the oomycete *Phytophthora capsici*, which mainly attacks the roots and is one of the most destructive diseases worldwide (Hausbeck and Lamour, 2004; Zhang et al., 2013), as it also infects tomato, eggplant, cucumber, watermelon, pumpkin, squash, cocoa, and other plants (Biles et al., 1995; Oelke and Bosland, 2003). The pathogen can affect the plant at any stage of development causing damping-off, seedling blight, and wilting, followed by plant death. Infected plants have rapidly expanding water-soaked lesions (Kousik et al., 2012). Analysis of *C. annuum* SBP-box (*CaSBP*) genes in response to *P. capsici* and hormones is therefore important for identification of candidate genes in pepper.

In the current study, we report the genome-wide identification and characterization of SBP-box genes in the pepper genome, including sequence alignment, phylogenetic analysis, intron-exon structure, chromosomal location, and synteny. Moreover, we investigated the expression patterns of *CaSBP* genes in various pepper tissues/organs, as well as the transcriptional responses of *CaSBP* genes in the roots of different *P. capsici*. Five *CaSBP* genes were selected based on their expression patterns after inoculation with *P. capsici*, and their expression profiles were assessed following treatment with different plant hormones and corresponding biosynthetic inhibitors. Our findings lay the foundation for future research into the functions of disease-related genes from the SBP-box gene family in pepper.

MATERIALS AND METHODS

Identification and Annotation of SBP-Box Genes in Pepper

A hidden Markov model (HMM) profile of the SBP domain (Accession no. PF03110) was downloaded from the Pfam database¹. This domain was used to query the CM334 (*C. annuum*) Genome Database and Zunla-1 (*C. annuum*) Genome Database² (V1.55) with the BLASTP program. All hits with an *E*-value < 1.5e-7 were identified. All non-redundant protein sequences were searched for the SBP domain using NCBI's conserved domain database³. Candidate *CaSBP* genes were aligned with DNAMAN software (Version 5.0), and genes with differing sequences between the two cultivars were identified (Guo et al., 2015). Primers (Supplementary Table S1) were designed to amplify the sequences with Primer Premier 5.0 (Premier Biosoft International, Palo Alto, CA, USA), and CM334 and Zunla-1 sequences for the same gene were then aligned to confirm the correct sequences. In order to compute the theoretical isoelectric point (pI) and protein molecular weight (MW), the deduced amino acid sequences were analyzed using DNAMAN Lasergene software (Version 7.1). Names of putative *CaSBP* genes were assigned based on chromosomal order.

Sequence Alignments, Phylogenetic Analysis, and Intron/Exon Structure Determination

Multiple amino acid sequence alignment was performed using DNAMAN software (Version 5.0). The sequence logo was obtained using the online platform Weblogo⁴ for conserved sequences. Phylogenetic trees were constructed using MEGA 6.0 with the maximum likelihood method and 1000 bootstrap replicates. Intron/exon structures were determined by aligning coding sequences to their corresponding genomic sequences. A diagram of intron/exon structures was obtained using the method described by Guo

¹<http://www.sanger.ac.uk>

²<http://peppergenome.snu.ac.kr/blast.php>

³<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>

⁴<http://weblogo.berkeley.edu>

et al. (2015), which depicts both exon positions and gene lengths.

Chromosomal Location and Duplication Analysis

Chromosomal location information was derived from the pepper genome⁵, and genes were mapped to chromosomes using MapDraw (Liu and Meng, 2003) and their physical chromosome positions. Identification of duplicate genes within the pepper genome and between pepper and *Arabidopsis* was performed using the following criteria described by Gu et al. (2002): (1) the FASTA-alignable region between the two proteins had to be greater than 80% of the longer protein, and (2) the identity (I) between the two proteins had to be $\geq 30\%$ if the alignable region was longer than 150 aa and $\geq 0.01n + 4.8 L^{-0.32(1+\exp(-L/1000))}$ (Rost, 1999) if otherwise, where $n = 6$ and L is the alignable length between the two proteins (Rost, 1999; Gu et al., 2002).

Plant Materials and Seedling Treatment

In this study, we used the pepper cultivar AA3 (provided by the pepper research group, College of Horticulture, Northwest A&F University, Yangling, China), which is susceptible to a compatible HX-9 strain and resistant to an incompatible PC strain of *P. capsici*. Plants were grown in a growth chamber at 22/18°C day/night temperature and 16/8 h day/night photoperiod. Various vegetative and reproductive tissues, including roots, stems, leaves, flowers, green fruits, and mature fruits were collected and stored at -80°C for tissue-specific experiments.

Pepper plants at the 8–10 true leaves stage were inoculated with compatible and incompatible strains of *P. capsici* using the root-drenching method, as described by Wang et al. (2013a), while control plants were inoculated with sterile distilled water. Root samples were taken at 0, 6, 12, 24, and 48 h and stored at -80°C . Seedlings were treated with 100 μM SA synthesis inhibitor (paclobutrazol, PBZ; Liu et al., 2006) or 50 μM MeJA synthesis inhibitor (salicylhydroxamic acid, SHAM; Dong et al., 2009). After 24 h of treatment, plants were treated with the corresponding inducer, 5 mM SA or 50 μM MeJA, using the method described by Yin et al. (2014). A mixture of 0.5% Tween and 0.1% alcohol was used as a control for PBZ and SHAM treatment, while PBZ and SHAM treatment alone (no inducer) was also used as an induction control. Leaves were harvested at 0, 3, 6, 9, 12, 24, and 48 h and were quickly frozen with liquid nitrogen and stored at -80°C .

RNA Extraction and Quantitative Real-Time PCR

Total RNA was isolated using the method described by Guo et al. (2012), and cDNA was synthesized according to the manufacturer's instructions of PrimeScript Kit (Takara, Dalian, China). The cDNA was then diluted to 50 ng/ μL with ddH₂O. For quantitative real-time PCR (qRT-PCR), primer pairs (Supplementary Table S2) for *CaSBP* genes were designed by Primer Premier 5.0, and their specificities were assessed using

NCBI Primer BLAST⁶. The ubiquitin binding-protein gene (*UBI-3*) from pepper was used as reference (Schmittgen and Livak, 2008). qRT-PCR was performed as described by Guo et al. (2015) on the iQ5.0 Bio-Rad iCycler thermocycler (Bio-Rad, Hercules, CA, USA) using SYBR Green Supermix (Takara, Dalian, China). qRT-PCR cycling conditions were as follows: pre-denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s. The fluorescent signal was measured at the end of each cycle, and melting curve analysis was performed by heating the PCR product from 56 to 95°C in order to verify the specificities of the primers. Three independent biological replicates were carried out. The relative expression levels of pepper *SBP* genes were calculated using the $-\Delta\Delta\text{CT}$ method (Schmittgen and Livak, 2008).

RESULTS

Genome-Wide Identification and Annotation of SBP-Box Genes in Pepper

The identification of SBP-box gene family members in pepper was performed in three steps. In the first step, the HMM profile of the SBP domain was used as a BLAST query against the pepper genome. A total of 15 and 16 candidate SBP-box genes were obtained from pepper cultivars CM334 and Zunla-1, respectively. In the second step, CM334 and Zunla-1 genes were compared, and sequences were re-amplified to verify the corresponding genes. One candidate gene (Gene ID: Capana03g002994) found in Zunla-1 was discarded due to poor identification in comparison with the corresponding sequence in CM334. In the final step, each predicted SBP-box protein sequence was confirmed to have a conserved SBP domain using an NCBI search. As a result, 15 candidate SBP-box genes were confirmed and named based on their chromosomal order in pepper (Table 1). The *CaSBP* coding sequences ranged from 336 bp (*CaSBP08*) to 3024 bp (*CaSBP06*), while deduced proteins ranged from 111 to 983 amino acids in length and from 13.11 to 108.67 kDa in MW. The predicted isoelectric points (pI) of the *CaSBPs* varied from 5.61 to 9.54.

Sequence Alignments, Phylogenetic Analysis, and Intron/Exon Structure Determination

Multiple sequence alignment of full-length protein sequences was performed to analyze the domain structures of *CaSBPs* in detail. The SBP domain is the only conserved domain shared by all *CaSBPs* (Figure 1A) and was highly similar across proteins, with high or complete conservation at certain positions (Figure 1B). All *CaSBPs* exhibit two zinc finger-like structures (C3H, C2HC) and a highly conserved bipartite nuclear localization signal (NLS), with the exception of *CaSBP08*, which lacks the C2HC and NLS. In addition, *CaSBP09* and *CaSBP15* are also lacking C3H, as

⁵<http://peppergenome.snu.ac.kr/>

⁶http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome

TABLE 1 | Characterization of SQUAMOSA promoter binding protein (SBP)-box family genes in pepper.

Gene	SGN locus	Chr.	Introns	AA	WT	PI
CaSBP01	Capana01g002647	1	3	463	50.32	8.84
CaSBP02	Capana01g002832	1	11	930	103.34	5.61
CaSBP03	Capana01g003073	1	9	796	89.15	6.73
CaSBP04	Capana01g003445	1	2	290	33.21	9.01
CaSBP05	Capana02g001917	2	1	136	15.72	8.27
CaSBP06	Capana05g002237	5	10	983	108.67	7.45
CaSBP07	Capana07g001731	7	1	183	20.79	9.54
CaSBP08	CA07g17550 ▲	7	0	111	13.11	7.72
CaSBP09	CA08g03640 ▲	8	0	144	16.32	9.04
CaSBP10	Capana10g000507	10	1	141	16.27	7.31
CaSBP11	Capana10g000709	10	2	507	55.17	8.81
CaSBP12	Capana10g000886	10	2	299	33.71	8.48
CaSBP13	Capana10g002379	10	2	367	39.57	8.53
CaSBP14	Capana11g002003	11	2	548	60.19	7.41
CaSBP15	CA11g04690 ▲	11	0	144	16.18	9.46

Chr, chromosome location; AA, amino acid; Mol. Wt., molecular weight (kDa); pl, isoelectric point. SGN loci marked with triangle (▲) are from CM334 genome, others are from Zunla-1 genome.

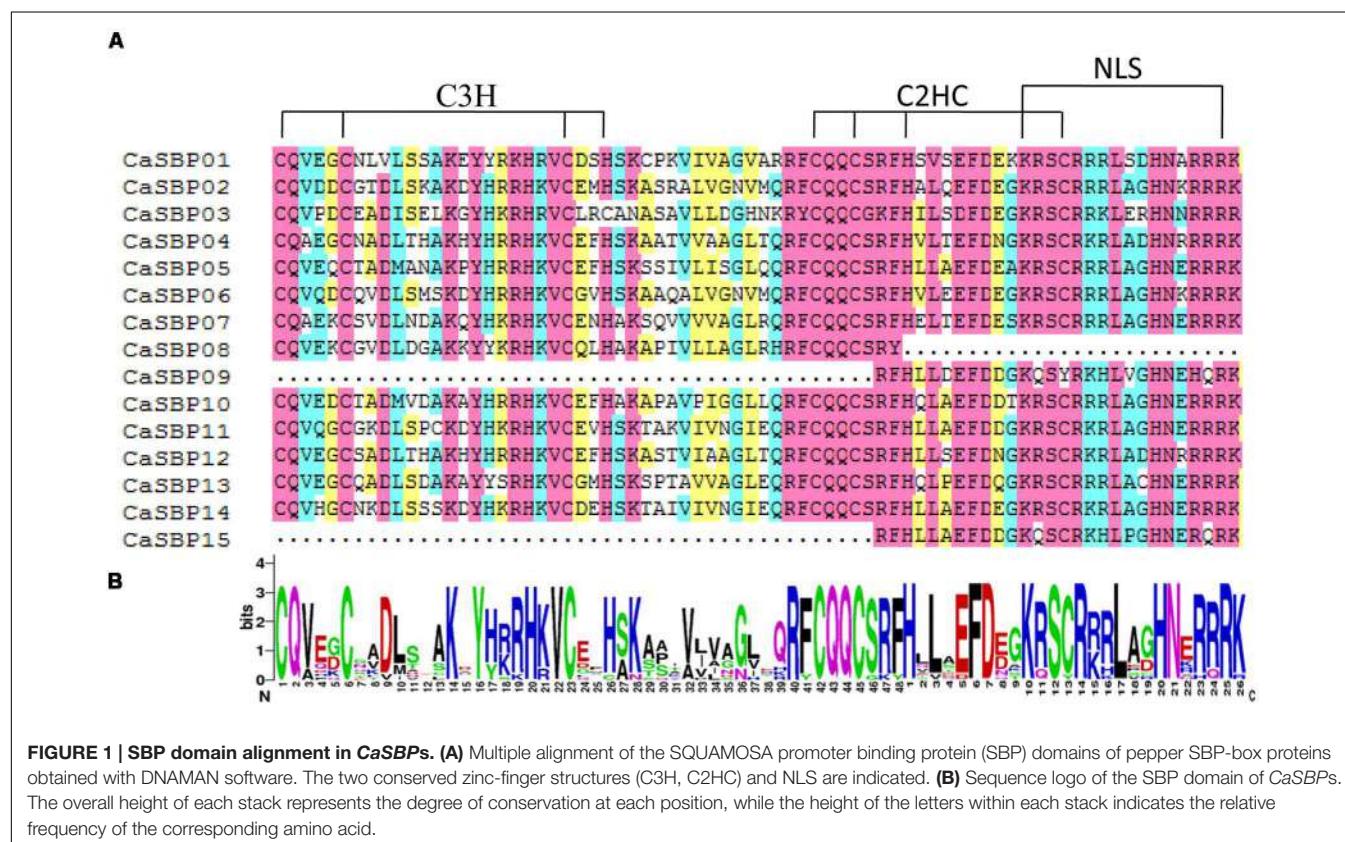
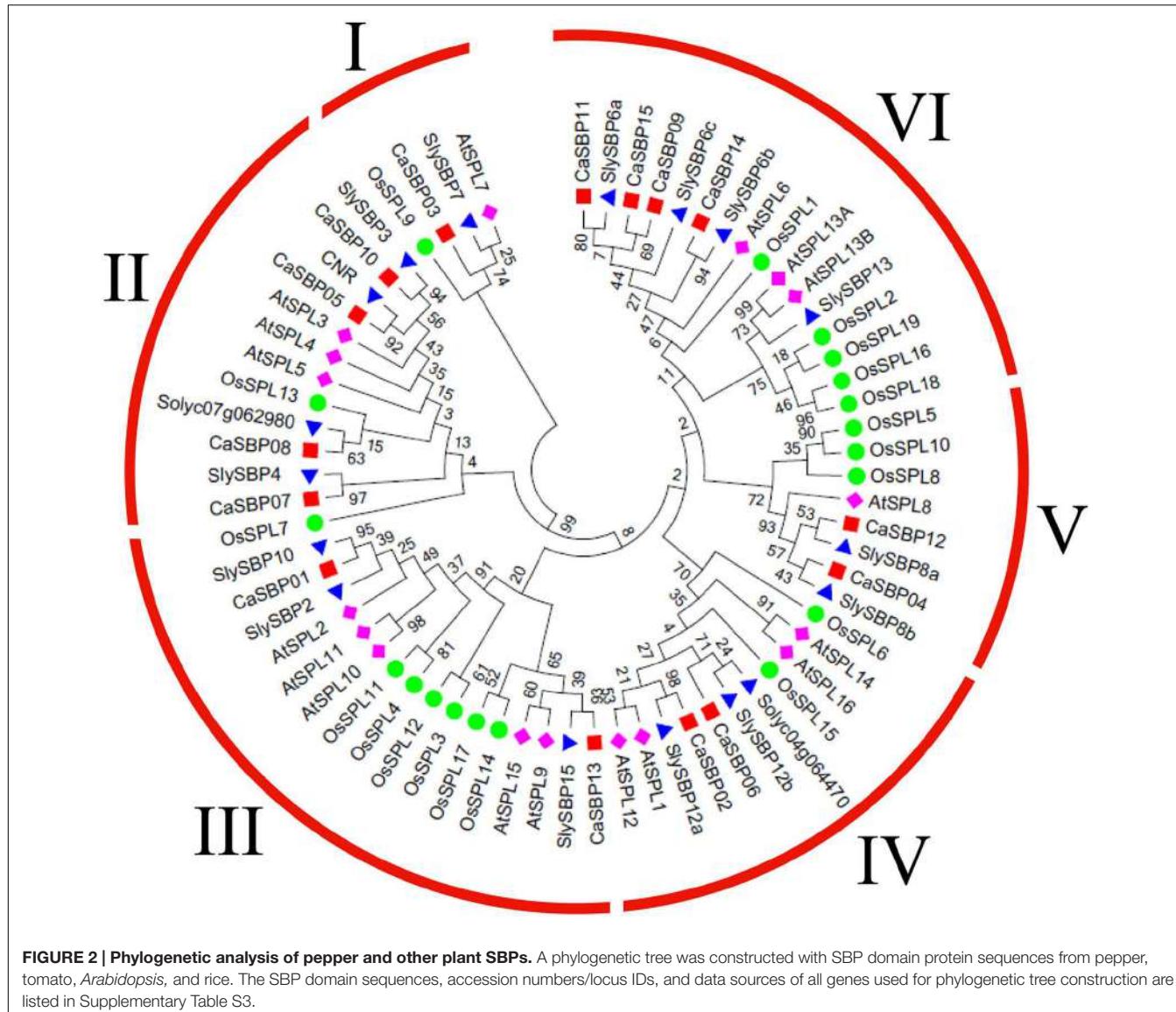


FIGURE 1 | SBP domain alignment in CaSBPs. (A) Multiple alignment of the SQUAMOSA promoter binding protein (SBP) domains of pepper SBP-box proteins obtained with DNAMAN software. The two conserved zinc-finger structures (C3H, C2HC) and NLS are indicated. **(B)** Sequence logo of the SBP domain of CaSBPs. The overall height of each stack represents the degree of conservation at each position, while the height of the letters within each stack indicates the relative frequency of the corresponding amino acid.

the second zinc finger-like structure partially overlaps the NLS, as previously reported (Birkenbihl et al., 2005).

To investigate the evolutionary relationship between *CaSBP* genes and SBP-box genes from *Arabidopsis*, tomato (*Solanum lycopersicum*), and rice (*Oryza sativa*), we constructed a phylogenetic tree using the maximum likelihood algorithm (Figure 2), with 17 *Arabidopsis* genes, 17 tomato genes, and

19 rice genes (Supplementary Table S3). Only the protein sequences of the highly conserved SBP domains were used for phylogenetic analysis, as alignment of the full-length protein sequences revealed that only the SBP domains were conserved (Hou et al., 2013a). According to the unrooted phylogenetic tree, *CaSBP* proteins clustered with those of the other species into six distinct groups (I–VI; Figure 2), with each group containing



at least one protein from each species. The plant SBP-box gene family is evolutionarily diversified. An unrooted phylogenetic tree was also constructed using only the SBP domains from CaSBPs (**Figure 3A**).

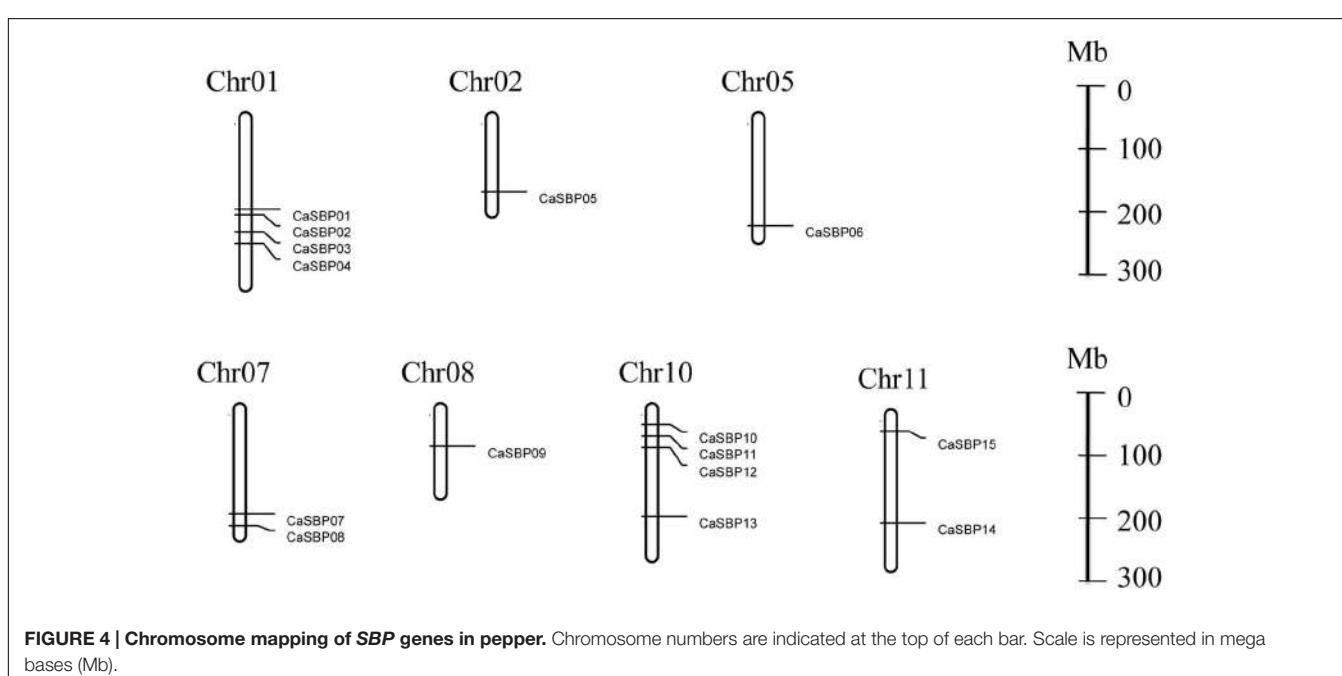
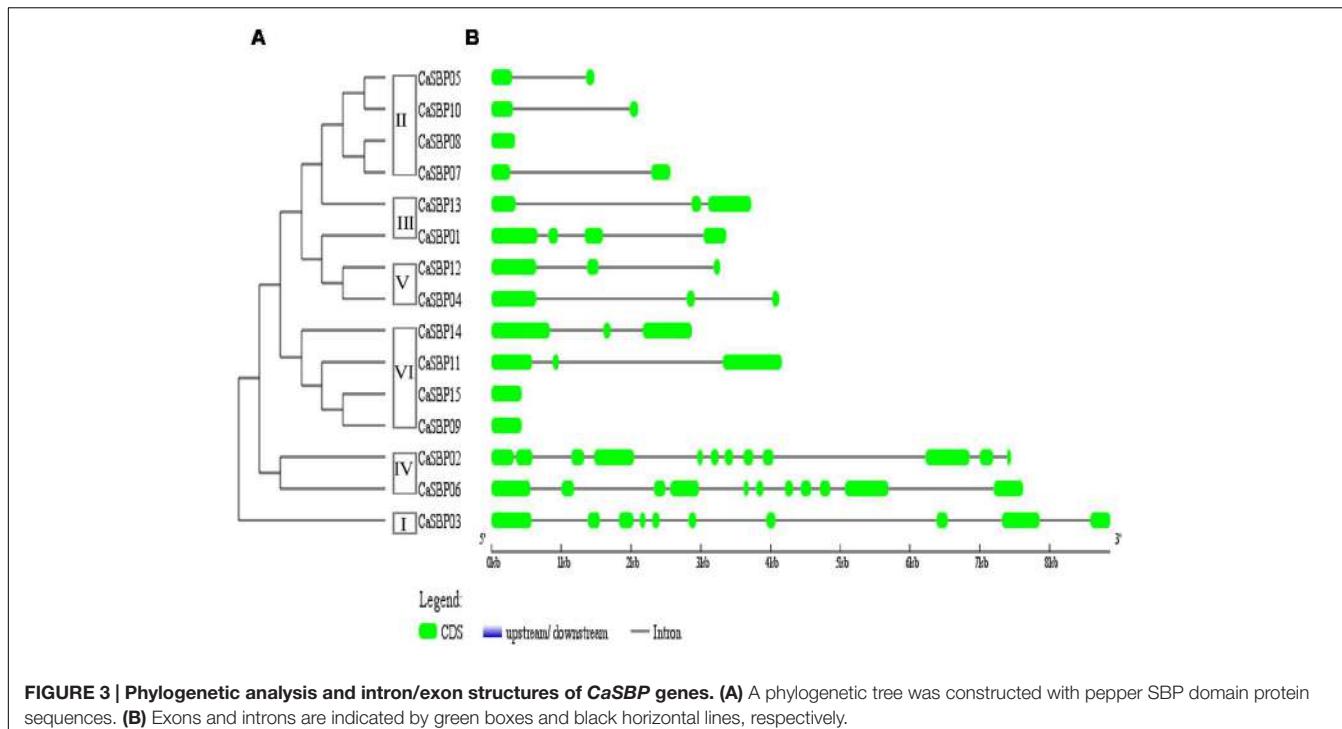
Intron/exon structures of all 15 *CaSBP* genes were generated based on genome sequences and corresponding coding sequences (**Figure 3B**). Intron/exon structure diagrams revealed high variation in the number of introns, from zero (*CaSBP08*, *CaSBP09*, and *CaSBP15*) to 11 (*CaSBP02*). Based on the *CaSBP* tree (**Figure 3A**), class I proteins contain nine introns, class II contains 0–1, class III contains 2–3, class IV contains 10–11, class V contains 2, and class VI contains 0–2 introns.

Chromosomal Location and Duplication Analysis

We found that *CaSBP* genes were located on seven of the twelve pepper chromosomes (**Figure 4**): chromosomes 1, 2, 5, 7, 8,

10, and 11 (**Table 1**). Chromosomes 1 and 10 contained the most *CaSBP* genes, with four genes each (*CaSBP01–CaSBP04* and *CaSBP10–CaSBP13*, respectively), followed by chromosomes 7 and 11, with two genes each (*CaSBP07–CaSBP08* and *CaSBP14–CaSBP15*, respectively).

Duplication analysis, using the criteria described by Gu et al. (2002), confirmed that four pairs of pepper SBP-box genes (*CaSBP02/06*, *CaSBP04/12*, *CaSBP05/10*, and *CaSBP09/15*) were the result of interchromosomal segmental duplications (**Figure 5**). Because *Arabidopsis* is a popular model plant and the functions of several *Arabidopsis* SBP-box genes have been well characterized, we also used the same criteria to identify SBP-box gene orthologs between the pepper and *Arabidopsis* genomes to further study the origin, evolutionary history, and putative function of the pepper SBP-box genes. Based on this analysis, we identified ten pairs of CaSBP-AtSPL orthologs (*CaSBP01-AtSPL2*, *CaSBP02-AtSPL1/12*,



CaSBP03-AtSPL7, *CaSBP04/12-AtSPL8*, *CaSBP05/10-AtSPL3*, and *CaSBP06-AtSPL1/12* (Figure 6), indicating that many of pepper SBP-box genes and their *Arabidopsis* counterparts appear to be derived from a common ancestor. According to these results, we were able to infer the functions of several pepper SBP-box genes based on their *Arabidopsis* homologs, facilitating research into the roles of SBP-box genes in pepper.

Expression Profiles of *CaSBP* Genes in Pepper Tissues

In order to provide additional information on the functions of SBP-box genes in pepper, we investigated their expression profiles in various organs and at different stages of fruit development in cultivar AA3 via qRT-PCR with transcript-specific primers (Supplementary Table S2). Generally, the expression patterns of *CaSBP* genes can be classified into two

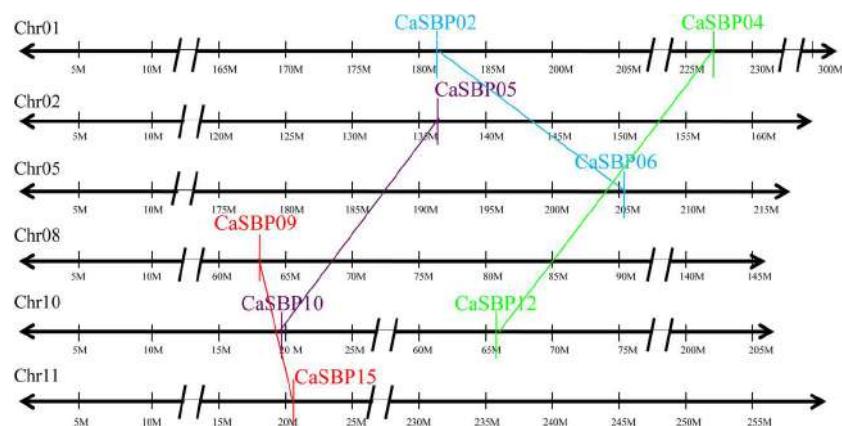


FIGURE 5 | Duplication analysis of pepper SBP-box genes. The positions of duplicated CaSBP genes are depicted on pepper chromosomes 1, 2, 5, 8, 10, and 11. Colored lines connecting two chromosomal regions indicate duplicated regions between pepper chromosomes.

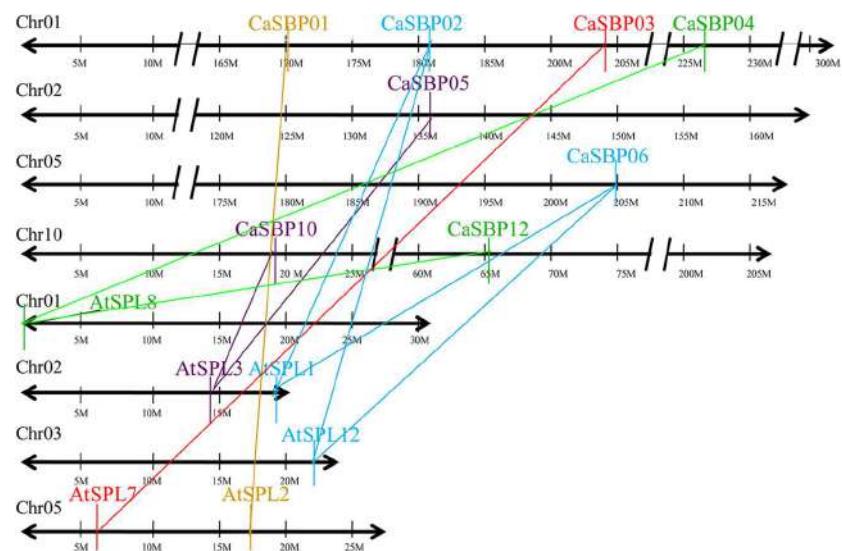


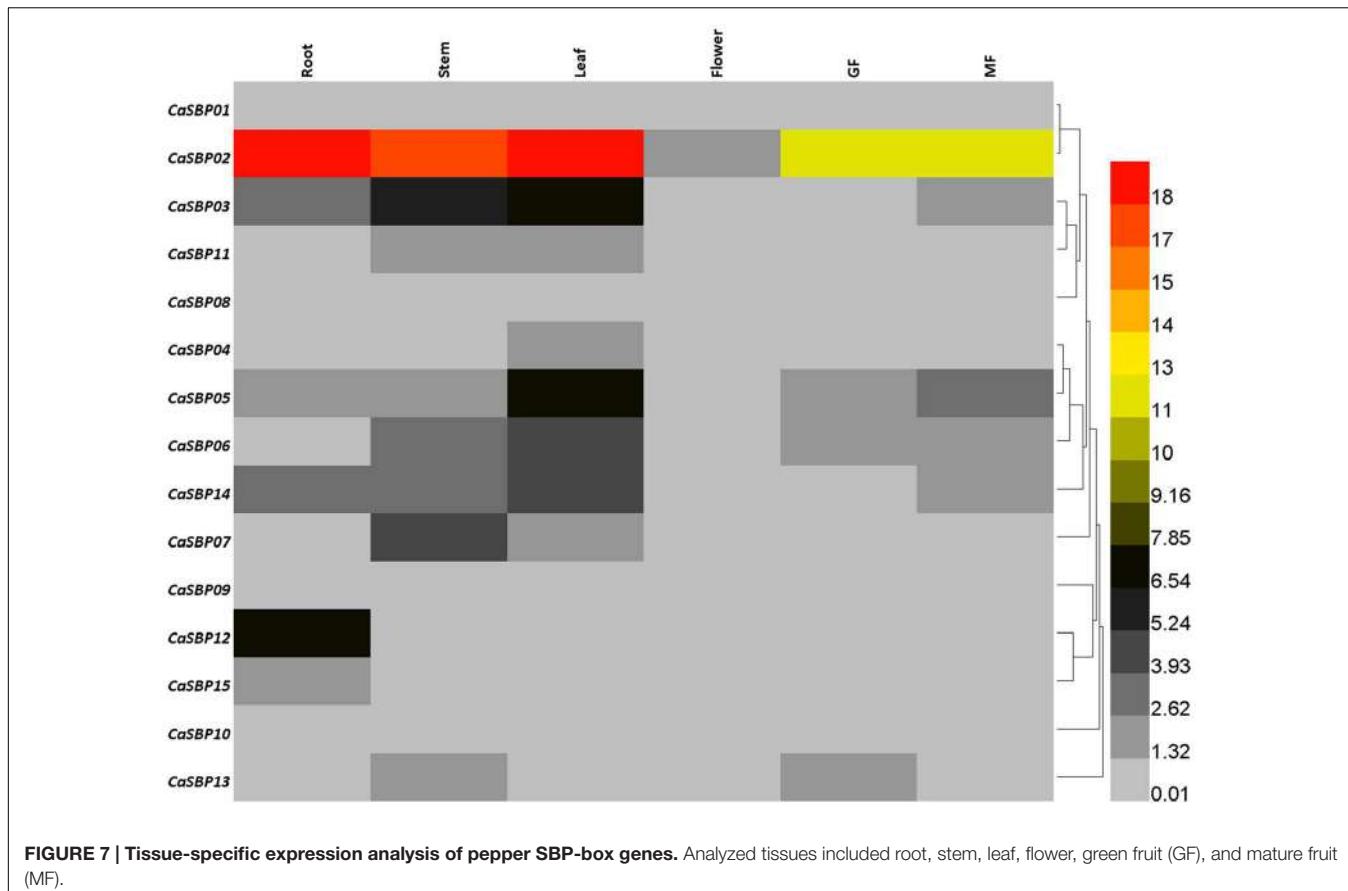
FIGURE 6 | Duplication analysis of SBP-box genes between pepper and *Arabidopsis* genomes. The positions of related CaSBP and AtSPL genes are depicted on pepper chromosomes 1, 2, 5, and 10 and *Arabidopsis* chromosomes 1, 2, 3, and 5. Colored lines connecting two chromosomal regions indicate duplicated regions between pepper and *Arabidopsis* chromosomes.

types (Figure 7). The minority of *CaSBP* genes, specifically *CaSBP01*, *CaSBP08*, *CaSBP09*, and *CaSBP10*, exhibited low-level, constitutive expression in all pepper tissues/organs examined. The remaining *CaSBP* genes were only expressed in certain tissues or organs. *CaSBP02* was the most highly expressed SBP-box gene in the examined tissues. In general, the expression of *CaSBP* genes was highest in the leaf, followed by the stem, root, green fruit, mature fruit, and flowers.

Expression Analysis of *CaSBP* Genes under *P. capsici* and Hormone Treatments

To investigate the effect of *P. capsici* infection on the expression of *CaSBP* genes, roots from the AA3 cultivar were inoculated

with compatible and incompatible *P. capsici* strains, and changes in gene expression were analyzed using qRT-PCR (Figure 8). The results indicate that after inoculation with either the compatible or incompatible strain, four *CaSBP* genes (*CaSBP02*, *CaSBP05*, *CaSBP06*, and *CaSBP13*) were up-regulated 0–24 h post-inoculation and subsequently down-regulated, while *CaSBP04* was up-regulated 0–12 h and then down-regulated. Similarly, *CaSBP14* was up-regulated 0–6 h post-inoculation and subsequently down-regulated. Following inoculation with just the incompatible strain, four genes (*CaSBP01*, *CaSBP03*, *CaSBP05*, and *CaSBP08*) exhibited down-regulation 0–12 h post-inoculation, followed by up-regulation to 24 h and subsequent down-regulation again. *CaSBP10* and *CaSBP11* exhibited the same pattern but following inoculation with the compatible strain only. Following compatible strain inoculation, four



genes (*CaSBP01*, *CaSBP02*, *CaSBP03*, and *CaSBP12*) were up-regulated 0–24 h and subsequently down-regulated, while two genes (*CaSBP07* and *CaSBP09*) were up-regulated 0–6 h after inoculation with the incompatible strain and then down-regulated. Moreover, *CaSBP09* exhibited consistent down-regulation following inoculation with the compatible strain, and *CaSBP12* exhibited up-regulation 0–48 h after inoculation with the incompatible strain. Generally, the expression patterns of *CaSBPs* after inoculation with *P. capsici* can be divided into five categories. The first and second categories contain one gene each, *CaSBP04* and *CaSBP10*, whose expression peaked at 12 and 48 h, respectively, after inoculation with either the compatible or incompatible strain. The third category contains seven genes (*CaSBP01*–*CaSBP03*, *CaSBP05*, *CaSBP06*, *CaSBP11*, and *CaSBP13*) whose expressions peaked 24 h after inoculation with either the compatible or incompatible strain. The fourth category contains two genes, *CaSBP08* and *CaSBP12*, whose expressions peaked earlier following inoculation with the compatible strain than following inoculation with the incompatible strain. The fifth category contains four genes (*CaSBP07*, *CaSBP09*, *CaSBP14*, and *CaSBP15*), whose expressions were down-regulated 12 h after inoculation with either the compatible or incompatible strain.

To investigate the expression patterns of *CaSBPs* in response to treatment with various signal molecules, five representative genes (*CaSBP04*, *CaSBP10*–*12*, and *CaSBP15*), one from each

of the five categories above, were treated with SA inhibitor (PBZ) or MeJA inhibitor (SHAM), and changes in gene expression were analyzed using qRT-PCR (Figure 9). Results showed that the expression of all five genes was rapidly down-regulated 0–6 h after treatment with SA inhibitor (PBZ) or MeJA inhibitor (SHAM), reaching the lowest level at 6 h. After 24 h of treatment, the corresponding inducer (SA or MeJA) was applied. Subsequently, the expression levels of the five genes after SA treatment peaked at 12 h, with the exception of *CaSBP11*, which peaked at 48 h. Following MeJA treatment, expression levels of the five genes peaked earlier than 12 h.

DISCUSSION

Most evidence suggests that SBP-box genes play central roles in plant development, signal transduction, and defense processes (Schwarz et al., 2008; Shikata et al., 2009; Hou et al., 2013b). Benefiting from the availability of genome sequences, the functions of SBP-box genes have been characterized in many plants, including *Arabidopsis*, *S. miltiorrhiza* (Zhang et al., 2014), rice (Yang et al., 2008), tomato (Yang et al., 2008), *Populus trichocarpa* (Li and Lu, 2014), grape (Hou et al., 2013a), apple (Li et al., 2013), *G. hirsutum* (Zhang et al., 2015), *Prunus mume* (Xu et al., 2015), castor bean (Zhang and Ling, 2014), and

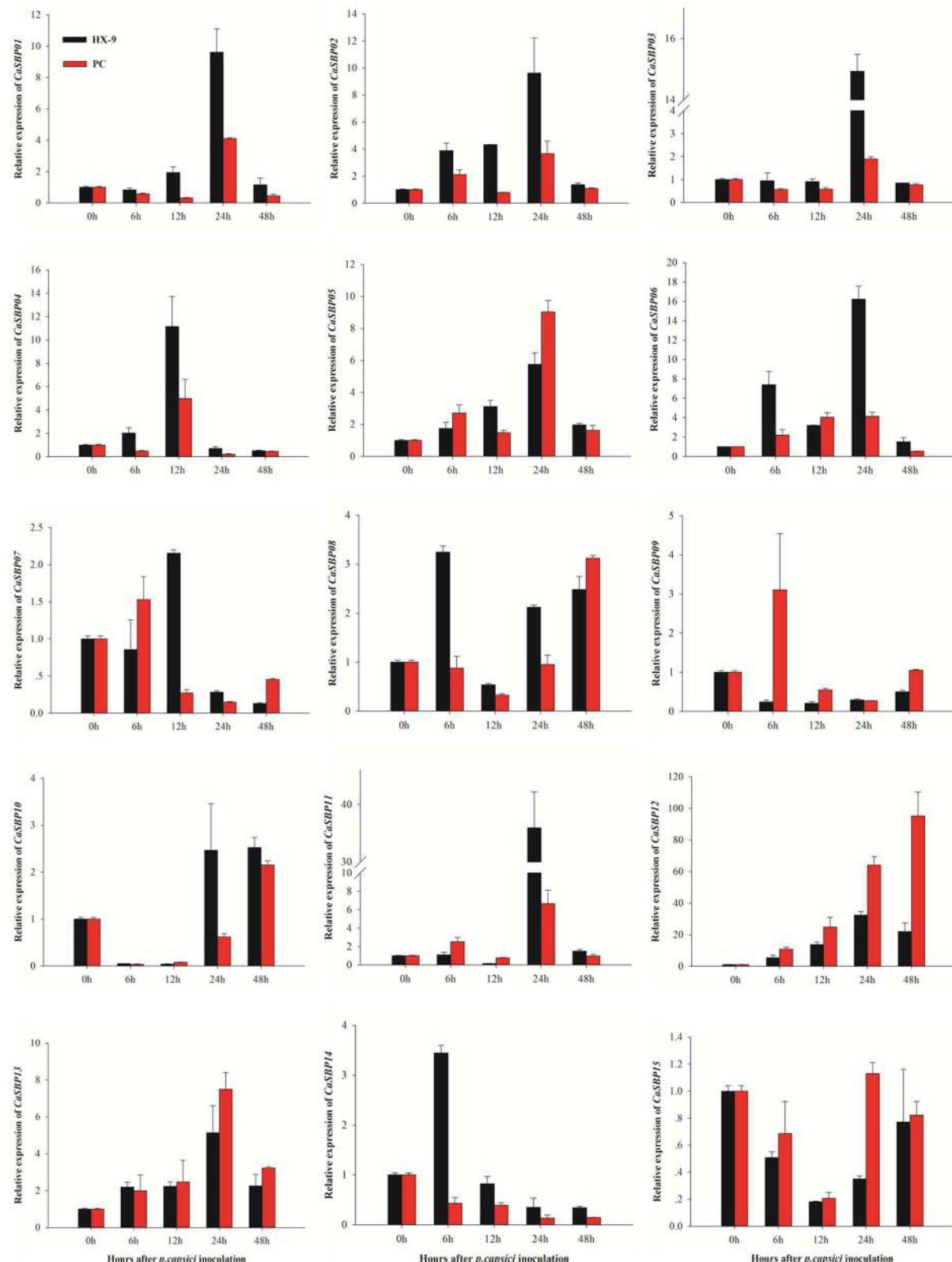


FIGURE 8 | Expression profiles of CaSBPs in response to inoculation with compatible or incompatible *Phytophthora capsici* strains. Mean values and SDs for three replicates are shown.

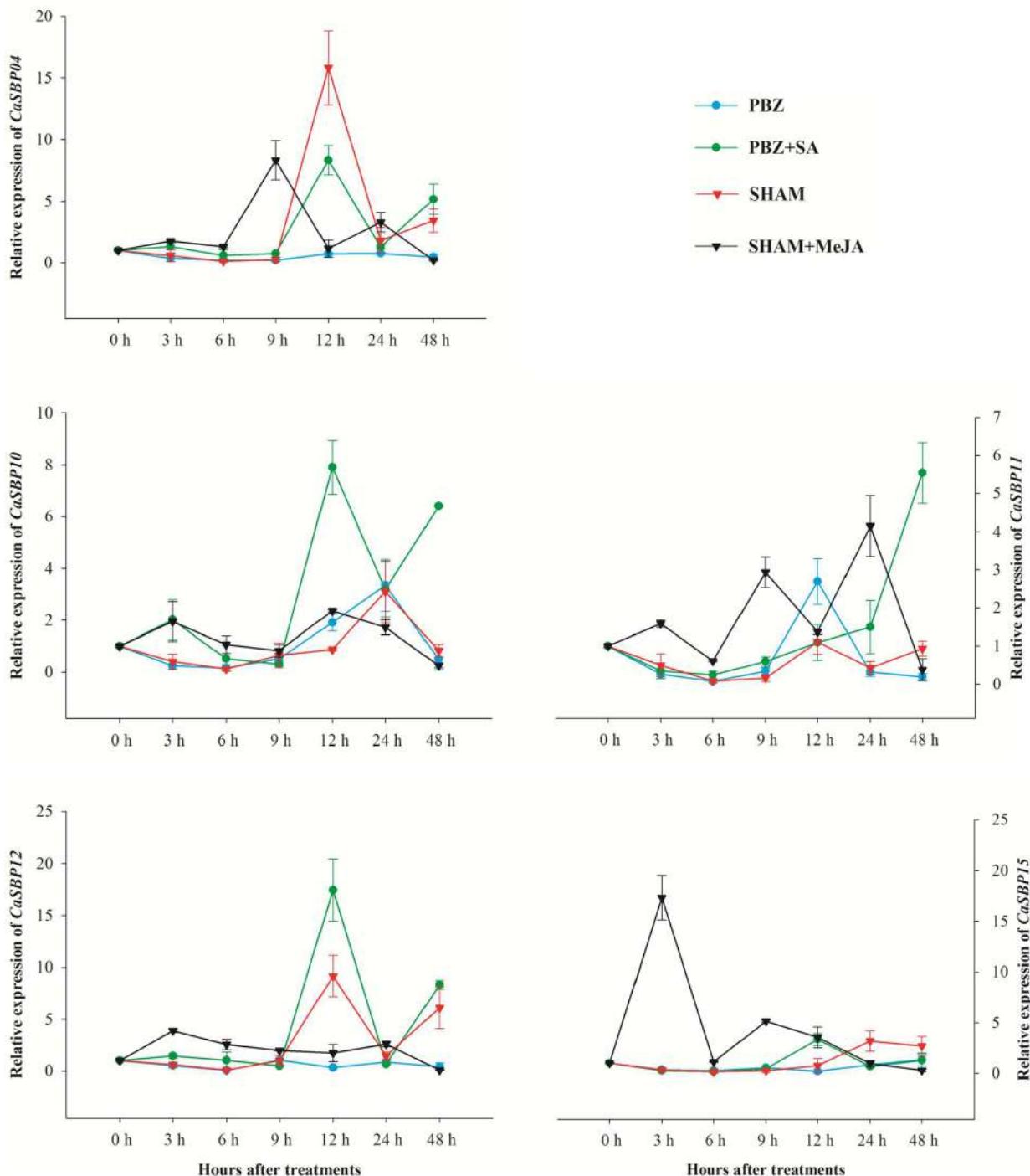


FIGURE 9 | Expression profiles of *CaSBPs* in response to treatment with SA or MeJA hormones and the corresponding inhibitors PBZ or SHAM.
Mean values and SDs for three replicates are shown.

citrus (Shalom et al., 2015). However, the functions of pepper SBP-box TFs are still unknown. In this study, through genome-wide identification and molecular cloning, we discovered the first set of *CaSBP* genes (Table 1). In total, we identified

15 *CaSBPs* in pepper, a number similar to that found in *S. miltiorrhiza* (Zhang et al., 2014), *P. mume* (Xu et al., 2015), castor bean (Zhang and Ling, 2014), and citrus (Shalom et al., 2015).

Phylogenetic tree analysis showed that SBPs from representative plants are clustered into six groups, with *CaSBP* genes distributed across all six groups (**Figure 2**). In addition, each group contains at least one gene from *Arabidopsis*, tomato, and rice. *CaSBP* genes are more closely related to genes from tomato or *Arabidopsis* than to rice SBP-box genes, reflecting the fact that *Arabidopsis*, tomato, and pepper are eudicots and diverged more recently from a common ancestor (Li et al., 2013). These results indicate that although plant SBP-box genes may be derived from a common ancestor, many have undergone distinct patterns of differentiation with the divergence of different lineages. Gene structure analyses showed that within the same phylogenetic group, most *CaSBP* genes shared similar intron/exon structures, indicating that the evolution of SBP domains may be closely related to the diversification of gene structures, as described previously in tomato (Wan et al., 2013), rice (Xie et al., 2006), apple (Li et al., 2013), and grape (Hou et al., 2013a). *CaSBP* genes are distributed across seven of the twelve pepper chromosomes, with no *CaSBP* genes on chromosomes 3, 4, 6, 9, or 12. Similarly, only chromosomes 6, 8, 9, and 11 lack SBP genes in tomato, suggesting that SBP genes may have been widely distributed across the genome of the *Solanaceae* common ancestor.

Gene duplication events include tandem, segmental, and whole-genome duplications, and they have played crucial roles in the evolution of various organisms (Xu et al., 2012). In the SBP-box gene family, there are two pairs of *Arabidopsis* (*AtSPL1/12* and *AtSPL4/5*), six pairs of rice genes (*OsSPL1/6*, *OsSPL3/12*, *OsSPL4/11*, *OsSPL5/10*, *OsSPL14/17*, and *OsSPL16/18*), eight pairs of apple genes (*MdSBP1B/9*, *MdSBP4A-B/20*, *MdSBP8/27A-B*, *MdSBP10/21*, *MdSBP10/22*, *MdSBP11/21*, *MdSBP12/23*, and *MdSBP13/15*), and six pairs of grape genes (*VvSBP2/15*, *VvSBP3/12*, *VvSBP5/7*, *VvSBP9/11*, *VvSBP9/18*, and *VvSBP11/18*) located within segmental duplications (Xie et al., 2006; Li et al., 2013; Hou et al., 2013a). Similarly, we used the criteria described by Gu et al. (2002) and confirmed that four pairs of pepper SBP-box genes (*CaSBP02/06*, *CaSBP04/12*, *CaSBP05/10*, and *CaSBP09/15*) are located in putative segmental duplications. Therefore, it is clear that segmental duplications have played an important role in the expansion of the plant SBP-box gene family.

Comparative genomic analysis is a relatively rapid and effective way to transfer genomic knowledge acquired in one taxon to another, whose genome structure, function, and/or evolution are less known (Lyons et al., 2008). Thus, putative functions of pepper SBP-box genes can be inferred via comparison with orthologs in well-studied model plants such as *Arabidopsis*. In this study, duplication analysis between pepper and *Arabidopsis* indicated that ten pairs of SBP-box genes (*CaSBP01/AtSPL02*, *CaSBP02-06/AtSPL1-12*, *CaSBP03/AtSPL7*, *CaSBP04-12/AtSPL8*, and *CaSBP05-10/AtSPL3*) are located in syntenic genomic regions and represent putative orthologs (**Figure 6**). To date, the majority of *Arabidopsis* SBP-box genes, including *AtSPL2* (Shikata et al., 2009), *AtSPL3* (Yamaguchi et al., 2009), *AtSPL4* (Jung et al., 2011), *AtSPL5* (Jung et al., 2011), *AtSPL6* (Padmanabhan et al., 2013), *AtSPL7* (Yamasaki et al., 2009), *AtSPL8* (Zhang et al., 2007; Xing et al., 2010), *AtSPL9* (Cui et al., 2014), *AtSPL10* (Shikata et al., 2009), *AtSPL11* (Shikata

et al., 2009), *AtSPL13* (Martin et al., 2010), *AtSPL14* (Stone et al., 2005), and *AtSPL15* (Schwarz et al., 2008) have been functionally characterized. Therefore, the functions of several *CaSBP* gene homologs, such as *CaSBP01-CaSBP05*, *CaSBP10*, and *CaSBP12*, can be predicted based on their *Arabidopsis* counterparts. Further experiments are necessary to confirm these functions.

In order to further reveal the possible roles of *CaSBP* genes in pepper growth and development, the expression profile of each *CaSBP* gene was investigated in six different tissues. Results indicate that *CaSBP* genes exhibit different expression patterns (**Figure 7**). While a few *CaSBP* genes (*CaSBP01*, *CaSBP08-CaSBP10*) demonstrated low-level, constitutive expression in all tissues or organs examined, the majority were limited to certain tissues/organs, with *CaSBP02* exhibiting the highest expression across all tissues. The transcription levels of *CaSBP03*, *CaSBP05*, and *CaSBP06* were also higher than other *CaSBP* genes in root, stem, and leaf, consistent with the results of previous sequencing in hot peppers (Kim et al., 2014). In addition, the expression of *CaSBP* genes in flowers and fruits was lower than that in roots, stems, and leaves, similar to results from grapes (Hou et al., 2013a), which may indicate that *CaSBP* genes play a role in the transition from vegetative to reproductive growth. Unlike *MdSBP* genes in apple (Li et al., 2013), however, *CaSBP* expression patterns were not correlated with gene location, gene length, gene structure, or gene sequence.

Most *CaSBP* genes were up-regulated after inoculation with compatible and incompatible *P. capsici*. Specifically, *CaSBP02*, *CaSBP05*, *CaSBP06*, *CaSBP11*, *CaSBP12*, and *CaSBP13* exhibited significantly higher expression under *P. capsici* stress conditions in pepper roots (**Figure 8**). In addition, the transcript levels of *CaSBP05*, *CaSBP12*, and *CaSBP13* were up-regulated more rapidly and more intensely following inoculation with the strain than with the compatible strain. Recent studies have indicated that a novel peroxidase (*CanPOD*) and oxysterol-binding protein (*CanOBP*) genes, which are involved in the defense response to *P. capsici* infection, exhibit expression patterns similar to these *CaSBPs* (Liu, 2009; Wang et al., 2013b). Moreover, similar expression patterns are also found in some defense-related genes – such as the disease-associated protein gene (*CABPR1*), β -1,3-glucanase gene (*CABGLU*), and peroxidase gene (*CAPO1*) – in pepper roots after inoculation with compatible and incompatible *P. capsici* (Wang, 2013). However, according to Kim and Hwang (2000), the expression of *CABPR1* is higher in the compatible interaction than in the incompatible interaction. While differences in expression changes between *CaSBP* and *CABPR1* genes may be due to differences in inoculation of the *P. capsici* strains or to differences in the compatibility systems, it suggests that these genes are related to the pepper's resistance to *P. capsici*. Phylogenetic tree analysis showed that *CaSBP02* and *CaSBP06* exhibited a close relationship with *AtSPL14*, which has been found to be involved in programmed cell death and plays a role in sensitivity to fumonisin B1 (Stone et al., 2005). Moreover, the ortholog of *AtSPL14* and *VpSBP5* is likely to participate in regulating resistance to *E. necator* (Hou et al., 2013a). It also has been reported that *AtSPL* genes are co-expressed with two TFs, *TGA1*, and *WRKY65*, which are induced by pathogens and

regulate the expression of several stress-responsive genes, such as pathogenesis-related 1 protein (*PR-1*) and GLUTATHIONE S-TRANSFERASE 6 (*GST6*; Wang et al., 2009). Based on the above results, we speculate that these *SBP* genes may be involved in disease resistance, but this will need to be verified.

The signal transduction pathway mediated by salicylic acid (SA) and methyl jasmonate (MeJA) is linked to the plant defense response (Thomma et al., 2001; An et al., 2008; Choi and Hwang, 2011). SA typically mediates basal defense to biotrophic pathogens (Thomma et al., 2001), while MeJA generally controls defensive reactions to necrotrophs (Glazebrook, 2005). Therefore, we investigated the responses of five representative *CaSBPs* (*CaSBP04*, *CaSBP10*, *CaSBP11*, *CaSBP12*, and *CaSBP15*) to plant hormone signals by examining their transcript levels in pepper leaves upon treatment with SA or MeJA and their corresponding biosynthesis inhibitors. The expression levels of most genes peaked at 12 h following SA treatment, the exception being *CaSBP11*, which peaked at 48 h. Following MeJA treatment, the maximum expression of all five genes occurred earlier than after SA treatment. It has been reported that SA and MeJA can induce the expression of defense-related gene *PR-1* in tobacco (Xu et al., 1994; Vidal et al., 1997). Moreover, SA induces the recruitment of *trans*-activating TGA factors to the promoter of a defense gene in *Arabidopsis* (Johnson et al., 2003). The *Arabidopsis* SBP-box gene *AtSPL2* and the grape SBP-box gene *VpSBP5* also exhibit responsiveness to biotic stress signaling hormones (Jung et al., 2007; Hou et al., 2013b). Therefore, we speculate that these genes may be involved in the response to various plant stress hormones, particularly the MeJA-induced necrotroph pathway.

CONCLUSION

In this study, we identified SBP-box genes in pepper and analyzed them via sequence alignment, phylogenetic analysis, intron/exon

REFERENCES

- An, S. H., Sohn, K. H., Choi, H. W., Hwang, I. S., Lee, S. C., and Hwang, B. K. (2008). Pepper pectin methylesterase inhibitor protein CaPMEI1 is required for antifungal activity, basal disease resistance and abiotic stress tolerance. *Planta* 228, 61–78. doi: 10.1007/s00425-008-0719-z
- Biles, C. L., Brunton, B. D., Wall, M. M., and Rivas, M. (1995). *Phytophthora capsici* zoospore infection of pepper fruit in various physical environments. *Proc. Okla. Acad. Sci.* 75, 1–5.
- Birkenbihl, R. P., Jach, G., Saedler, H., and Huijser, P. (2005). Functional dissection of the plant-specific SBP-Domain: overlap of the DNA-binding and nuclearlocalization domains. *J. Mol. Biol.* 352, 585–596. doi: 10.1016/j.jmb.2005.07.013
- Cardon, G., Höhmann, S., Klein, J., Nettesheim, K., Saedler, H., and Huijser, P. (1999). Molecular characterisation of the *Arabidopsis* SBP-box genes. *Gene* 237, 91–104. doi: 10.1016/S0378-1119(99)00308-X
- Cardon, G. H., Höhmann, S., Nettesheim, K., Saedler, H., and Huijser, P. (1997). Functional analysis of the *Arabidopsis thaliana* SBP-box gene SPL3 a novel gene involved in the floral transition. *Plant J.* 12, 367–377. doi: 10.1046/j.1365-313X.1997.12020367.x
- Choi, D. S., and Hwang, B. K. (2011). Proteomics and functional analyses of pepper abscisic acid-responsive 1 (ABR1), which is involved in cell death and defense signaling. *Plant Cell* 23, 823–842. doi: 10.1105/tpc.110.082081
- Chuck, G., Whipple, C., Jackson, D., and Hake, S. (2010). The maize SBP-box transcription factor encoded by tasselsheath4 regulates bract development and the establishment of meristem boundaries. *Development* 137, 1243–1250. doi: 10.1242/dev.048348
- Cui, L. G., Shan, J. X., Shi, M., Gao, J. P., and Lin, H. X. (2014). The miR156-SPL9-DFR pathway coordinates the relationship between development and abiotic stress tolerance in plants. *Plant J.* 80, 1108–1117. doi: 10.1111/tpj.12712
- Dong, T. X., Cai, K. Z., and Zeng, R. S. (2009). Effects of methyl jasmonate(MeJA)on photosynthetic traits of rice seedlings under drought stress. *Ecol. Environ.* 18, 1872–1876.
- Glazebrook, J. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* 43, 205–227. doi: 10.1146/annurev.phyto.43.040204.135923
- Gu, Z. L., Cavalcanti, A., Chen, F. C., Bouman, P., and Li, W. H. (2002). Extent of gene duplication in the genomes of drosophila, nematode, and yeast. *Mol. Biol. Evol.* 19, 256–262. doi: 10.1093/oxfordjournals.molbev.a004079
- Guo, M., Lu, J. P., Zhai, Y. F., Chai, W. G., Gong, Z. H., and Lu, M. H. (2015). Genome-wide analysis, expression profile of heat shock factor gene family structure, chromosomal location, and duplication analysis. We also assessed the expression profiles of pepper *SBP* genes across different tissues (root, stem, leaf, flower, and fruit) and under infection with both compatible and incompatible *P. capsici* strains and hormone treatment. Most *CaSBP* genes are expressed at low levels under normal circumstances and are induced by *P. capsici* and hormones, indicating that these genes may be involved in the resistance pathways mediated by *P. capsici*, SA, and MeJA. Candidate pepper SBP-box genes from this analysis should be further functionally characterized for deeper understanding of the precise regulatory checkpoints that operate during stress responses.

AUTHOR CONTRIBUTIONS

H-XZ, W-GC, and Z-HG conceived and designed the experiments. H-XZ, J-HJ, Y-MH, D-WL, B-YL, and AK performed the experiments. H-XZ analyzed the data. W-GC and Z-HG contributed reagents/materials/analysis tools. H-XZ wrote the paper.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00504>

- (CaHsfs) and characterisation of CaHsfA2 in pepper (*Capsicum annuum* L.). *BMC Plant Biol.* 15:151. doi: 10.1186/s12870-015-0512-7
- Guo, W. L., Chen, R. G., Gong, Z. H., Yin, Y. X., Ahmed, S. S., and He, Y. M. (2012). Exogenous abscisic acid increases antioxidant enzymes and related gene expression in pepper (*Capsicum annuum*) leaves subjected to chilling stress. *Genet. Mol. Res.* 11, 4063–4080. doi: 10.4238/2012.September.10.5
- Hausbeck, M. K., and Lamour, K. H. (2004). *Phytophthora capsici* on vegetable crops: research progress and management challenges. *Plant Dis.* 88, 1292–1303. doi: 10.1094/PDIS.2004.88.12.1292
- Hou, H., Li, J., Gao, M., Singer, S. D., Wang, H., Mao, L., et al. (2013a). Genomic organization, phylogenetic comparison and differential expression of the SBP-box family genes in grape. *PLoS ONE* 8:e59358. doi: 10.1371/journal.pone.0059358
- Hou, H., Yan, Q., Wang, X. P., and Xu, H. (2013b). A SBP-Box gene VpSBP5 from Chinese wild vitis species responds to erysipelas necator and defense signaling molecules. *Plant Mol. Biol.* 91, 1261–1270. doi: 10.1007/s11105-013-0591-2
- Johnson, C., Boden, E., and Arias, J. (2003). Salicylic acid induces the recruitment of trans-activating TGA factors to a defense gene promoter in *Arabidopsis*. *Plant Cell* 15, 1846–1858. doi: 10.1105/tpc.012211
- Jung, C., Yeo, S. Y., Koo, Y. J., Kim, M., Choi, Y. D., and Cheong, J. J. (2007). Transcript profile of transgenic *Arabidopsis* constitutively producing methyl jasmonate. *J. Plant Biol.* 50, 12–17. doi: 10.1007/BF03030594
- Jung, J. H., Seo, P. J., Kang, S. K., and Park, C. M. (2011). miR172 signals are incorporated into the miR156 signaling pathway at the SPL3/4/5 genes in *Arabidopsis* developmental transitions. *Plant Mol. Biol.* 76, 35–45. doi: 10.1007/s11103-011-9759-z
- Kim, S., Park, M., Yeom, S. I., Kim, Y. M., Lee, J. M., Lee, H. A., et al. (2014). Genome sequence of the hot pepper provides insights into the evolution of pungency in *Capsicum* species. *Nat. Genet.* 46, 270–278. doi: 10.1038/ng.2877
- Kim, Y. J., and Hwang, B. K. (2000). Pepper gene encoding a basic pathogenesis-related 1 protein is pathogen and ethylene inducible. *Physiol. Plant.* 108, 51–60. doi: 10.1034/j.1399-3054.2000.108001051x
- Klein, J., Saedler, H., and Huijser, P. (1996). A new family of DNA binding proteins includes putative transcriptional regulators of the *Antirrhinum majus* floral meristem identity gene SQUAMOSA. *Mol. Gen. Genet.* 250, 7–16. doi: 10.1007/BF02191820
- Kousik, C. S., Donahoo, R. S., and Hassell, R. (2012). Resistance in watermelon rootstocks to crown rot caused by *Phytophthora capsici*. *Crop Prot.* 39, 18–25. doi: 10.1016/j.cropro.2012.04.004
- Lannenpaa, M., Janonen, I., Holtta-Vuori, M., Gardemeister, M., Porali, I., and Sopanen, T. (2004). A new SBP-box gene BpSPL1 in silver birch (*Betula pendula*). *Physiol. Plant.* 120, 491–500. doi: 10.1111/j.0031-9317.2004.00254.x
- Li, C. L., and Lu, S. F. (2014). Molecular characterization of the SPL gene family in *Populus trichocarpa*. *BMC Plant Biol.* 14:131. doi: 10.1186/1471-2229-14-131
- Li, J., Hou, H. M., Li, X. Q., Xiang, J., Yin, X. J., Gao, H., et al. (2013). Genome-wide identification and analysis of the SBP-box family genes in apple (*Malus x domestica* Borkh.). *Plant Physiol. Biochem.* 70, 100–114. doi: 10.1016/j.plaphy.2013.05.021
- Liu, H. T., Liu, Y. Y., Pan, Q. H., Yang, H. R., Zhan, J. C., and Huang, W. D. (2006). Novel interrelationship between salicylic acid, abscisic acid, and PIP2-specific phospholipase C in heat acclimation-induced thermotolerance in pea leaves. *J. Exp. Bot.* 57, 3337–3347. doi: 10.1093/jxb/erl098
- Liu, K. K. (2009). *Studies on the Resistance and its Mechanisms of Pepper to Phytophthora capsici*. Ph.D. thesis, Northwest A & F University, Yangling District.
- Liu, R. H., and Meng, J. L. (2003). MapDraw: a Microsoft Excel macro for drawing genetic linkage maps based on given genetic linkage data. *Hereditas* 25, 317–321.
- Lyons, E., Pedersen, B., Kane, J., Alam, M., Ming, R., Tang, H. B., et al. (2008). Finding and comparing synteny regions among *Arabidopsis* and the outgroupspapaya, poplar, and grape: coge with rosids. *Plant Physiol.* 148, 1772–1781. doi: 10.1104/pp.108.124867
- Martin, R. C., Asahina, M., Liu, P. P., Kristof, J. R., Coppersmith, J. L., Pluskota, W. E., et al. (2010). The regulation of post-germinative transition from the cotyledon- to vegetative-leaf stages by microRNA-targeted SQUAMOSA PROMOTER-BINDING PROTEIN LIKE13 in *Arabidopsis*. *Seed Sci. Res.* 20, 89–96. doi: 10.1017/S0960258510000073
- Olde, L. M., and Bosland, P. W. (2003). Differentiation of race specific resistance to *Phytophthora* root rot and foliar blight in *Capsicum annuum*. *J. Am. Soc. Hortic. Sci.* 128, 213–218.
- Padmanabhan, M. S., Ma, S., Burch-Smith, T. M., Czymbek, K., Huijser, P., and Dinesh-Kumar, S. P. (2013). Novel positive regulatory role for the SPL6 transcription factor in the N TIR-NB-LRR receptor-mediated plant innate immunity. *PLoS Pathog.* 9:e1003235. doi: 10.1371/journal.ppat.1003235
- Rost, B. (1999). Twilight zone of protein sequence alignments. *Protein Eng.* 12, 85–94. doi: 10.1093/protein/12.2.85
- Salinas, M., Xing, S. P., Hohmann, S., Berndtgen, R., and Huijser, P. (2012). Genomic organization, phylogenetic comparison and differential expression of the SBP-box family of transcription factors in tomato. *Planta* 235, 1171–1184. doi: 10.1007/s00425-011-1565-y
- Schmittgen, T. D., and Livak, K. J. (2008). Analyzing real-time PCR data by the comparative C-T method. *Nat. Protoc.* 3, 1101–1108. doi: 10.1038/nprot.2008.73
- Schwarz, S., Grande, A. V., Bujdoso, N., Saedler, H., and Huijser, P. (2008). The microRNA regulated SBP-box genes SPL9 and SPL15 control shoot maturation in *Arabidopsis*. *Plant Mol. Biol.* 67, 183–195. doi: 10.1007/s11103-008-9310-z
- Shalom, L., Shlizerman, L., Zur, N., Doron-Faigenboim, A., Blumwald, E., and Sadka, A. (2015). Molecular characterization of SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) gene family from *Citrus* and the effect of fruit load on their expression. *Front. Plant Sci.* 6:389. doi: 10.3389/fpls.2015.00389
- Shikata, M., Koyama, T., Mitsuda, N., and Ohme-Takagi, M. (2009). *Arabidopsis* SBP-box genes SPL10, SPL11 and SPL2 control morphological change in association with shoot maturation in the reproductive phase. *Plant Cell Physiol.* 50, 2133–2145. doi: 10.1093/pcp/pcp148
- Stone, J. M., Liang, X., Nekl, E. R., and Stiers, J. J. (2005). *Arabidopsis* AtSPL14, a plant-specific SBP-domain transcription factor, participates in plant development and sensitivity to fumonisins B1. *Plant J.* 41, 744–754. doi: 10.1111/j.1365-313X.2005.02334.x
- Thomma, B. P., Penninckx, I. A., Broekaert, W. F., and Cammue, B. P. (2001). The complexity of disease signaling in *Arabidopsis*. *Curr. Opin. Immunol.* 13, 63–68. doi: 10.1016/S0952-7915(00)00183-7
- Vidal, S., Leon, I. P. D., Denecke, J., and Palva, E. T. (1997). Salicylic acid and the plant pathogen *Erwinia carotovora* induce defense genes via antagonistic pathway. *Plant J.* 11, 115–123. doi: 10.1046/j.1365-313X.1997.110115.x
- Wan, H. J., Yuan, W., Yu, K., Liu, Y. F., Li, Z. M., Ye, Q. J., et al. (2013). Genome-wide identification, structure characterization and expression analysis of SBP gene family in tomato. *Mol. Plant Breed.* 11, 299–306.
- Wang, J. E. (2013). *Expression Analysis and Functional Identification of CaRGA1 and CaPOD Genes Induced by Phytophthora capsici in Pepper*. Ph.D. thesis, Northwest A&F University, Yangling District.
- Wang, J. E., Li, D. W., Zhang, Y. L., Zhao, Q., He, Y. M., and Gong, Z. H. (2013a). Defence responses of pepper (*Capsicum annuum* L.) infected with incompatible and compatible strains of *Phytophthora capsici*. *Eur. J. Plant Pathol.* 136, 625–638. doi: 10.1007/s10658-013-0193-8
- Wang, J. E., Liu, K. K., Li, D. W., Zhang, Y. L., Zhao, Q., He, Y. M., et al. (2013b). A novel peroxidase CanPOD gene of pepper is involved in defense responses to *Phytophthora capsici* infection as well as abiotic stress tolerance. *Int. J. Mol. Sci.* 14, 3158–3177. doi: 10.3390/ijms14023158
- Wang, Y., Hu, Z. L., Yang, Y. X., Chen, X. Q., and Chen, G. P. (2009). Function annotation of an SBP-box gene in *Arabidopsis* based on analysis of co-expression networks and promoters. *Int. J. Mol. Sci.* 10, 116–132. doi: 10.3390/ijms10010116
- Xie, K. B., Wu, C. Q., and Xiong, L. Z. (2006). Genomic organization, differential expression, and interaction of SQUAMOSA promoter-binding-like transcription factors and microRNA156 in rice. *Plant Physiol.* 142, 280–293. doi: 10.1104/pp.106.084475
- Xing, S. P., Salinas, M., Hohmann, S., Berndtgen, R., and Huijser, P. (2010). miR156-targeted and nontargeted SBP-box transcription factors act in concert to secure male fertility in *Arabidopsis*. *Plant Cell* 22, 3935–3950. doi: 10.1105/tpc.110.079343
- Xu, G. X., Guo, C. C., Shan, H. Y., and Kong, H. Z. (2012). Divergence of duplicate genes in exon-intron structure. *Proc. Natl. Acad. Sci. U.S.A.* 109, 1187–1192. doi: 10.1073/pnas.1109047109

- Xu, Y., Chang, P. F. L., Liu, D., Narasimhan, M. N., Raghothama, K. G., Hasegawa, P. M., et al. (1994). Plant defense genes are synergistically induced by ethylene and methyl Jasmonate. *Plant Cell* 6, 1077–1085. doi: 10.1105/tpc.6.8.1077
- Xu, Z. D., Sun, L. D., Zhou, Y. Z., Yang, W. R., Cheng, T. R., Wang, J., et al. (2015). Identification and expression analysis of the SQUAMOSA promoter-binding protein (SBP)-box gene family in *Prunus mume*. *Mol. Genet. Genomics* 290, 1701–1715. doi: 10.1007/s00438-015-1029-3
- Yamaguchi, A., Wu, M. F., Yang, L., Wu, G., Poethig, R. S., and Wagner, D. (2009). The microRNA-regulated SBP-Box transcription factor SPL3 is a direct upstream activator of LEAFY, FRUITFULL, and APETALA1. *Dev. Cell* 17, 268–278. doi: 10.1016/j.devcel.2009.06.007
- Yamasaki, H., Hayashi, M., Fukazawa, M., Kobayashi, Y., and Shikanai, T. (2009). SQUAMOSA promoter binding protein-like7 is a central regulator for copper homeostasis in *Arabidopsis*. *Plant Cell* 21, 347–361. doi: 10.1105/tpc.108.060137
- Yamasaki, K., Kigawa, T., Inoue, M., Tateno, M., Yamasaki, T., Yabuki, T., et al. (2004). A novel zinc-binding motif revealed by solution structures of DNA-binding domains of *Arabidopsis* SBP-family transcription factors. *J. Mol. Biol.* 337, 49–63. doi: 10.1016/j.jmb.2004.01.015
- Yang, Z. F., Wang, X. F., Gu, S. L., Hu, Z. Q., Xu, H., and Xu, C. W. (2008). Comparative study of SBP-box gene family in *Arabidopsis* and rice. *Gene* 407, 1–11. doi: 10.1016/j.gene.2007.02.034
- Yin, Y. X., Guo, W. L., Zhang, Y. L., Ji, J. J., Xiao, H. J., Yan, F., et al. (2014). Cloning and characterisation of a pepper aquaporin, CaAQP, which reduces chilling stress in transgenic tobacco plants. *Plant Cell Tissue Organ Cult.* 118, 431–444. doi: 10.1007/s11240-014-0495-3
- Zhang, L. S., Wu, B., Zhao, D. G., Li, C. L., Shao, F. G., and Lu, S. F. (2014). Genome-wide analysis and molecular dissection of the SPL gene family in *Salvia miltiorrhiza*. *J. Integr. Plant Biol.* 56, 38–50. doi: 10.1111/jipb.12111
- Zhang, S. D., and Ling, L. Z. (2014). Genome-wide identification and evolutionary analysis of the SBP-box gene family in castor bean. *PLoS ONE* 9:e86688. doi: 10.1371/journal.pone.0086688
- Zhang, X. H., Dou, L. L., Pang, C. Y., Song, M. Z., Wei, H. L., Fan, S. L., et al. (2015). Genomic organization, differential expression, and functional analysis of the SPL gene family in *Gossypium hirsutum*. *Mol. Genet. Genomics* 290, 115–126. doi: 10.1007/s00438-014-0901-x
- Zhang, Y., Schwarz, S., Saedler, H., and Huijser, P. (2007). SPL8, a local regulator in a subset of gibberellin-mediated developmental processes in *Arabidopsis*. *Plant Mol. Biol.* 63, 429–439. doi: 10.1007/s11103-006-9099-6
- Zhang, Y. L., Jia, Q. L., Li, D. W., Wang, J. E., Yin, Y. X., and Gong, Z. H. (2013). Characteristic of the pepper CaRGA2 gene indefense responses against *Phytophthora capsici* leonian. *Int. J. Mol. Sci.* 14, 8985–9004. doi: 10.3390/ijms14058985

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Resistance evaluation of Chinese wild *Vitis* genotypes against *Botrytis cinerea* and different responses of resistant and susceptible hosts to the infection

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The necrotrophic fungus *Botrytis cinerea* is a major threat to grapevine cultivation worldwide. A screen of 41 *Vitis* genotypes for leaf resistance to *B. cinerea* suggested species independent variation and revealed 18 resistant Chinese wild *Vitis* genotypes, while most investigated *V. vinifera*, or its hybrids, were susceptible. A particularly resistant Chinese wild *Vitis*, "Pingli-5" (*V. sp.* [Qinling grape]) and a very susceptible *V. vinifera* cultivar, "Red Globe" were selected for further study. Microscopic analysis demonstrated that *B. cinerea* growth was limited during early infection on "Pingli-5" before 24 h post-inoculation (hpi) but not on Red Globe. It was found that reactive oxygen species (ROS) and antioxidative system were associated with fungal growth. O_2^- accumulated similarly in *B. cinerea* 4 hpi on both *Vitis* genotypes. Lower levels of O_2^- (not H_2O_2) were detected 4 hpi and ROS (H_2O_2 and O_2^-) accumulation from 8 hpi onwards was also lower in "Pingli-5" leaves than in "Red Globe" leaves. *B. cinerea* triggered sustained ROS production in "Red Globe" but not in "Pingli-5" with subsequent infection progresses. Red Globe displayed little change in antioxidative activities in response to *B. cinerea* infection, instead, antioxidative activities were highly and timely elevated in resistant "Pingli-5" which correlated with its minimal ROS increases and its high resistance. These findings not only enhance our understanding of the resistance of Chinese wild *Vitis* species to *B. cinerea*, but also lay the foundation for breeding *B. cinerea* resistant grapes in the future.

Keywords: antioxidative system, *Botrytis-Vitis* interactions, Chinese wild *Vitis*, ROS, resistance evaluation

INTRODUCTION

The necrotrophic fungal pathogen *Botrytis cinerea* causes gray mold disease in a broad range of plant species, including grape. Grape production, of great economic importance in China, relies almost exclusively on European grapevine varieties (Iv, 2013); however, these are currently threatened by gray mold disease, especially with the rapid development of protected cultivation (Zhang, 2011; Iv, 2013). Although agronomic, genetic, and biological approaches have been

proposed to limit yield losses caused by gray mold, disease management is still largely based on chemical control (Angelini et al., 2014), which is not sustainable.

B. cinerea is one of the most comprehensively studied necrotrophic plant pathogens which can produce ROS and simultaneously induce host oxidative burst (van Kan, 2006). ROS, such as superoxide and hydrogen peroxide, can delay, or accelerate pathogen proliferation (Temme and Tudzynski, 2009; Afzal et al., 2014), and participate in cell wall modification, programmed cell death and the integration of many different signaling networks (Serrano et al., 2014). In addition, it has also been proposed that they may work as dynamic signaling molecules (Torres et al., 2006; Mittler et al., 2011). Thus, ROS play important and multifaceted roles during the interaction between *B. cinerea* and its plant hosts (Lamb and Dixon, 1997; De Tullio, 2010).

There is considerable evidence that *B. cinerea* can overturn the ROS stress induced in planta to assist its invasion of plant tissues (Govrin and Levine, 2000; Temme and Tudzynski, 2009). ROS have been reported to reduce resistance and accelerate expansion of disease lesions during *B. cinerea-Nicotiana benthamiana* interactions (Asai and Yoshioka, 2009). Tomato (*Solanum lycopersicum*) plants overexpressing the transcription factor *SISHINE3*, which regulates cuticle production, were observed to be more resistant to *B. cinerea* with lower levels of ROS production and more cuticles than wild-type plants (Buxdorf et al., 2014). Nevertheless, the roles of ROS in the interaction between *B. cinerea* and its hosts remain controversial. For example, an induction of oxidative burst resulted in enhanced resistance against *B. cinerea* in *A. thaliana* with the application of the herbicide paraquat (Tierens et al., 2002), and a timely hyperinduction of H₂O₂ in the *sitiens* tomato mutant (deficient in abscisic acid (ABA) synthesis) effectively blocked infection by the pathogen (Asselbergh et al., 2007). Moreover, *A. thaliana* ABA or wax biosynthesis mutants, accompanied by an increased cuticular permeability, were reported to produce ROS earlier and in higher amounts, also showing increased resistance (L'Haridon et al., 2011; Serrano et al., 2014). In another study using bean (*Glycine max*) leaves, it was shown that the secondary oxidative burst was much stronger following challenge by a non-aggressive *B. cinerea* strain than by an aggressive strain, indicating that ROS-mediated responses have the capacity to block infection by the pathogen (Urbanek et al., 1996).

Despite numerous studies those have been conducted regarding the role of ROS in plant-*B. cinerea* interactions, the importance of ROS generation during *B. cinerea* invasion of grapevine has not been extensively examined. The application of bacterial rhamnolipids or BcPG1 (an endopolygalacturonase from *B. cinerea*) to *V. vinifera* was reported to improve resistance to *B. cinerea* by inducing ROS production and the expression of genes involved in defense through different signal pathways (Vandelle et al., 2006; Varnier et al., 2009). Similarly, treatment of grape cells with oligogalacturonides (Aziz et al., 2004) or bacteria, such as *Pseudomonas fluorescens* and *Pantoea agglomerans*, or extracts from these bacteria (Verhagen et al., 2010, 2011), triggered an oxidative burst in tandem with improving resistance to *B. cinerea* to varying degrees. Moreover, Gabler et al. (2003)

found that *V. rotundifolia* and *V. labrusca* were highly resistant, while cultivars of *V. vinifera* were highly susceptible to *B. cinerea*. However, little is known about the potential sources and mechanisms of resistance in grapevines to *B. cinerea*. China is one of the major centers of origin of *Vitis* species (Wang et al., 1995, 1998), and the rich Chinese wild *Vitis* germplasm has been largely utilized for grape breeding programs due to its many desirable characteristics, such as resistance to a variety of fungal diseases and its ability to be easily crossed with *V. vinifera* than the multi-disease resistant *Muscadinia rotundifolia* (Luo and He, 2004).

In this study, *B. cinerea* resistance levels of Chinese wild *Vitis* are reported and the time course of colonization by *B. cinerea* on the leaves of highly resistant and susceptible *Vitis* genotypes is described. Histochemical and physiological evidence for the role of ROS and antioxidative systems in *Vitis-B. cinerea* interactions is presented. Taken together, our data provide a foundation for elucidating the events leading to resistance of Chinese wild *Vitis* to *B. cinerea* and for the future breeding of grape genotypes resistant to this pathogen.

MATERIALS AND METHODS

Plant and Fungal Material

Eleven Chinese wild *Vitis* species and four other *Vitis* species, totaling 41 genotypes, including 30 Chinese wild *Vitis* species, seven *V. vinifera* species, as well as *V. riparia Michaux*. "Hear-3," two *V. vinifera* × *V. labrusca* cv. "Kyoho" and "NO. 8 Hutai" and *V. vinifera* × *V. amurensis* cv. "Beichun," were evaluated from 2011 to 2013 (Table 1). The germplasm was maintained in the vineyard overseen by the grape germplasm and breeding program of Northwest A&F University, Shaanxi, China.

B. cinerea was isolated from "Red Globe" (*V. vinifera*) in the greenhouse and was maintained on Potato Glucose Agar medium in the dark at 22°C. After 21 days, conidia were washed down with distilled water, counted, and added to the inoculation solution at concentrations detailed in the following sections. Conidia were pre-germinated for 2 h at 22°C before inoculations were performed (Asselbergh et al., 2007).

Detached Leaf Evaluation, Fungal Colonization Experiments, and ROS Measurements

Detached leaf assays were carried out using leaves of a similar age and size (leaves at nodes 3 and 4, counted from the top) selected randomly from vines. Detached leaves were washed carefully, first under tap water and then distilled water, and were then quickly transferred to a bed of 0.8% agar in trays and then uniformly sprayed with *B. cinerea* conidia suspension. Trays were covered with preservative film to ensure a relative humidity of 90–100%. All leaves from control (sprayed with distilled water) and inoculation treatments were incubated in the dark for the first 24 h and then in a light/dark (12/12 h) regime at 22°C (Audenaert et al., 2002; Windram et al., 2012).

To evaluate detached leaves (laboratory evaluation), at least 18 leaves from three biological replicates of each genotype

TABLE 1 | Laboratory evaluation results (including macroscopic and light microscopic examination) of 41 *Vitis* genotypes against *Botrytis Cinerea* from 2011 to 2013.

Species	Names of genotypes	Disease Severity ^c	Scores ^d	Rank of scores	Resistance levels ^e	Rates of germination (%)	Rates of infection (%)	Macroscopic mycelium	New sporulation
<i>V. amurensis</i> Rupr	Huaxian-11	5.91 ± 1.86	1.56	35	R	2.91	0.67	— ^f	—
<i>V. amurensis</i> Rupr	Taishan-11	4.58 ± 0.79	1.44	36	HR	12.07	3.45	—	—
<i>V. amurensis</i> Rupr	Zuoshan-1	38.99 ± 1.31	3.78	20	S	63.05	28.14	✓ ^g	—
<i>V. amurensis</i> Rupr	Tonghua-3	0.20 ± 0.05	1.00	40	HR	13.10	6.35	—	—
<i>V. amurensis</i> Rupr	Shuangyou ^b	0.18 ± 0.05	1.00	41	HR	13.17	7.32	—	—
<i>V. romanetii</i> Roman.	Pingli-2	46.98 ± 1.20	4.89	11	S	63.71	47.18	✓	✓
<i>V. romanetii</i> Roman.	Baihe-22	29.71 ± 2.73	3.44	21	S	56.14	38.16	—	—
<i>V. romanetii</i> Roman.	Liuba-11	46.59 ± 2.09	4.78	13	S	46.67	42.22	✓	—
<i>V. romanetii</i> Roman.	Jiangxi-2	49.33 ± 3.36	4.89	12	S	61.90	52.86	✓	✓
<i>V. quinquangularis</i> Rehd.	Shang-24	70.48 ± 5.81	6.00	3	HS	51.85	43.70	✓	—
<i>V. quinquangularis</i> Rehd.	Taishan-12	21.96 ± 2.18	3.00	25	R	19.38	9.69	—	—
<i>V. quinquangularis</i> Rehd.	83-4-85 ^a	21.38 ± 2.95	2.89	27	R	12.39	7.34	✓	—
<i>V. quinquangularis</i> Rehd.	83-4-96 ^a	42.9 ± 2.73	4.33	17	S	42.35	14.12	—	—
<i>V. piasezkii</i> Maxim	Liuba-6	18.04 ± 0.59	3.00	24	R	29.74	21.24	—	—
<i>V. piasezkii</i> Maxim	Liuba-7	16.68 ± 1.19	2.78	29	R	21.91	16.36	—	—
<i>V. piasezkii</i> Maxim	Gansu-91	12.64 ± 0.66	2.11	33	R	30.26	20.61	—	—
<i>V. adstricta</i> Hance	Taishan-1	15.14 ± 1.14	2.56	31	R	23.69	16.47	—	—
<i>V. adstricta</i> Hance	Taishan-2	2.08 ± 0.43	1.00	38	HR	23.49	15.36	—	—
<i>V. adstricta</i> Hance	Anlin-3	16.74 ± 1.65	2.78	30	R	46.88	38.92	✓	—
<i>V. davidii</i> Foex	Lueyang-4	55.63 ± 2.60	5.11	10	S	64.58	55.56	✓	✓
<i>V. davidii</i> Foex	Ningqiang-6	59.79 ± 1.10	5.56	7	HS	77.03	70.27	✓	✓
<i>V. davidii</i> Foex	Tangwei ^b	7.00 ± 1.52	1.89	34	R	74.65	62.50	✓	—
<i>V. davidii</i> Foex	Fujian-4	46.32 ± 3.09	4.56	15	S	54.01	36.36	✓	—
<i>V. pseudoreticulata</i> W.T. Wang	Guangxi-1	22.97 ± 2.57	3.11	22	R	26.06	15.49	—	—
<i>V. pseudoreticulata</i> W.T. Wang	Hunan-1	61.40 ± 3.97	5.67	5	HS	82.95	60.08	✓	✓
<i>V. sp.</i> (Maihuang grape)	Baihe-41	28.04 ± 0.86	3.00	26	R	38.10	20.95	—	—
<i>V. sp.</i> (Maihuang grape)	Baihe-36-2	16.54 ± 1.37	2.89	28	R	37.67	22.26	✓	—
<i>V. davidii</i> var. <i>cyanocarpa</i> Sarg.	Zhenan-3	40.43 ± 2.12	4.00	19	S	43.27	33.82	✓	—
<i>V. sp.</i> (Qinling grape)	Pingli-5	3.70 ± 0.90	1.22	37	HR	28.06	12.23	—	—
<i>V. yenshanensis</i>	Yanshan-1	0.36 ± 0.16	1.00	38	HR	29.19	9.81	—	—
<i>V. vinifera</i> L.	NO19 Xinong	38.27 ± 2.35	4.00	18	S	58.70	51.09	✓	✓
<i>V. vinifera</i> L.	Rizamat	24.14 ± 2.62	3.00	23	R	25.58	21.14	✓	—
<i>V. vinifera</i> L.	Hongmu Nage	46.63 ± 3.46	4.67	14	S	87.50	82.95	✓	✓
<i>V. vinifera</i> L.	Zao Jinxiang	13.06 ± 0.89	2.11	32	R	46.54	37.11	—	—
<i>V. vinifera</i> L.	Muscat Hamburg	59.69 ± 6.12	5.44	9	S	86.08	64.64	✓	✓
<i>V. vinifera</i> L.	Red Face Seedless	60.59 ± 2.17	5.56	6	HS	72.40	61.99	✓	✓
<i>V. vinifera</i> L.	Red Globe	72.25 ± 3.57	6.11	2	HS	88.77	70.01	✓	✓
<i>V. riparia</i> Michaux	Hean-3	43.57 ± 2.13	4.33	16	S	84.85	55.56	✓	✓
<i>V. vinifera</i> L. × <i>V. labrusca</i> L.	Kyoto	58.11 ± 6.49	5.56	8	HS	72.88	54.95	✓	✓
<i>V. vinifera</i> L. × <i>V. labrusca</i> L.	NO8 Hutai	77.82 ± 6.17	6.33	1	HS	79.54	70.96	✓	✓
<i>V. vinifera</i> L. × <i>V. amurensis</i> Rupr	Beichun	66.90 ± 6.17	5.89	4	HS	65.26	54.21	✓	✓

^aThe genotypes were selected from seedlings of *V. qinqangularis* (Wang et al., 1995).^bThe flower type of the genotypes were hermaphrodites under natural conditions (Wang et al., 1995).^cDisease Severity: the average percentage of spreading lesions determined by observing at least 10 leaves in each repeated experiment from 2011 to 2013.^dScore: disease severity was scored as previously described (Liu et al., 2003; Patykowski, 2006; Foyer and Noctor, 2013).^eResistance level: Highly Resistant (HR: scores of 0–1.50); Resistant (R: scores of 1.51–3.50); Susceptible (S: scores of 3.51–5.50); Highly Susceptible (HS: scores of 5.51–7).^f✓/Mycelium or sporulation were observed by the naked eye on leaf surfaces.^g—No mycelium or sporulation was observed by the naked eye on leaf surfaces.

were tested. Four days after inoculation, the infection was evaluated by counting the percentage of spreading lesions on each leaf. Before evaluation, the optimal inoculation solution

and conidia concentration of *B. cinerea* were determined. The conidia germination in solutions with different glucose (Glc) and phosphate concentrations was determined under a light

microscope after 6 and 24 h. The four solutions tested in this study were: (i) sterile; (ii) 1×10^6 spores mL $^{-1}$, 0.1 M Glc, 67 mM KH₂PO₄; (iii) 1×10^6 spores mL $^{-1}$, 0.05 M Glc, 33 mM KH₂PO₄; and (iv) 1×10^6 spores mL $^{-1}$, 0.01 M Glc, 6.7 mM KH₂PO₄ (Audenaert et al., 2002). Detached leaves of Red Globe and four Chinese wild grapevines, "Shang-24" (*V. quinquangularis*), "Hunan-1" (*V. pseudoreticulata*), "Taishan-2" (*V. adstrica*), "Baihe-41" (*V. sp. [Maihuang grape]*) were evaluated after infection with conidia suspensions of different concentrations (1×10^7 spores mL $^{-1}$; 1.5×10^6 spores mL $^{-1}$; 5×10^5 spores mL $^{-1}$ and 5×10^4 spores mL $^{-1}$).

For time series experiments, single inoculated, and control leaves were sampled 4, 8, 12, 18, 24, 36, 48, 72, and 96 hpi (hours post-inoculation) in a randomized manner from each of three biological replicates, except in the case of samples used for DAB (diaminobenzidine) staining.

Rating of Disease Severity

Disease severity was evaluated from 2011 to 2013 and scored as previously described (Liu et al., 2003; Poolsawat et al., 2012). Disease resistance levels of the different genotypes were classified as: Highly Resistant (HR: scores of 0–1.50); Resistant (R: scores of 1.51–3.50); Susceptible (S: scores of 3.51–5.50); or Highly Susceptible (HS: scores of 5.51–7.0).

Light Microscopy and Scanning Electron Microscopy

To characterize the colonization of "Pingli-5" (HR, Highly Resistant) and "Red Globe" (HS, Highly Susceptible) by *B. cinerea*, 2–3 cm 2 leaf pieces were collected at 4, 8, 12, 18, 24, 36, 48, 72, and 96 hpi, fixed, and decolorized in ethanol/trichloromethane (3:1, v/v) containing 0.15% (w/v) trichloroacetic acid, before clearing in saturated chloral hydrate, and were then stored in 20% glycerol. Samples were subsequently stained with aniline blue solution (for staining fungal tissues a blue color) and examined with an Olympus BX-51 microscope (Olympus Corporation, Japan). For each sample, fungal germination, and infection percentages were examined. For scanning electron microscopy (SEM), leaf tissues were cut into small pieces (0.5–1 cm 2), fixed in 4% (v/v) glutaraldehyde in phosphate buffer (0.1 M, pH 6.8) for 12 h at 4°C, and rinsed in the same buffer four times for 10–15 min. After dehydration in a graded ethanol series (30, 50, 70, 80, 90, 100%, v/v), the samples were then critical-point dried, coated with gold in a sputter coater, and examined with a JEOL FESEM S-4800 scanning electron microscope at 15 kV (Cheng et al., 2012).

Histochemical Analysis of ROS Responses

H₂O₂ and O₂⁻ were respectively detected by DAB and NBT (nitro blue tetrazolium) staining protocols, as previously described (ThordalChristensen et al., 1997; Wang et al., 2007) to compare the ROS responses of the two genotypes defined as HR and HS. Two to three centimeter 2 leaf segments were immersed under direct light in a DAB solution (1 mg/mL with HCl acidifying to pH 3.8) 8 h before sample collection except for that samples 4 hpi were directly immersed in DAB solution once inoculated. The leaves were prepared for observation as described above. Leaf

segments of the same size were collected directly into 0.1% (w/v) NBT solution in 10 mM phosphate buffer (pH 7.8) prior to a vacuum infiltration for 30 min and an exposure to direct light for 20 min. The NBT stained samples were then observed as above, except for the omission of aniline blue staining. The percentages of conidia, germ tubes, and infection sites exhibiting O₂⁻ or H₂O₂ accumulation were evaluated.

Antioxidant Enzyme Extraction and Activity Assays

Crude protein extracts to assess superoxide dismutase (SOD) (Mittler et al., 2011) and peroxidase (POD) (Atkinson and Urwin, 2012) activities were isolated from approximately 0.5 g leaves using protocols described by Giannopolitis and Ries (1977). For SOD activity, briefly, 3.4 mL reaction mixtures comprising 50 mM sodium phosphate buffer (pH 7.0), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100 μl crude protein extract were illuminated for 20 min at 4000 Lux and then measured at 560 nm. POD activity was assayed as previously described (Maehly and Chance, 1954). Six-hundred Microliter crude protein extract added to a 3 mL reaction mixture comprising 0.05 M guaiacol and 2% H₂O₂ was measured at 470 nm.

Crude protein extracts for measuring catalase (CAT) (Atkinson and Urwin, 2012) activity were obtained from approximately 2.5 g leaves that was ground in 25 mL cold 0.2 M PBS buffer (pH 7.8). CAT activity was determined by measuring the consumption of H₂O₂ by KMnO₄. The mixture of 3 mL crude protein extract, 2.5 mL 10% H₂SO₄ and 2.5 mL 0.1 M H₂O₂ were incubated for 10 min at 30°C and then titrated with 0.1 M KMnO₄. Samples with 3 mL boiled extract in the reaction mixtures were used as controls. The consumption of 1.7 mL 0.1 M KMnO₄ was assumed to be equal to 1.7 mg H₂O₂. The KMnO₄ solution of 0.1 M was critically determined by 0.1 M oxalic acid GR (Maehly and Chance, 1954).

Statistical Analyses

All experiments were performed using three biological replicates. At least 300 conidia from eight to ten leaf sections per time point were examined in histopathological and histochemical sections. Means and standard errors were calculated from three independent experiments by Microsoft Excel (Microsoft Corporation) and significant differences and Duncan LSD analysis by a completely random design and correlation analyses of resistance evaluation data from 2011 to 2013 were performed using SPSS Statistics (Gabler et al., 2003; Poolsawat et al., 2012). All pictures were combined by Adobe Photoshop (Adobe Systems Incorporated).

RESULTS

The Optimum Inoculum and Concentration of *B. cinerea*

Since some *B. cinerea* isolates germinate readily in distilled water, while others require sugars to initiate an infection (Schumacher and Tudzynski, 2012), a comparative assay was performed to

determine the optimal inoculation solution, as well as a moderate concentration of *B. cinerea* conidia to be used in the subsequent experiments (Figure S1). *V. vinifera* cv. "Red Globe" and *V. adstricta* "Taishan-2" have previously been tested and found to be HS and HR species, respectively. Three other genotypes "Shang-24" (*V. quinquangularis*), "Hunan-1" (*V. pseudoreticulata*) and "Baihe-41" (*V. sp.* [Maihuang grape]), were also randomly selected for the comparative assay with different concentrations of spores in sterile water (Figure S1A). The *B. cinerea* used in the present study performed substantially better for higher spore germination rate after 24 h in sterile water than in solutions of different Glc and KH₂PO₄ concentrations (Figure S1B). Inoculation of "Red Globe," "Shang-24," "Hunan-1" leaves with a 1×10^7 mL⁻¹ spore suspension all caused brownish spreading lesions that almost colonized the whole leaf area. When 5×10^4 spores mL⁻¹ was used, no spreading lesions were observed on "Baihe-41" and "Taishan-2." Assay conditions should result in a moderately aggressive infection to distinguish different levels of resistance. Thus, 1×10^7 spores mL⁻¹ was evidently too aggressive, while 5×10^4 spores mL⁻¹ was too mild. Therefore, an inoculation with 1.5×10^6 spores mL⁻¹ in sterile water was opted for the subsequent analyses, which allowed us to detect both increases and decreases in disease severity, for its larger range of the percentages of spreading lesions on the different genotypes than 5×10^5 spores mL⁻¹.

Chinese Wild *Vitis* Species Exhibit Different Levels of Resistance to *B. cinerea*

It has been established that the detached leaf assay in the laboratory gives similar results to field evaluations and that it is a reliable method for screening resistance of grapevine cultivars/lines and their hybrids (Wang et al., 1995; Liu et al., 2003; Poolsawat et al., 2012). According to our laboratory resistance evaluation of *B. cinerea*, whereby spreading leaf lesions (disease severities) were counted 4 days post-inoculation, Chinese wild *Vitis* species generally exhibited a greater degree of variation in their resistance to *B. cinerea* than other species did (Table 1). The data showed similarity in repeated tests and average disease severities varied significantly ($P \leq 0.05$) among the different genotypes through completely random Duncan LSD analysis (Table 2), but no significant difference ($P > 0.05$) was observed between years (2011 and 2013) using correlation analyses (Table S1).

Among the 20 genotypes that were classified as resistant at least (scores between 0 and 3.50), 18 were Chinese wild *Vitis* genotypes, which was approximately 70% of all 41 genotypes tested. The remaining 21 were susceptible genotypes at least (scores between 3.51 and 7.0) in which only 10 belonged to Chinese wild *Vitis* species (Table 1). The disease severity of the three most highly resistant genotypes (HR, scores between 0.00 and 1.50) was less than 0.5%, and infection lesions were rarely to be observed (Table 1). In contrast, leaves of the most susceptible genotypes (HS, scores between 5.51 and 7.0) showed soft-rot and new sporulation (Table 1).

Variation in the resistance levels of Chinese wild *Vitis* to *B. cinerea* is shown in Figure 1, indicating that resistance diversity is reasonably species independent at least to an extent.

Little or no resistance was observed in the widely grown *V. vinifera* cultivars. Indeed, five of the eight HS genotypes were cultivars of *V. vinifera* or its hybrids. Four *V. romanetii* genotypes and three *V. davidii* genotypes but "Tangwei" were classified as susceptible at least, while four of five *V. amurensis* genotypes were resistant at least. Furthermore, all three *V. piasezkii*, three *V. adstricta* and two *V. sp.* genotypes were classified as R (scores between 1.51 and 3.5) or HR, as were *V. sp.* (Qinling grape) and *V. yenshanensis*, although there was only one representative. It is noteworthy that all six genotypes identified as HR were Chinese wild *Vitis*: "Pingli-5" (*V. sp.* [Qinling grape]); "Yanshan-1" (*V. yenshanensis*); "Taishan-2" (*V. adstricta*); and three *V. amurensis* genotypes ("Shuangyou," "Tonghua-3" and "Taishan-11").

All the susceptible genotypes of *V. vinifera* or Chinese wild *Vitis* showed macroscopic mycelium 4 days after infection (Table 1). However, there were also five genotypes classified as R that showed minimal formation of mycelia, and the spreading lesions were far smaller than those seen in the susceptible genotypes. The fungus underwent new sporulation on 14 genotypes, half of which was classified as S (scores between 3.51 and 5.5) and the other half as HS, and neither mycelia nor sporulation were observed on leaves of any HR genotype. Germination and infection rates of all 41 evaluated genotypes were also measured, with germination rates referring to the percentages of germinated conidia of total counted conidia, and infection rates indicating the percentages of successful infection of total counted germinated conidia (Table 1). Most germination and infection rates on HR leaves were less than 20%, while those on R leaves were typically 15–50% and 20–40%, respectively. The rates with S genotypes were at least 50 and 20–60%, respectively, while on HS plants they were more than 60 and 50–80%, respectively. However, there were some conflicting observations: for example, although the germination rate on leaves of the susceptible "83-4-96" (*V. quinquangularis*) was 42.4% and spreading lesions reached 42.9%, the infection rate was only 14.1% that was even lower than the R genotype "Gansu-91" (*V. piasezkii*) (Table 1). Since disease development is not only related to infection rates but also to post-penetration processes (Elad, 1997), the latter genotype was suggested being more sensitive to *B. cinerea* because lower infection rates caused more lesions. Despite of that, the data from the different analyses were generally corroborated with each other, so the laboratory analysis combining with the macroscopic and microscopic evaluation should give important insights into the resistance levels of the tested genotypes.

Two representative genotypes from the HR, R, S, and HS classes were selected to further compare the macroscopic and microscopic growth of *B. cinerea* 4 days after inoculation (Figure 2). The leaves of "Red Globe" (Figures 2A,B) and "Beichun" (Figures 2E,M), two HS genotypes, had entirely decayed and were covered with mycelium, and new conidia with infection rates were 70 and 50%, respectively (Table 1). The S genotypes, *V. davidii* var. "Zhenan-3" (Figures 2B,J) and *V. romanetii* "Pingli-2" (Figures 2F,N) had numerous spreading lesions with mycelia and few new conidia, and with infection rates of 34 and 47%, respectively, and spreading lesions of 47 and 40%, respectively (Table 1). The R genotypes

TABLE 2 | Means ± standard deviations of 3 years of lesions percent ages on the leaves of 41 Vitis genotypes infected with *B. cinerea* over 3 years, along with significance analysis of disease severities.

Species	Names of genotypes	Means ± Deviation of lesion %			<i>P</i> < 0.05*	<i>P</i> < 0.01**
		2011	2012	2013		
<i>V. amurensis</i> Rupr	Huaxian-11	6.11 ± 1.21	4.33 ± 2.44	7.29 ± 1.93	o	QR
<i>V. amurensis</i> Rupr	Taishan-11	4.49 ± 1.00	4.71 ± 0.52	4.53 ± 0.85	o	RS
<i>V. amurensis</i> Rupr	Zuoshan-1	39.7 ± 0.89	38.8 ± 1.12	38.47 ± 1.92	j	KL
<i>V. amurensis</i> Rupr	Tonghua-3	0.18 ± 0.06	0.18 ± 0.09	0.23 ± 0.13	p	S
<i>V. amurensis</i> Rupr	Shuangyou	0.20 ± 0.04	0.12 ± 0.03	0.21 ± 0.07	p	S
<i>V. romanetii</i> Roman.	Pingli-2	45.44 ± 0.85	47.89 ± 1.39	47.61 ± 1.35	fg	GH
<i>V. romanetii</i> Roman.	Baihe-22	30.30 ± 2.38	28.38 ± 3.89	30.45 ± 1.91	k	LM
<i>V. romanetii</i> Roman.	Liuba-11	45.56 ± 3.20	46.54 ± 1.77	47.66 ± 1.29	fg	GHI
<i>V. romanetii</i> Roman.	Jiangxi-2	50.93 ± 1.04	50.35 ± 2.57	46.71 ± 6.48	fg	GH
<i>V. quinquangularis</i> Rehd.	Shang-24	68.27 ± 2.67	66.93 ± 8.13	76.23 ± 6.62	abc	ABC
<i>V. quinquangularis</i> Rehd.	Taishan-12	20.81 ± 1.58	20.38 ± 2.34	24.69 ± 2.63	l	MNO
<i>V. quinquangularis</i> Rehd.	83-4-85	21.52 ± 1.72	23.50 ± 2.61	19.12 ± 4.52	lm	NO
<i>V. quinquangularis</i> Rehd.	83-4-96	43.44 ± 2.02	43.80 ± 2.87	41.46 ± 3.30	hi	IJ
<i>V. piasezkii</i> Maxim	Liuba-6	18.53 ± 0.55	17.28 ± 0.62	18.38 ± 0.59	l	MNO
<i>V. piasezkii</i> Maxim	Liuba-7	16.12 ± 0.29	17.14 ± 2.10	16.79 ± 1.19	lm	NO
<i>V. piasezkii</i> Maxim	Gansu-91	14.13 ± 0.81	12.38 ± 0.68	11.42 ± 0.49	n	P
<i>V. adstricta</i> Hance	Taishan-1	14.30 ± 1.30	15.27 ± 1.69	15.84 ± 0.44	m	O
<i>V. adstricta</i> Hance	Taishan-2	1.87 ± 0.17	2.23 ± 0.51	2.13 ± 0.62	p	S
<i>V. adstricta</i> Hance	Anlin-3	16.39 ± 1.08	17.65 ± 1.61	16.18 ± 2.25	lm	NO
<i>V. davidii</i> Foex	Lueyang-4	56.35 ± 1.59	53.98 ± 3.08	56.57 ± 3.12	f	EFG
<i>V. davidii</i> Foex	Ningqiang-6	60.65 ± 0.54	59.38 ± 1.92	59.34 ± 0.83	de	CDE
<i>V. davidii</i> Foex	Tangwei	6.38 ± 0.57	5.70 ± 2.33	8.92 ± 1.65	n	PQ
<i>V. davidii</i> Foex	Fujian-4	45.88 ± 2.95	47.54 ± 5.31	45.53 ± 1.02	gh	HI
<i>V. pseudoreticulata</i> W.T. Wang	Guangxi-1	21.78 ± 2.34	20.75 ± 1.82	26.39 ± 3.54	l	MN
	Hunan-1	61.50 ± 2.46	60.12 ± 4.25	62.59 ± 5.20	cde	BCD
<i>V. sp.</i> (Maihuang grape)	Baihe-41	26.68 ± 1.72	28.37 ± 0.36	29.06 ± 0.49	l	MNO
<i>V. sp.</i> (Maihuang grape)	Baihe-36-2	17.16 ± 1.58	16.79 ± 1.80	15.68 ± 0.72	lm	NO
<i>V. davidii</i> var. <i>cyanocarpa</i> Sarg.	Zhenan-3	40.68 ± 2.13	38.95 ± 2.80	41.67 ± 1.42	ij	JK
<i>V. sp.</i> (Qinling grape)	Pingli-5	3.91 ± 1.12	3.57 ± 0.62	3.61 ± 0.95	op	RS
<i>V. yenshanensis</i>	Yanshan-1	0.26 ± 0.14	0.34 ± 0.23	0.48 ± 0.11	p	S
<i>V. vinifera</i> L.	NO. 19 Xinong	39.91 ± 2.91	40.53 ± 1.71	34.37 ± 2.44	ij	JK
<i>V. vinifera</i> L.	Rizamat	25.04 ± 1.78	24.40 ± 3.40	22.97 ± 2.69	l	MNO
<i>V. vinifera</i> L.	Hongmu Nage	45.68 ± 0.74	44.93 ± 5.36	49.27 ± 4.28	gh	GHI
<i>V. vinifera</i> L.	Zao Jinxiang	12.70 ± 0.33	13.00 ± 1.60	13.48 ± 0.74	n	P
<i>V. vinifera</i> L.	Muscat Hamburg	61.55 ± 3.75	63.30 ± 5.60	54.22 ± 9.02	e	
<i>V. vinifera</i> L.	Red Face Seedless	61.10 ± 3.48	58.97 ± 1.13	61.70 ± 1.89	de	CDE
<i>V. vinifera</i> L.	Red Globe	71.90 ± 1.26	69.37 ± 2.91	75.47 ± 6.55	ab	AB
<i>V. riparia</i> Michaux	Hean-3	47.85 ± 2.33	43.95 ± 3.13	38.90 ± 0.92	hi	IJ
<i>V. vinifera</i> L. × <i>V. labrusca</i> L.	NO. 8 Hutai	69.98 ± 6.78	76.33 ± 7.82	87.15 ± 3.90	a	A
<i>V. vinifera</i> L. × <i>V. labrusca</i> L.	Kyoho	61.33 ± 6.22	55.53 ± 5.13	57.47 ± 8.12	de	CDE
<i>V. vinifera</i> L. × <i>V. amurensis</i> Rupr	Beichun	65.39 ± 3.54	67.45 ± 13.36	67.86 ± 1.62	bcd	ABCD

* , ** Significance at $P \leq 0.05$ or $P \leq 0.01$, respectively. Different letters associated with each level of disease severity indicates significant differences at $P \leq 0.05$ or $P \leq 0.01$.

V. quinquangularis “83-4-85” (**Figures 2C,K**) and *V. piasezkii* Gansu-91 (**Figures 2G,O**) produced considerably fewer limited necrotic lesions than the S and HS genotypes. The conidia on their leaves were observed to penetrate with rates of 7 and 21%, respectively (**Table 1**); however, the secondary hyphae either did not develop or were very short, indicating restricted *B. cinerea* proliferation. Finally, leaves of the HR genotypes, *V. amurensis*

“Tonghua-3” (**Figures 2D,L**) and *V. sp.* (Qinling grape) “Pingli-5” (**Figures 2H,P**), had few lesions with the percentages of 0.2 and 4%, respectively. Germination rates of 13 and 28% and infection rates of 6 and 12% were also extremely low (**Table 1**). Abnormal germ tubes (**Figure 2P**) that were extremely short as well as hollow or collapsed conidia were observed to varying degrees on the leaves of almost all the HR genotypes analyzed.

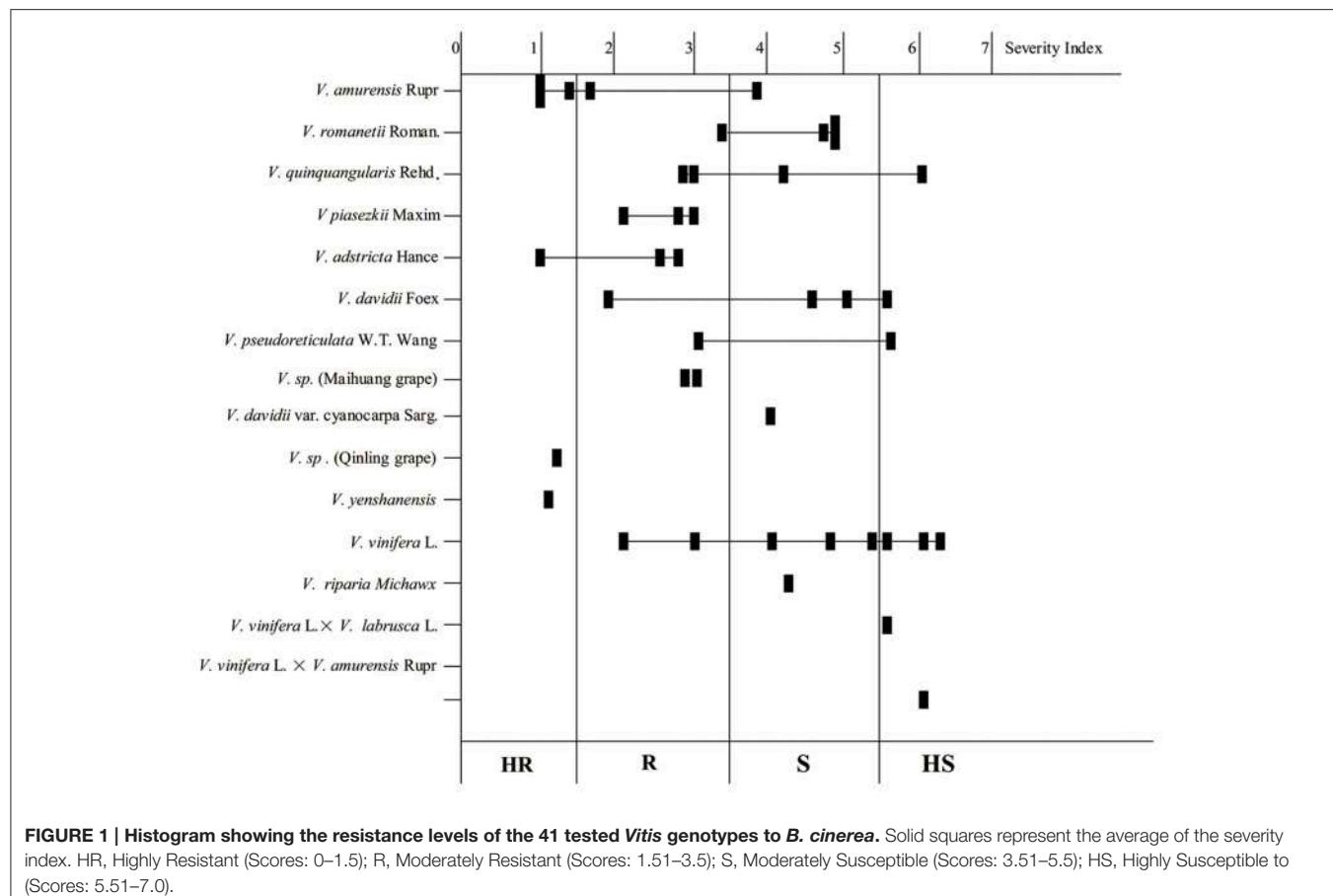


FIGURE 1 | Histogram showing the resistance levels of the 41 tested *Vitis* genotypes to *B. cinerea*. Solid squares represent the average of the severity index. HR, Highly Resistant (Scores: 0–1.5); R, Moderately Resistant (Scores: 1.51–3.5); S, Moderately Susceptible (Scores: 3.51–5.5); HS, Highly Susceptible to (Scores: 5.51–7.0).

***B. cinerea* Growth on the HS “Red Globe” and the HR Chinese Wild *Vitis* “Pingli-5”**

In this study, one of the most resistant Chinese wild *Vitis* genotypes, “Pingli-5,” and one of the most susceptible *V. vinifera*, “Red Globe,” were selected to characterize differences in their infection by *B. cinerea*. The first different visual symptoms were small, dark needle-like lesions 18 hpi on the upper leaf surface of “Red Globe” that were not present on “Pingli-5.” These subsequently developed into small necrotic lesions 24 hpi that expanded rapidly until 96 hpi, resulting in extensive tissue rot and new sporulation. Conversely, only a few necrotic spots were observed on “Pingli-5” leaves and these showed minimal expansion (Figure 3A), with about 5% necrosis compared to 95% on “Red Globe” leaves (Figure 3C).

The SEM time series observations indicated that the infection of “Red Globe” was more substantial and aggressive (Figures 4A–I), while the germination was delayed and fungal growth was mostly blocked on “Pingli-5” leaves at the early time points of the initial 24 h infection (Figures 4J–R). No difference was observed 4 hpi (Figures 3B, 4A,J). On “Red Globe,” germination rate increased rapidly to 39% 8 hpi when appressoria were observed (Figure 4B) and to 47% 12 hpi when penetrations were apparent (Figure 4C), after that, infection rate

increased to 30% 18 hpi when infection pegs were apparent (Figures 3B, 4D). Then, infection rate increased to 38% 24 hpi, while germination increased slowly (Figure 3B), and this was accompanied by germ tube elongation and the appearance of necrotic spots (Figures 3, 4). During this period, *B. cinerea* failed to capture “Pingli-5” (Figures 4K–N) and germination and infection rates were far lower than “Red Globe” (Figure 3B). The presence of appressoria surrounded by sheaths was first noted 18 hpi (Figure 4M) which seemed to peel away from leaf surfaces (Figures 4N,P), suggesting an even lower rate of infection on “Pingli-5” than that was observed by light microscopy. Infections on “Pingli-5” increased slowly with 6% 18 hpi and 10% 48 hpi (Figure 3B). From 24 hpi, *B. cinerea* germination, and infection on “Red Globe” leaves increased steadily until 96 hpi (Figure 3B). Many hyphae branched (Figure 4G), and a collapse of plant cells around infection sites (Figures 4E,F) and obvious lesion spreading accompanied. From 48 hpi onwards, the fungus grew rudely and sporulated on “Red Globe” (Figures 4G,I). In contrast, *B. cinerea* growth was blocked at an early stage on “Pingli-5” and subsequently the infection was almost completely abolished (Figures 4N–R). The hollow conidia described above were present as early as 36 hpi (Figures 4A–O) and were observed in increasing numbers until 96 hpi (Figures 4A–R).

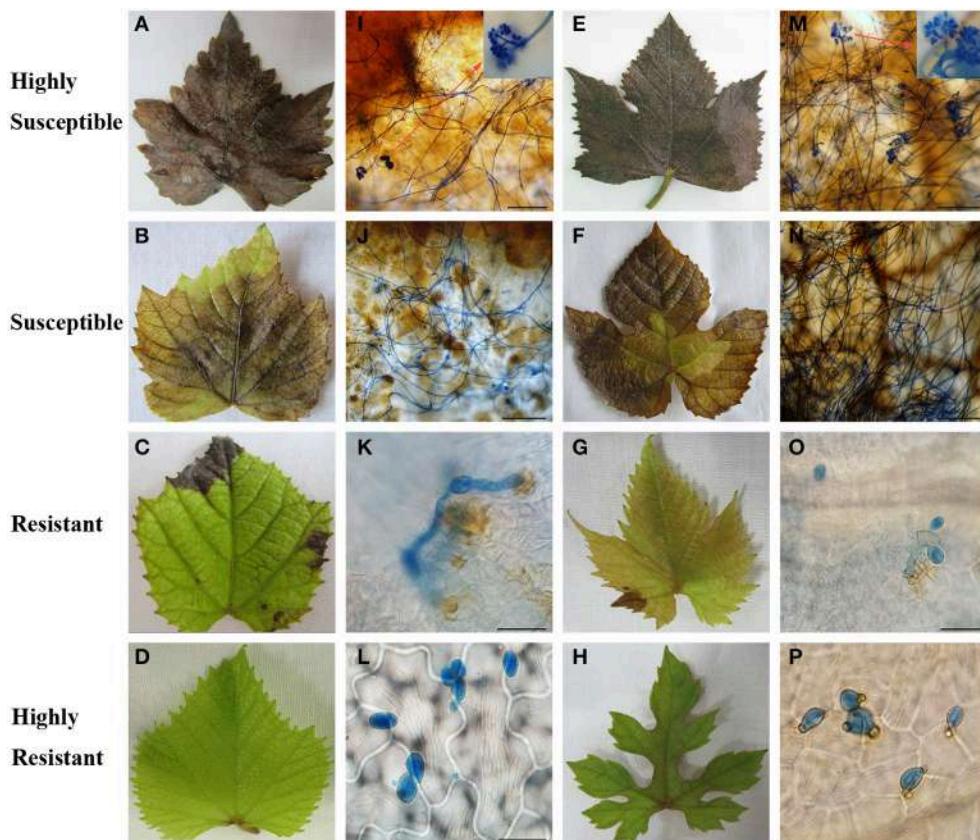


FIGURE 2 | Macroscopic (A–H) and microscopic (I–P) evaluation of two representative *Vitis* genotypes from each level of *B. cinerea* resistance, respectively. Highly susceptible “Red Globe” (*V. Vinifera*) and “Beichun” (*V. Vinifera* × *V. amurensis* Rupr) are shown in (A,I) and (E,M), respectively. Red arrows in (I,M) show new sporulation events at sites indicated. Susceptible *V. davidii* var. “Zhenan-3” and *V. romanetii* “Pingli-2” are shown in (B,J) and (F,N), respectively. *V. quinquangularis* “83-4-85” and *V. piasezkii* “Gansu-91” represent resistant genotypes and are shown in (C,K) and (G,O), respectively. *V. amurensis* “Tonghua-3” and *V. sp.* (Qinling grape) “Pingli-5” are highly resistant and are shown in (D,L) and (H,P), respectively. Scale bars: (I,J,M,N): 50 μm; (K,L,O,P): 20 μm. One representative leaf of three biological replicates is shown for each time point. Samples were collected 4 days after inoculation.

H₂O₂ Accumulation in the Interactions of *B. cinerea* with HS “Red Globe” and HR Chinese Wild *Vitis* “Pingli-5”

Since one of the earliest defense responses in plant–*B. cinerea* interactions is ROS production (van Kan, 2006; Asselbergh et al., 2007), H₂O₂ accumulation was measured during the interactions of *B. cinerea* with HS “Red Globe” and HR Chinese wild *Vitis* “Pingli-5” through DAB staining: brown precipitates at the sites of H₂O₂ accumulation due to DAB polymerization (ThordalChristensen et al., 1997). “Red Globe” and “Pingli-5” leaves were sampled 4, 8, 12, 18, 24, 36, 48, 72, and 96 hpi. No staining or germination was observed 4 hpi with either genotype (Figures 5A,J). H₂O₂ accumulation was evident 12 hpi in “Red Globe” epidermal cell walls that were in close contact with 31% of the infecting appressoria (Figure 5T), and was also observed in the interspaces between appressoria and epidermal cell walls (Figure 5C). From 12 to 18 hpi, H₂O₂ accumulation expanded from the sites of fungal contact, resulting in intense DAB staining in all epidermal cell walls surrounding approximately 55% of the infection sites (Figures 5D,T). Intracellular H₂O₂

also accumulated adjacent to “Red Globe” epidermal cell walls (Figure 5D). None of these reactions were visible in “Pingli-5” at these early time points (Figures 5K–M).

H₂O₂ generation in *B. cinerea* conidia, germinating spores, and infection structures was also indicated by DAB staining from 8 hpi onwards (Figure 5T), with gradual increases observed over time. However, much lower values were detected for “Pingli-5.” On “Red Globe,” low levels of DAB staining were detected 8 hpi in approximately 13% of the appressoria (Figures 5B,T), and subsequently, H₂O₂ accumulation increased at fungal infection sites with an increase of 34% on “Red Globe.” Instead, H₂O₂ accumulation was apparent in or around 21% of the germ tubes and 15% of the initial appressoria on “Pingli-5” (Figures 5M,T). On both genotypes, H₂O₂ accumulation was observed from 8 hpi onwards, with the largest changes from 8 to 18 hpi: with an increase of 39% on “Red Globe,” and 15% on “Pingli-5” (Figure 5T). DAB staining was especially strong in the top ends of germ tubes and appressoria associated with infection sites, and was much stronger on “Red Globe” (Figures 5C,D) than “Pingli-5” (Figures 5K–M).

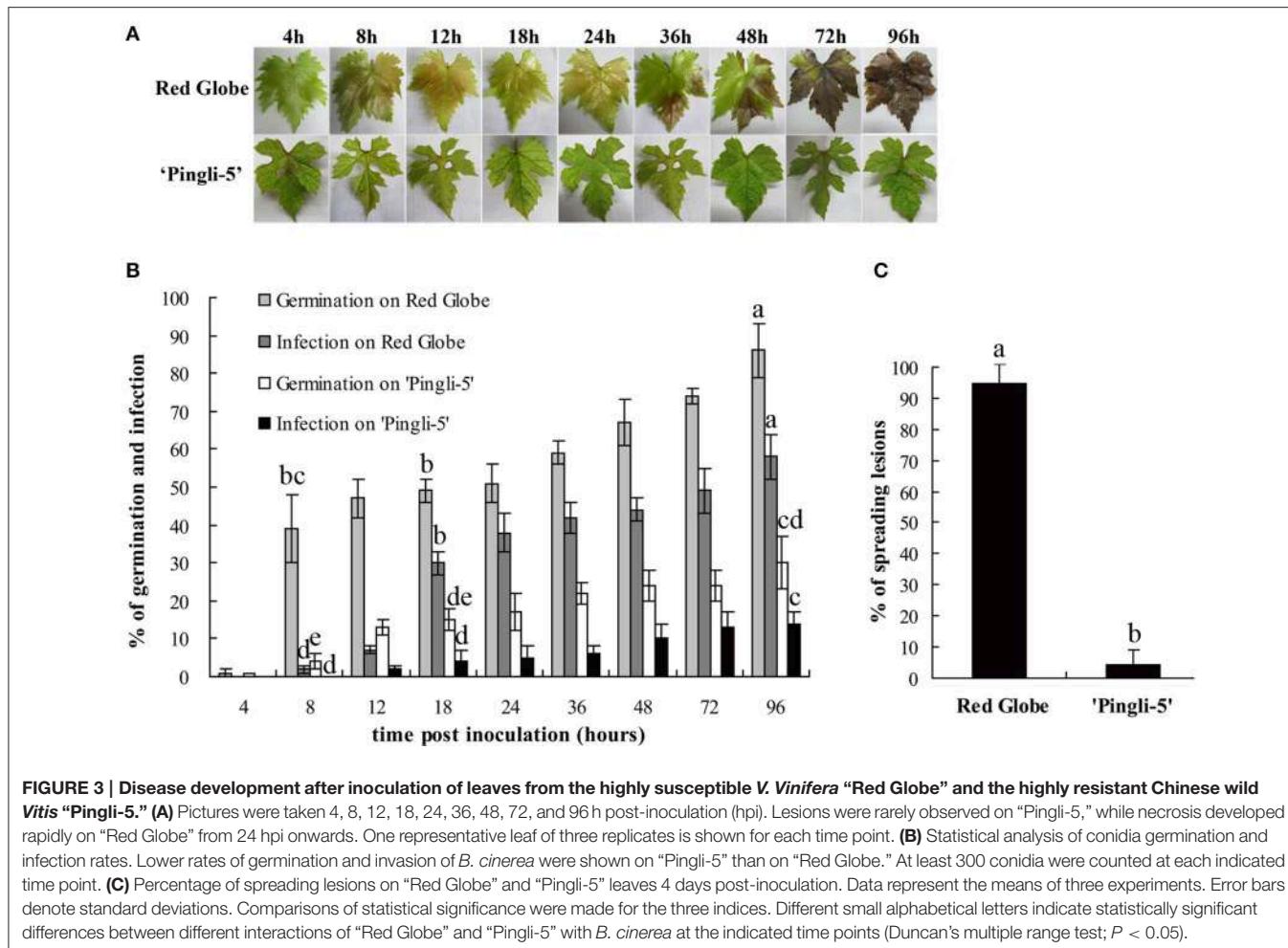


FIGURE 3 | Disease development after inoculation of leaves from the highly susceptible *V. Vinifera* "Red Globe" and the highly resistant Chinese wild *Vitis* "Pingli-5." (A) Pictures were taken 4, 8, 12, 18, 24, 36, 48, 72, and 96 h post-inoculation (hpi). Lesions were rarely observed on "Pingli-5," while necrosis developed rapidly on "Red Globe" from 24 hpi onwards. One representative leaf of three replicates is shown for each time point. (B) Statistical analysis of conidia germination and infection rates. Lower rates of germination and invasion of *B. cinerea* were shown on "Pingli-5" than on "Red Globe." At least 300 conidia were counted at each indicated time point. (C) Percentage of spreading lesions on "Red Globe" and "Pingli-5" leaves 4 days post-inoculation. Data represent the means of three experiments. Error bars denote standard deviations. Comparisons of statistical significance were made for the three indices. Different small alphabetical letters indicate statistically significant differences between different interactions of "Red Globe" and "Pingli-5" with *B. cinerea* at the indicated time points (Duncan's multiple range test; $P < 0.05$).

From 18 to 48 hpi, the extent of H_2O_2 distribution in the epidermal cells of "Red Globe" decreased gradually and more intense DAB staining was detected in the infection pegs, the elongating and branching hyphae as necrosis spread (Figures 5E–G). At later time points, during the period of cell death and rapid rot of "Red Globe" leaves, DAB staining of extracellular, and intracellular plant tissue, as well as *B. cinerea* sporulation structures, was very intense (Figures 5H,I). In contrast, appressoria associated with infection sites on "Pingli-5" exhibited increased H_2O_2 accumulation of only about 7%. Even though some appressoria on "Pingli-5" were strongly stained, only a few successful infections and limited H_2O_2 accumulation at the infection sites were observed (Figures 5N–R).

O_2^- Accumulation in the Interactions of *B. cinerea* with HS "Red Globe" and HR Chinese Wild *Vitis* "Pingli-5"

The accumulation of O_2^- was assessed by NBT staining (Wang et al., 2007), which forms a bluish violet precipitate at the sites of O_2^- accumulation. Leaf samples of HS "Red Globe" and HR Chinese wild *Vitis* "Pingli-5" were collected at the indicated time

points. O_2^- generation indicated by small wispy spots of NBT staining occurred over larger areas in "Red Globe" (Figure 6T) than in "Pingli-5" 4 hpi (Figure 6U) whether conidia were present or not. These almost disappeared in "Pingli-5" from 8 hpi onwards (Figures 6K–R). The patterns of O_2^- accumulation in "Red Globe" from 8 hpi onwards were very different from those 4 hpi: the *B. cinerea*–"Red Globe" interactions resulted in dark and concentrated NBT staining in the epidermal cell walls in close contact to 47% of infection appressoria, and in the interspaces of epidermal cells and appressoria (Figure 6B). By 12 hpi, O_2^- accumulation weakened in the majority of infection appressoria and the epidermal cells around 74% of them when infection sites formed (Figure 6C). However, by 18 hpi, the spreading of O_2^- accumulation from the sites of fungal contact resulted in more intense NBT staining of the entire cell walls of many layers of cells around about 73% of the infection sites (Figure 6D and Figure S2); however, O_2^- accumulation in "Red Globe" cells declined 24 hpi and was absent 36 hpi (Figures 6E–I), while O_2^- accumulated rapidly in infection pegs, hyphae, mycelium, and new sporulation from 36 to 96 hpi (Figures 5E–G). None of these reactions in "Red Globe" from 8 hpi was observed in "Pingli-5" (Figures 6J–R). Contrastingly, the proportion of conidia that did not germinate but showed NBT staining increased 8 hpi following

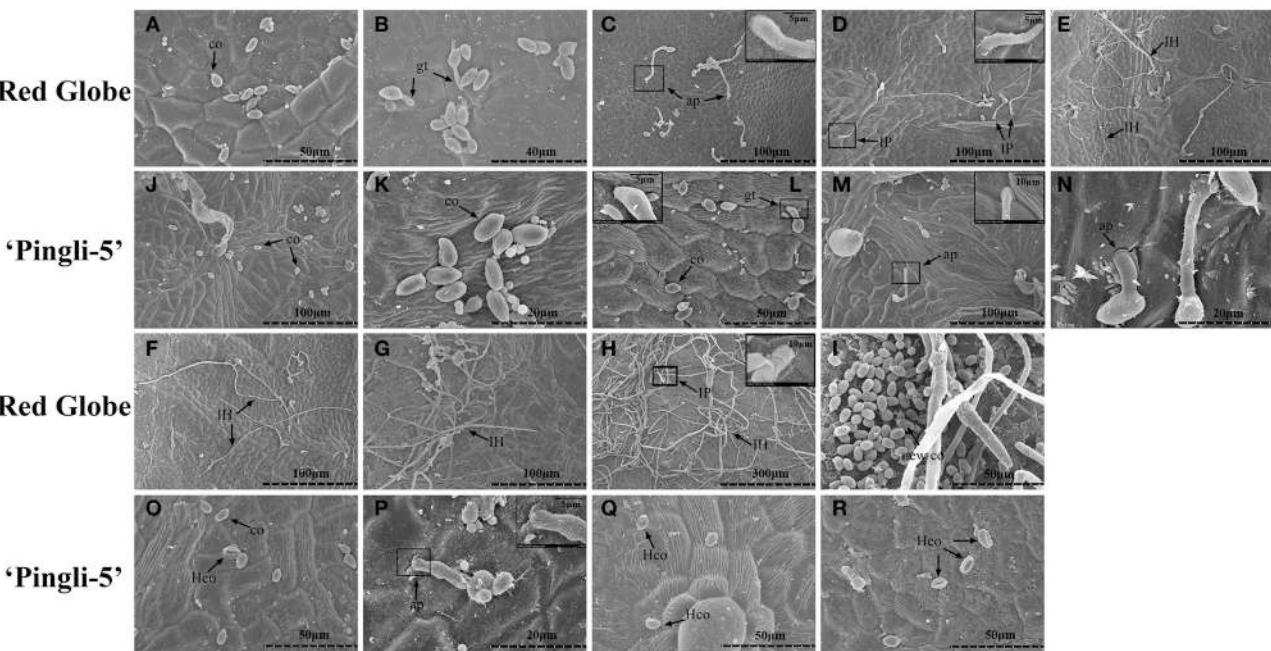


FIGURE 4 | Comparison of *B. cinerea* conidia development on “Pingli-5” and “Red Globe” leaves using scanning electron microscopy. Progression of *B. cinerea* colonization on “Red Globe” (A–I) and “Pingli-5” (J–R). Leaves were harvested 4, 8, 12, 18, 24, 36, 48, 72, and 96 h post-inoculation (hpi) and the experiments were repeated three times. Arrows indicate a co, conidium; gt, germ tube; ap, appressorium; IP, infection peg; IH, infection hypha; new co, new conidium; and Hco, hollow conidium. Large black blocks indicate magnifications at the sites of small black blocks. Scale bars: (A,I,O,Q,R): 50 µm; (B): 40 µm; (C–G,J,M): 100 µm; (H): 300 µm; (K,N,L,P): 20 µm; Magnification pictures in (C,D, L,P): 5 µm; Magnification pictures in (H,M): 10 µm.

a gradually decline until 96 hpi. At last, 18% infection structures showed NBT staining (Figure S2) and “Pingli-5” cells beneath these infection sites showed only limited and indistinct staining (Figures 6Q,R).

The percentages of *B. cinerea* conidia, germinating spores, and infection sites generating O₂⁻ were analyzed in “Pingli-5” and “Red Globe” (Figure S2). These three indices from “Pingli-5” decreased, except for that NBT staining conidia increased marginally from 90% 4 hpi to 96% 8 hpi and that germinating spores with NBT staining increased from 26% 8 hpi to 35% 12 hpi. In the case of “Red Globe,” conidia showing NBT staining decreased from 92% 4 hpi to the lowest percent of 29% 72 hpi, and then increased to 39% 96 hpi as a consequence of new sporulation. Germination conidia showing NBT staining first occurred with the percent of 73% 8 hpi and declined to the lowest percent of 51% 48 hpi, before increasing to 74% 96 hpi. A total of 67% infection sites with NBT staining first appeared 8 hpi and 74% showed staining 24 hpi, before the number decreased to 55% 36 hpi and increased again to 72% 96 hpi.

Activities of Peroxidase, Catalase, and Superoxide Dismutase in HS “Red Globe” and HR Chinese Wild Vitis “Pingli-5” Infected by *B. cinerea*

Antioxidant enzymes protect plants from oxidative stress and maintain redox equilibria through scavenging of ROS produced

during pathogen attack (Pallavi Sharma et al., 2012). Peroxidase (POD), catalase (CAT) and superoxide dismutase (SOD) activity levels in the leaves of HR “Pingli-5” and HS “Red Globe” were tested to assess the dynamics of the antioxidant system following challenge with *B. cinerea*. Protein extracts from leaves of “Pingli-5” control as well as “Red Globe” inoculation and control all exhibited similar CAT or POD background activities with basal invariant (Figures 7A,B). However, in inoculated “Pingli-5” leaves, CAT activity gradually increased to approximately three-fold the background value 24 hpi, followed by a small drop 36 hpi with another four-fold increase 48 hpi compared to the background value and by 96 hpi, the activity decreased to a value two-fold higher than that of the background (Figure 7A); POD activity increased to a peak of eight-fold higher activity than the background value 48 hpi, followed by a decrease 72 hpi and a final increase of about six times higher than the background 96 hpi (Figure 7B).

Interestingly, background SOD activity in “Red Globe” was approximately twice that of “Pingli-5” in the control assays (Figure 7C). Moreover, SOD activity in inoculated “Pingli-5” leaves was similar to that of the control, except for an almost three-fold increase 4 hpi to nearly the same value with the background activity in “Red Globe” leaves (Figure 7C). In contrast, SOD activity in “Red Globe” leaves increased following *B. cinerea* infection to a maximum of 3.6-fold that of the background activity 18 hpi, but then decreased rapidly to the background levels by 36 hpi with no further increases detected (Figure 7C).

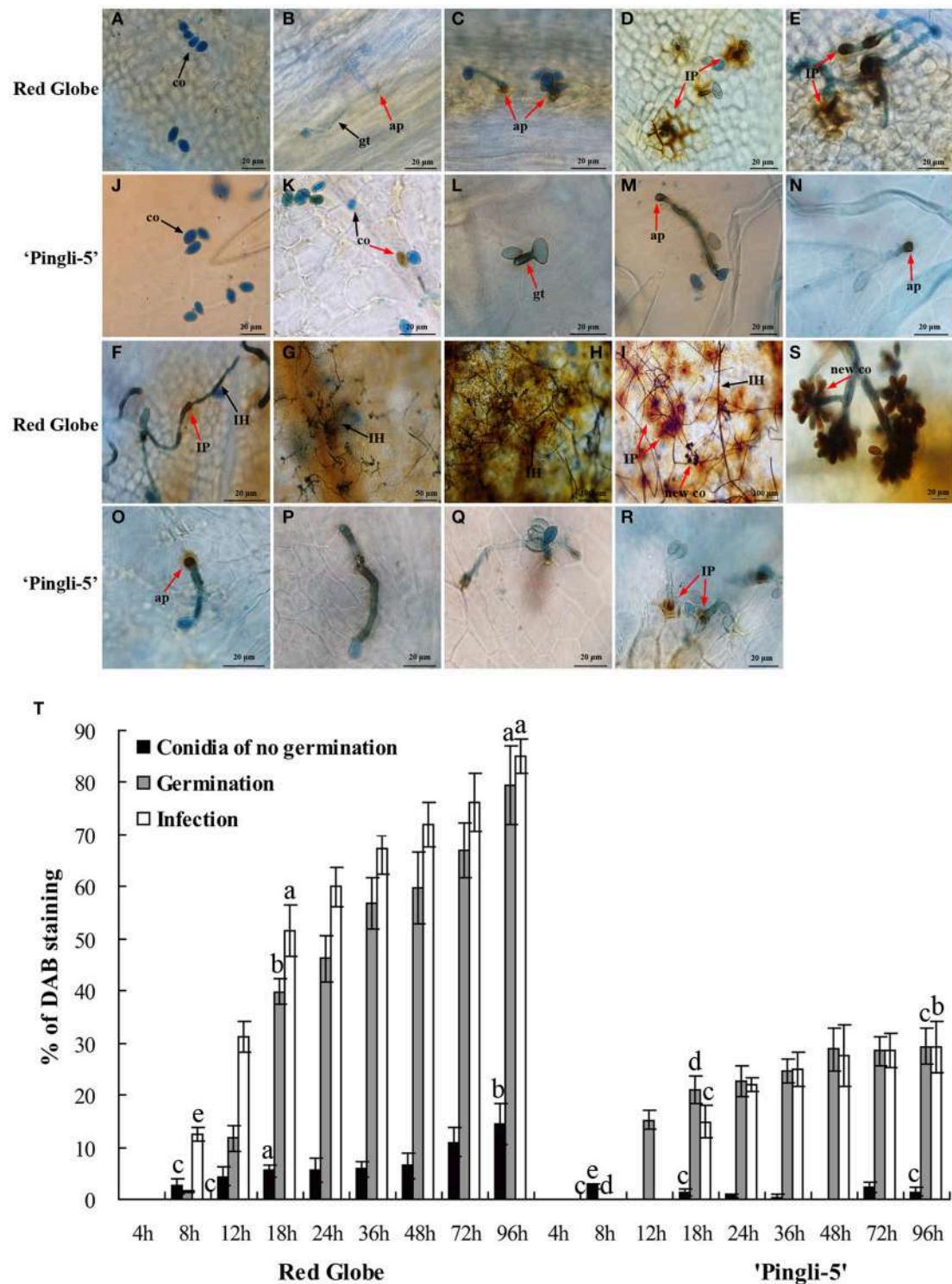


FIGURE 5 | Temporal evolution of H₂O₂ accumulation in the leaves of “Red Globe” and “Pingli-5” as well as in *B. cinerea* following inoculation. Aniline blue stains the fungus a bluish color while DAB (3-diaminobenzidine) stains H₂O₂ purple. H₂O₂ accumulation was assessed in the interactions of “Red Globe” (A–I) and “Pingli-5” (J–R) with *B. cinerea* 4, 8, 12, 18, 24, 36, 48, 72, and 96 h post-inoculation (hpi). Arrows indicate a co, conidium; gt, germ tube; ap, appressorium; IP, infection peg; IH, infection hypha; and new co, new conidium. Black arrows indicate no DAB staining and red arrows indicate DAB staining. (S) Higher magnification of the site of the red arrow in (I), showing the DAB stained sporulation. Scale bars: (A–F): 20 μ m; (G): 50 μ m; (H, I): 100 μ m; (J–R): 20 μ m; (S): 5 μ m. (T) Percentages of *B. cinerea* conidia, germ tubes and infection sites exhibiting H₂O₂ accumulation at the indicated times. At least 300 conidia were counted at each time point. Experiments were repeated three times with similar results. Comparisons of statistical significance were made for the three indices. Different small alphabetical letters indicate statistically significant differences between different interactions of “Red Globe” and “Pingli-5” with *B. cinerea* at the indicated time points (Duncan’s multiple range test; $P < 0.05$).

DISCUSSION

Chinese Wild *Vitis* Species Represent Valuable *B. cinerea* Resistant Germplasm

It has been reported that most popular *V. vinifera* berries are susceptible to *B. cinerea*, while *V. rotundifolia*, *V. labrusca*, or other complex hybrids are highly resistant (Gabler et al., 2003). Although *B. cinerea* predominantly infects grape flowers, leaves are the second most infected organs (Holz et al., 2003; van Kan, 2006). To our knowledge, the current study is the first to document the resistance of Chinese wild *Vitis* to *B. cinerea* where leaf resistance levels of 41 genotypes, including 30 Chinese wild *Vitis* species, were described (Tables 1, 2). Intraspecific variation was found since the resistance levels did not correlate perfectly with individual species (Figure 1). Eighteen of the 30 Chinese wild *Vitis* were resistant to the fungus, while little or no resistance was seen in most *V. vinifera* and its hybrids such as “Red Globe,” “Muscat Hamburg,” “No. 8 Hutai” and “Kyoho” (Table 1). Indeed, the six most highly resistant genotypes with extremely less lesion events and relatively low infection rates were all Chinese wild *Vitis* species: three *V. amurensis* Rupr; one ‘*V. yenshanensis*; one *V. sp.* (Qinling grape), and one *V. adstricta* (Table 1 and Figure 2).

Wang et al. (1995, 1998) described Chinese wild *Vitis* as a valuable resource for future disease resistance breeding programs. Many Chinese wild *Vitis* species exhibit synchronous multi-fungal disease defense: *V. amurensis* is known for its tolerance of cold and anthracnose, as a multi-resistant rootstock (Liu and Li, 2013); “Pingli-5” of *V. sp.* (Qinling grape) is resistant to anthracnose, powdery and downy mildew (Wang et al., 1995, 1998). Consequently, Chinese wild *Vitis* of high resistance to *B. cinerea* like “Shuangyou,” “Tonghua-3,” and “Pingli-5” may have the potential to decrease the gray mold in vineyards and protected grapevine cultivation systems, and may therefore represent valuable germplasm for breeding new varieties with resistance to multiple fungal diseases.

B. cinerea Growth is Blocked in the Early Infection Stages on the Highly Resistant Chinese Wild *Vitis* “Pingli-5”

In the present study, the distinct colonization of *B. cinerea* on grapevine leaves was first revealed by SEM over a time series. On “Red Globe,” penetration of *B. cinerea* was direct and the pathogen established a primary restricted infection as necrosis occurred before 24 hpi (Figures 3, 4). Subsequently, *B. cinerea* initiated a massive outgrowth and sporulation (Figures 3, 4). Conversely, during the early infection steps before 24 hpi, penetration on “Pingli-5” showed a substantial delay resulting in markedly lower germination and infection rates (Figures 3B, 4). Most appressorium on “Pingli-5” leaves had a sheath (Figure 4) possibly composed of disassembled polysaccharides and secondary metabolites (Viret et al., 2004; van Kan, 2006; Choquer et al., 2007), but they rarely developed into infection pegs like those present on “Red Globe” leaves (Figure 4). Therefore, it seems that the colonization of *B. cinerea* was blocked on Chinese wild *Vitis* “Pingli-5” during these early

infection stages, possibly due to its physical and chemical barriers such as cell wall reinforcement and phytoalexin synthesis (Elad, 1997; Adrian and Jeandet, 2012; Cheng et al., 2012) or defense responses such as the timely deployment of ROS (Foyer and Noctor, 2013).

Reactive Oxygen Species and Antioxidative Activities were Differentially Induced Depending on the Susceptibility of the *Vitis* Genotype to *B. cinerea* Infection

After establishing that HR Chinese wild *Vitis* “Pingli-5” could effectively block *B. cinerea* and that HS “Red Globe” was a favorable host, the underlying possible mechanisms of resistance in “Pingli-5” and susceptibility in “Red Globe” were investigated. Since ROS are implicated in plant responses to pathogen attacks (Torres et al., 2006; Foyer and Noctor, 2013) and a detailed time point series evaluation of ROS accumulation during the interactions with *B. cinerea* were conducted, and the potential participation of antioxidant enzymes were assessed.

It has been previously shown that H_2O_2 induced in plant cells, accompanied by O_2^- generation, can promote programmed cell death in the host and expansion of disease lesions to facilitate *B. cinerea* infection (Govrin and Levine, 2000; Patykowski, 2006; Asai and Yoshioka, 2009; Simon et al., 2013; Zhang et al., 2014). Other studies with *A. thaliana*, tomato and other plants species (Asselbergh et al., 2007; L’Haridon et al., 2011; Windram et al., 2012; Serrano et al., 2014) have also suggested the importance of increased ROS levels in defense against *B. cinerea*. Elicitors and bacteria have been shown to contribute to the ROS based defense mechanism in grapevines (Aziz et al., 2004; Varnier et al., 2009; Verhagen et al., 2010, 2011; Benikhlef et al., 2013). Here, ROS accumulation was not observed in control leaves (data not shown). Overall, high levels of ROS accumulated in the host-fungal interfaces, infection structures, and many layers of epidermal cells surrounding the infection sites between 8 and 18 hpi when infection initiated on HS “Red Globe” (Figures 4–6). Then ROS accumulated continuously in “Red Globe” and *B. cinerea* concurrent with the infection progression and lesion spreading. Conversely, only consistently low levels of ROS accumulation were observed following inoculation of resistant “Pingli-5” with *B. cinerea* (Figures 5, 6). Therefore, it seems that the reliably high level of ROS production seen in “Red Globe” could, at least in part, promote its susceptibility to *B. cinerea* infection and colonization, while the weak ROS induction seen following *B. cinerea* inoculation of “Pingli-5” may contribute to its resistance.

With regards to antioxidant activity, we found that “Red Globe” leaves inoculated with *B. cinerea* exhibited little change in CAT and POD activities as lesions spread. However, they did display increased SOD activity between 8 and 18 hpi (Figure 7), which correlates well with the increase in H_2O_2 levels and diminishment of O_2^- from 24 hpi onwards (Figures 5, 6). However, CAT and POD activities in resistant “Pingli-5” increased throughout the experiment, but virtually no change in SOD activity was observed with the exception of an increase 4 hpi (Figure 7), which was consistent with its minimal induction

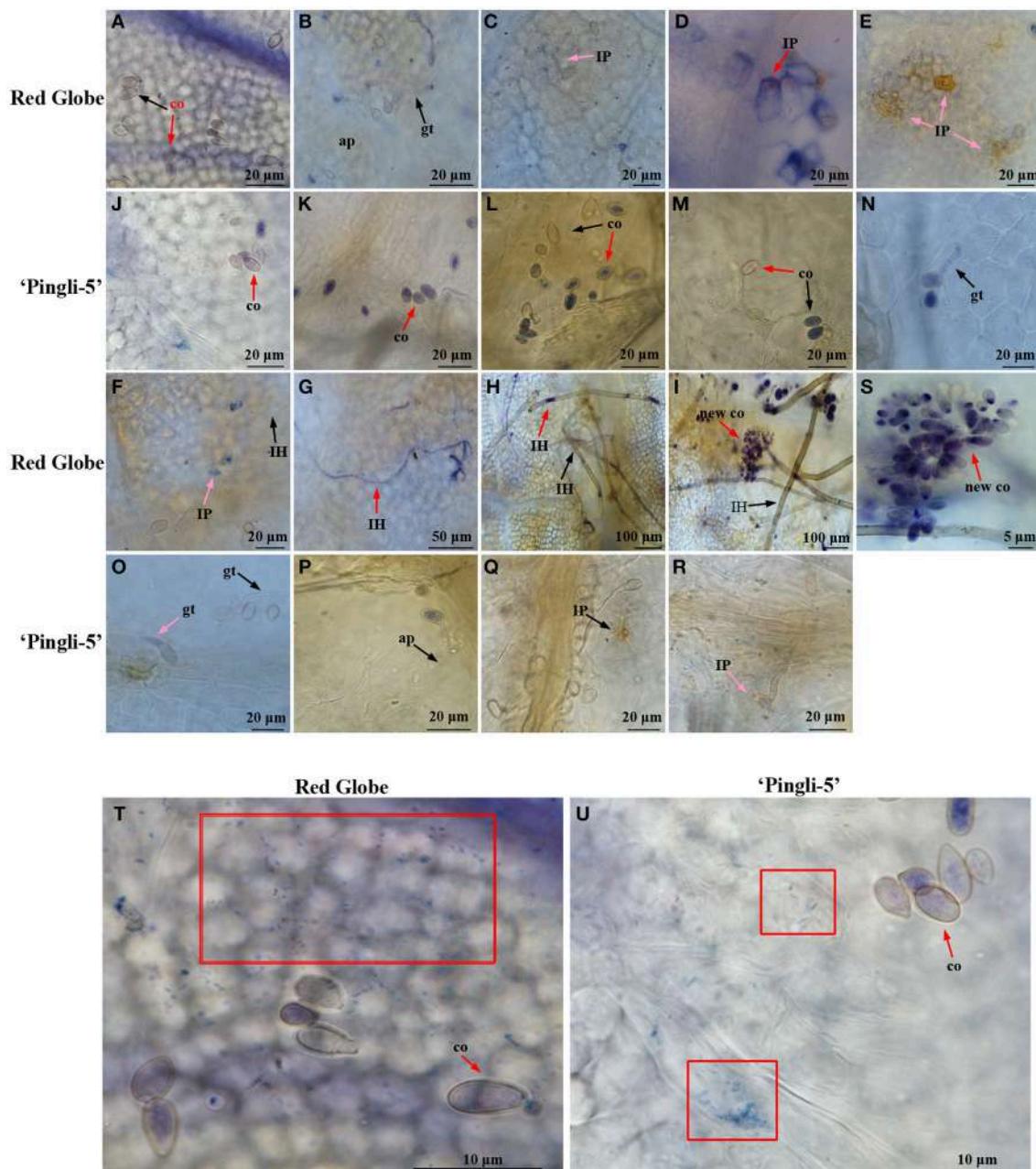


FIGURE 6 | Temporal evolution of O_2^- accumulation in the leaves of “Red Globe” and “Pingli-5” as well as in *B. cinerea* following inoculation. Nitroblue tetrazolium (NBT) stains O_2^- purple and was used to assess O_2^- accumulation in the interactions between *B. cinerea* and “Red Globe” (A–I) or “Pingli-5” (J–R) with *B. cinerea* 4, 8, 12, 18, 24, 36, 48, 72, and 96 h post-inoculation (hpi). Arrows indicate a co, conidium; gt, germ tube; ap, appressorium; IP, infection peg, IH, infection hypha; and new co, new conidium. Black arrows indicate no NBT staining and red arrows indicate means DAB staining. (S) Higher magnification of the site of the red arrow in (I), showing the NBT stained sporulation. (T,U) O_2^- accumulation 4 hpi in the leaves of “Red Globe” (T) and “Pingli-5” (U) infected with *B. cinerea*. Red blocks indicate wispy and small NBT stained spots in cells of both *Vitis* genotypes. Arrows indicate a NBT stained conidium (co). Scale bar: (A–F): 20 μm ; (G): 50 μm ; (H,I): 100 μm ; (J–R): 20 μm ; (S): 5 μm ; (T,U): 10 μm .

of ROS (Figures 5, 6). Antioxidative systems are critical for controlling timing and strength of ROS production to maintain redox homeostasis (Torres et al., 2006; Mittler et al., 2011) and for protecting cells from ROS damage (Pallavi Sharma et al., 2012). It has been reported that after *B. cinerea* infection,

A. thaliana (Govrin and Levine, 2000; Simon et al., 2013) and tomato (Asselbergh et al., 2007; Zhang et al., 2014) and *Phaseolus vulgaris* (Muckenschnabel et al., 1954) continuously accumulate ROS and lesions develop for their insufficient antioxidative systems, and it is necessary that plants timely modulated its own

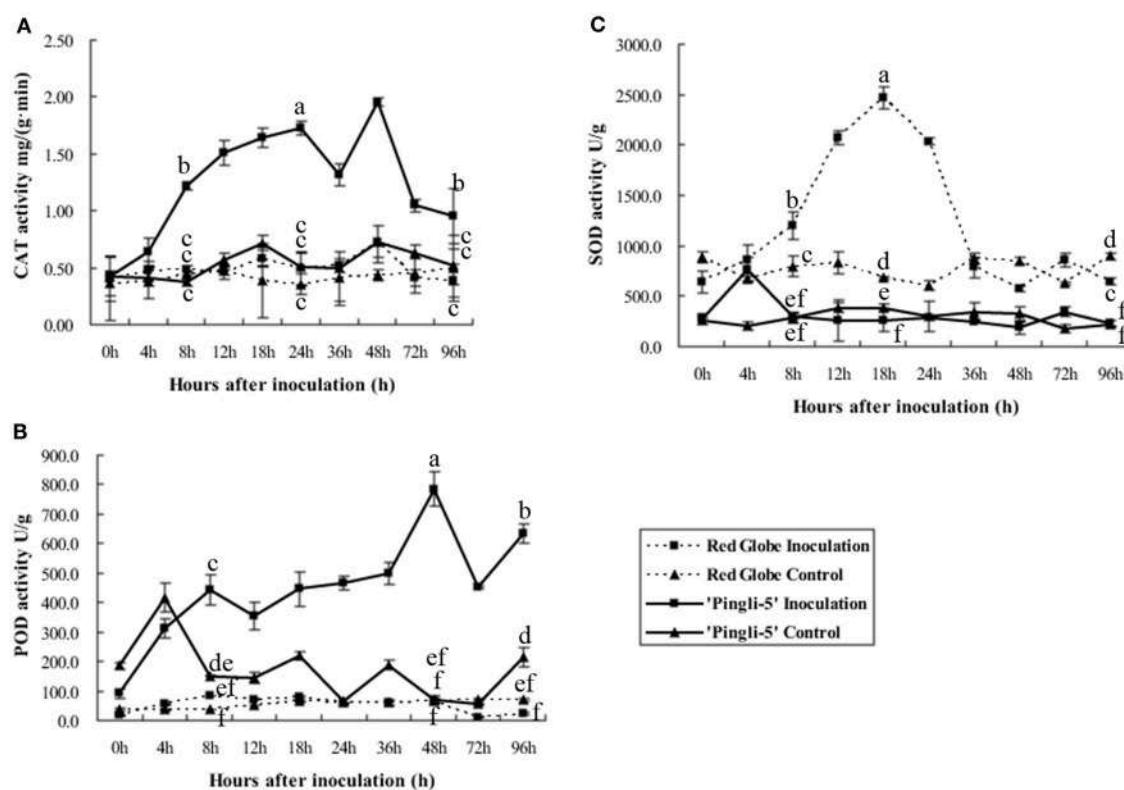


FIGURE 7 | Activities of catalase (CAT; A), superoxide dismutase (SOD; B) and peroxidases (POD; C) in proteins extracts from “Red Globe” and “Pingli-5” leaves 4, 8, 12, 18, 24, 36, 48, 72, and 96 h post-inoculation (hpi) with *B. cinerea* and sterile water as the control. The means and standard deviations of three independent experiments are shown. Comparisons of statistical significance were made for the three indices. Different small alphabetical letters indicate statistically significant differences between different interactions of “Red Globe” and “Pingli-5” with *B. cinerea* at the indicated time points (Duncan’s multiple range test; $P < 0.05$).

ROS accumulation to low levels through antioxidative system to maintain redox equilibrium (Mittler et al., 2011; Foyer and Noctor, 2013). In line with this, we found that when challenged by *B. cinerea*, susceptible “Red Globe” indeed experienced the effects of an insufficient antioxidative system, resulting in consistently high ROS levels, while “Pingli-5” rapidly upregulated its antioxidative capacity following inoculation (particularly CAT and POD activities) and thus experienced less ROS-induced stress. Since substantial ROS was induced in “Red Globe” but not in “Pingli-5,” the precise coordination of ROS production and associated scavenging mechanisms by antioxidative system during combined biotic and abiotic stress (Atkinson and Urwin, 2012) is likely to be important for Chinese wild *Vitis* “Pingli-5” to defense itself against *B. cinerea*.

It has been proved *B. cinerea* itself also generates ROS (Rolle et al., 2004) and adapts this high oxidative stress (Choquer et al., 2007; Temme and Tudzynski, 2009) but perturbs the redox status in and around the infected tissue, thereby promoting infection, which is important for pathogenicity (van Kan, 2005, 2006). We observed ROS accumulation within the pathogen *B. cinerea* on both grapevine leaves, higher in fungi present on “Red Globe” than “Pingli-5.” In any case, regardless of whether the low antioxidative capacity in “Red Globe” was inherent or caused by the infecting *B. cinerea*, it is clear that “Red Globe”

suffered seriously from its sustained ROS accumulation. Instead, “Pingli-5” did not have to contend with huge oxidative stress for its highly and timely elevated antioxidative capacity.

Much attention has been paid to H_2O_2 induction in plants, which has been conflictingly found to contribute to either increased resistance or susceptibility toward *B. cinerea*, and on the other hand, O_2^- has generally been suggested to act as a primary substrate to form H_2O_2 (Govrin and Levine, 2000; Torres et al., 2006; van Kan, 2006; Asselbergh et al., 2007; Serrano et al., 2014). Some reports have suggested that O_2^- plays a role in promoting *B. cinerea* invasion (Urbanek et al., 1996; Patykowski, 2006; Zhang et al., 2014); however in studies of infected and mock infected tomato leaves, no O_2^- accumulation was observed (Asselbergh et al., 2007). In bean, the induction of O_2^- production in leaves is thought to be one of the key factors that differentiate the interactions with the compatible and incompatible pathogens: *B. fabae* and *B. cinerea*, respectively (Urbanek et al., 1996). Furthermore, it has been proposed that if strong oxidative damage at an early stage is insufficient to arrest the pathogen, its subsequent development will be less sensitive to oxidizing agents and so a relatively weak oxidative burst may serve to promote antioxidant systems, ultimately increasing its tolerance to subsequent oxidative stress (Gessler et al., 2007). Here, O_2^- accumulating was detected earlier than

H_2O_2 in inoculated leaves of both hosts (with or without conidia on). This accumulation began 4 hpi, at the earliest stage of the infection (**Figures 6T,U**), but was present to a lesser extent in the highly resistant “Pingli-5” and all disappeared from 8 hpi onwards in “Pingli-5” when more O_2^- began to accumulate around the infection sites in “Red Globe” (**Figure 6**). At this same time point, O_2^- also accumulated in more than 85% of conidia themselves on both hosts (**Figure 6**), which was also earlier than H_2O_2 production began within the fungus (**Figure 5**).

Taken together, we assume that at the earliest stages of the different interaction systems, similar O_2^- levels generated by *B. cinerea* may provide the same attack signal both to “Red Globe” and “Pingli-5,” but could induce distinct O_2^- responses in hosts. This might in turn effect subsequent ROS accumulation, antioxidative system levels and infection progression. An induction of O_2^- generation, earlier than H_2O_2 production, may be among the first consequences of an interaction between *B. cinerea* with grapevines. The higher levels of O_2^- induced in HS “Red Globe” compared to “Pingli-5” at the earliest infection stages (before 8 hpi) could potentially result in much higher and sustained ROS levels with its insufficient antioxidative protection during subsequent infection periods and could ultimately lead to oxidative damage and cell death. In comparison, the lower levels of O_2^- in “Pingli-5” at the earliest infection stages (before 8 hpi) may represent a low/moderate concentration for a recognition process for timely elevating antioxidative capacity to prevent the subsequent sustained ROS production and arrest the attachment and development of *B. cinerea*. However, at present, this is a simply conjecture and would require further research to provide definitive answers with regards to the importance of the timing of O_2^- and H_2O_2 accumulation. Thus, the spatiotemporal relationship between ROS and antioxidative systems and other signaling molecules remains an interesting area to better understand the resistance of Chinese wild *Vitis* against *B. cinerea* and allow the development of *B. cinerea* resistant grapes.

In conclusion, we explored germplasm resources from Chinese wild *Vitis* species for resistance to *B. cinerea* that causes the gray mold disease. A lack of resistance in most cultivated genotypes was confirmed and a substantial amount of resistance in Chinese wild *Vitis* species was identified using detached leaf assays. The events leading to *B. cinerea*

resistance in Chinese wild *Vitis* species were further investigated by contrasting fungal growth, reactive oxygen species (ROS) responses and antioxidative system changes between the highly susceptible *Vitis vinifera* “Red Globe” and the highly resistant Chinese wild *Vitis* “Pingli-5” [V. sp. (Qinling grape)] after the infection with this pathogen. Our results demonstrated that minimal fungal development as well as minimal production of ROS and a timely elevation in antioxidative capacity were correlated with a high level of resistance in “Pingli-5,” while highly susceptible “Red Globe” suffered massive infection and sustained ROS production due to relatively unchanged antioxidative activities. Moreover, we speculated O_2^- induction, which occurred earlier than H_2O_2 production, may be among the first consequences of an interaction between *B. cinerea* with grapevines, suggesting a potential ROS response responsible for the timely recognition and defense of Chinese wild *Vitis* “Pingli-5” to *B. cinerea*. However, this remains to be resolved through further experiments on spatiotemporal relationship of ROS and molecular mechanism.

AUTHOR CONTRIBUTIONS

XPW and RW designed the study. RW, XH, and XHW contributed to the experiments. RW, XH, and JQ performed data analysis. RW, XH, and SS assisted with the interpretation of the results. XPW and YW provided guidance throughout the study. RW, XH, SS, and XPW wrote and revised the manuscript. All authors approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00854>

REFERENCES

- Adrian, M., and Jeandet, P. (2012). Effects of resveratrol on the ultrastructure of *Botrytis cinerea* conidia and biological significance in plant/pathogen interactions. *Fitoterapia* 83, 1345–1350. doi: 10.1016/j.fitote.2012.04.004
- Afzal, F., Khurshid, R., Ashraf, M., and Kazi, A. G. (2014). *Reactive Oxygen Species and Antioxidants in Response to Pathogens and Wounding. Oxidative Damage to Plants: Antioxidant Networks and Signaling*. London: Elsevier Science press, 397–424.
- Angelini, R. M. D. M., Rotolo, C., Masiello, M., Gerin, D., Pollastro, S., and Faretra, F. (2014). Occurrence of fungicide resistance in populations of *Botryotinia fuckeliana* (*Botrytis cinerea*) on table grape and strawberry in southern Italy. *Pest Manag Sci.* 70, 1785–1796. doi: 10.1002/ps.3711
- Asai, S., and Yoshioka, H. (2009). Nitric oxide as a partner of reactive oxygen species participates in disease resistance to necrotrophic pathogen *Botrytis cinerea* in *Nicotiana benthamiana*. *Mol. Plant Microbe Interact.* 22, 619–629. doi: 10.1094/MPMI-22-6-0619
- Asselbergh, B., Curvers, K., Franca, S. C., Audenaert, K., Vuylsteke, M., van Breusegem, F., et al. (2007). Resistance to *Botrytis cinerea* in sitiens, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiol.* 144, 1863–1877. doi: 10.1104/pp.107.099226
- Atkinson, N. J., and Urwin, P. E. (2012). The interaction of plant biotic and abiotic stresses: from genes to the field. *J. Exp. Bot.* 63, 3523–3543. doi: 10.1093/jxb/ers100

- Audenaert, K., De Meyer, G. B., and Höfte, M. M. (2002). Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. *Plant Physiol.* 128, 491–501. doi: 10.1104/pp.010605
- Aziz, A., Heyraud, A., and Lambert, B. (2004). Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *B. cinerea*. *Planta* 218, 767–774. doi: 10.1007/s00425-003-1153-x
- Benikhlef, L., L'Haridon, F., Abou-Mansour, E., Serrano, M., Binda, M., Costa, A., et al. (2013). Perception of soft mechanical stress in *Arabidopsis* leaves activates disease resistance. *BMC Plant Biol.* 13:133. doi: 10.1186/1471-2229-13-133
- Buxdorf, K., Rubinsky, G., Barda, O., Burdman, S., Aharoni, A., and Levy, M. (2014). The transcription factor *SISHINE3* modulates defense responses in tomato plants. *Plant Mol Biol.* 84, 37–47. doi: 10.1007/s11103-013-0117-1
- Cheng, Y., Zhang, H., Yao, J., Wang, X., Xu, J., Han, Q., et al. (2012). Characterization of non-host resistance in broad bean to the wheat stripe rust pathogen. *BMC Plant Biol.* 12:96. doi: 10.1186/1471-2229-12-96
- Choquer, M., Fournier, E., Kunz, C., Levis, C., Pradier, J.-M., Simon, A., et al. (2007). *Botrytis cinerea* virulence factors: new insights into a necrotrophic and polyphagous pathogen. *Fems Microbiol Lett.* 277, 1–10. doi: 10.1111/j.1574-6968.2007.00930.x
- De Tullio, M. C. (2010). Antioxidants and redox regulation: changing notions in a changing world. *Plant Physiol Biochem.* 48, 289–291. doi: 10.1016/j.plaphy.2010.02.011
- Elad, Y. (1997). Responses of plants to infection by *Botrytis cinerea* and novel means involved in reducing their susceptibility to infection. *Biol. Rev.* 72, 381–422.
- Foyer, C. H., and Noctor, G. (2013). Redox signaling in plants. *Antioxid. Redox Signal.* 18, 2087–2090. doi: 10.1089/ars.2013.5278
- Gabler, F. M., Smilanick, J. L., Mansour, M., Ramming, D. W., and Mackey, B. E. (2003). Correlations of morphological, anatomical, and chemical features of grape berries with resistance to *Botrytis cinerea*. *Phytopathology* 93, 1263–1273. doi: 10.1094/PHYTO.2003.93.10.1263
- Gessler, N. N., Aver'yanov, A. A., and Belozerskaya, T. A. (2007). Reactive oxygen species in regulation of fungal development. *Biochem. Moscow* 72, 1091–1109. doi: 10.1134/S0006297907100070
- Giannopolitis, C. N., and Ries, S. K. (1977). Superoxide dismutases: I. Occurrence in higher plants. *Plant Physiol.* 59, 309–314. doi: 10.1104/pp.59.2.309
- Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr Biol.* 10, 751–757. doi: 10.1016/S0960-9822(00)00560-1
- Holz, G., Gutschow, M., Coertze, S., and Calitz, F. J. (2003). Occurrence of *Botrytis cinerea* and subsequent disease expression at different positions on leaves and bunches of grape. *Plant Dis.* 87, 351–358. doi: 10.1094/PDIS.2003.87.4.351
- Lamb, C., and Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 48, 251–275. doi: 10.1146/annurev.arplant.48.1.251
- L'Haridon, F., Besson-Bard, A., Binda, M., Serrano, M., Abou-Mansour, E., Balet, F., et al. (2011). A permeable cuticle is associated with the release of reactive oxygen species and induction of innate immunity. *PLoS Pathog.* 7:e1002148. doi: 10.1371/journal.ppat.1002148
- Liu, L., and Li, H. (2013). Review: research progress in amur grape, *Vitis amurensis* Rupr. *Can. J. Plant Sci.* 93, 565–575. doi: 10.4141/cjps2012-202
- Liu, S. M., Sykes, S. R., and Clingeleffer, P. R. (2003). A method using leafed single-node cuttings to evaluate downy mildew resistance in grapevine. *Vitis* 42, 173–180. Available online at: <http://www.vitis-vea.de/admin/volltext/e049263.pdf>
- Luo, S. L., and He, P. H. (2004). The inheritances of fruit skin and must colors, in a series of interspecific and intraspecific crosses between *V. vinifera* and the wild grape species native to China. *Sci. Horticul.* 99, 29–40. doi: 10.1016/S0304-4238(03)00085-2
- lv, Q. (2013). *Research on Modern China's Wine Industry Development*. Dissertation, Northwest A & F University.
- Maehty, A. C., and Chance, B. (1954). The assay of catalases and peroxidases. *Meth. Biochem. Anal.* 1, 357–424.
- Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., et al. (2011). ROS signaling: the new wave? *Trends Plant Sci.* 16, 300–309. doi: 10.1016/j.tplants.2011.03.007
- Muckenschnabel, I., Williamson, B., Goodman, B. A., Lydon, G. D., Stewart, D., and Deighton, N. (1954). Markers for oxidative stress associated with soft rots in French beans (*Phaseolus vulgaris*) infected by *Botrytis cinerea*. *Planta* 212, 376–381. doi: 10.1007/s004250000401
- Pallavi Sharma, Jha, A. B., Dubey, R. S., and Pessarakli, M. (2012). Reactive oxygen species, oxidative damage, and antioxidative defense mechanism in plants under stressful conditions. *J. Bot.* 2012:217037. doi: 10.1155/2012/217037
- Patykowski, J. (2006). Role of hydrogen peroxide and apoplastic peroxidase in tomato—*Botrytis cinerea* interaction. *Acta Physiol. Plant.* 28, 589–598. doi: 10.1007/s11738-006-0054-6
- Poolsawat, O., Tharapreukpong, A., Wongkaew, S., Chaowiset, W., and Tantasawat, P. (2012). Laboratory and field evaluations of resistance to *Sphaceloma ampelinum* causing anthracnose in grapevine. *Aust. Plant Pathol.* 41, 263–269. doi: 10.1007/s13313-012-0127-5
- Rolle, Y., Liu, S. J., Quidde, T., Williamson, B., Schouten, A., Weltring, K. M., et al. (2004). Functional analysis of H_2O_2 -generating systems in *Botrytis cinerea*: the major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable. *Mol. Plant Path.* 5, 17–27. doi: 10.1111/j.1364-3703.2004.00201.x
- Schumacher, J., and Tudzynski, P. (2012). “Morphogenesis and pathogenicity in fungi,” in *Topics in Current Genetics*, eds J. Pérez Martín and A. Di Pietro (Heidelberg: Springer), 225–241.
- Serrano, M., Coluccia, F., Torres, M., L'Haridon, F., and Metraux, J.-P. (2014). The cuticle and plant defense to pathogens. *Front. Plant Sci.* 5:274. doi: 10.3389/fpls.2014.00274
- Simon, U. K., Polanschütz, L. M., Koffler, B. E., and Zechmann, B. (2013). High resolution imaging of temporal and spatial changes of subcellular ascorbate, glutathione and H_2O_2 distribution during *Botrytis cinerea* infection in *Arabidopsis*. *PLoS ONE* 8:e65811. doi: 10.1371/journal.pone.0065811
- Temme, N., and Tudzynski, P. (2009). Does *Botrytis cinerea* ignore H_2O_2 -induced oxidative stress during infection? characterization of *Botrytis* activator protein 1. *Mol. Plant Microbe Interact.* 22, 987–998. doi: 10.1094/MPMI-22-8-0987
- Thordal-Christensen, H., Zhang, Z. G., Wei, Y. D., and Collinge, D. B. (1997). Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. *Plant J.* 11, 1187–1194. doi: 10.1046/j.1365-313X.1997.11061187.x
- Tierens, K., Thomma, B., Bari, R. P., Garmier, M., Eggermont, K., Brouwer, M., et al. (2002). Esa1, an *Arabidopsis* mutant with enhanced susceptibility to a range of necrotrophic fungal pathogens, shows a distorted induction of defense responses by reactive oxygen generating compounds. *Plant J.* 29, 131–140. doi: 10.1046/j.1365-313X.2002.01199.x
- Torres, M. A., Jones, J. D. G., and Dangl, J. L. (2006). Reactive oxygen species signaling in response to pathogens. *Plant Physiol.* 141, 373–378. doi: 10.1104/pp.106.079467
- Urbanek, H., Gajewska, E., Karwowska, R., and Wielanek, M. (1996). Generation of superoxide anion and induction of superoxide dismutase and peroxidase in bean leaves infected with pathogenic fungi. *Acta Biochim. Pol.* 43, 679–685.
- Vandelle, E., Poinsot, B., Wendehenne, D., Bentéjac, M., and Alain, P. (2006). Integrated signaling network involving calcium, nitric oxide, and active oxygen species but not mitogen-activated protein kin in BcPG1-elicited grapevine defenses. *Mol. Plant Microbe Interact.* 19, 429–440. doi: 10.1094/MPMI-19-0429
- van Kan, J. A. L. (2005). “Infection strategies of *Botrytis cinerea*,” in *Proceedings of the VIith International Symposium on Postharvest Physiology of Ornamental Plants*, Vol. 669, eds N. Marissen, W. G. VanDoorn, and U. VanMeeteren (Wageningen: Acta Hortic, Pressed in Doorwerth), 77–89. doi: 10.17660/ActaHortic.2005.669.9
- van Kan, J. A. L. (2006). Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends Plant Sci.* 11, 247–253. doi: 10.1016/j.tplants.2006.03.005
- Varnier, A.-L., Sanchez, L., Vatsa, P., Boudesocque, L., Garcia-Brunner, A., Rabenoelina, F., et al. (2009). Bacterial rhamnolipids are novel MAMPs conferring resistance to *Botrytis cinerea* in grapevine. *Plant Cell Environ.* 32, 178–193. doi: 10.1111/j.1365-3040.2008.01911.x
- Verhagen, B., Trotel-Aziz, P., Jeandet, P., Baillieul, F., and Aziz, A. (2011). Improved Resistance Against *Botrytis cinerea* by Grapevine-Associated Bacteria that induce a prime oxidative burst and phytoalexin production. *Phytopathology* 101, 768–777. doi: 10.1094/PHYTO-09-10-0242
- Verhagen, B. W. M., Trotel-Aziz, P., Couderchet, M., Höfte, M., and Aziz, A. (2010). *Pseudomonas* spp.-induced systemic resistance to *Botrytis cinerea* is

- associated with induction and priming of defence responses in grapevine. *J. Exp. Bot.* 61, 249–260. doi: 10.1093/jxb/erp295
- Viret, O., Keller, M., Jaudzems, V. G., and Cole, F. M. (2004). *Botrytis cinerea* infection of grape flowers: light and electron microscopical studies of infection sites. *Phytopathology* 94, 850–857. doi: 10.1094/PHYTO.2004.94.8.850
- Wang, C.-F., Huang, L.-L., Buchenauer, H., Han, Q.-M., Zhang, H.-C., and Kang, Z.-S. (2007). Histochemical studies on the accumulation of reactive oxygen species (O_2^- and H_2O_2) in the incompatible and compatible interaction of wheat—*Puccinia striiformis* f. sp. *tritici*. *Physiol. Mol. Plant P.* 71, 230–239. doi: 10.1016/j.pmpp.2008.02.006
- Wang, Y., Liu, Y., He, P., Chen, J., Lamikanra, O., and Lu, J. (1995). Evaluation of foliar resistance to *Uncinula Necator* in Chinese wild *Vitis* species. *Vitis* 34, 159–164.
- Wang, Y., Liu, Y., He, P., Lamikanra, O., and Lu, J. (1998). Resistance of Chinese *Vitis* species to *Elsinoe ampelina* (de Bary) Shear. *Hortscience* 33, 123–126.
- Windram, O., Madhou, P., McHattie, S., Hill, C., Hickman, R., et al. (2012). *Arabidopsis* Defense against *Botrytis cinerea*: chronology and regulation deciphered by high-resolution temporal transcriptomic analysis. *Plant Cell* 24, 3530–3557. doi: 10.1105/tpc.112.102046
- Zhang, P. (2011). *Study on Occuring Regularity and Control Techniques of Grape Gray Mould*. Dissertation, Chinese Academy of Agricultural Sciences.
- Zhang, Y., Liu, B., Li, X., Ouyang, Z., Huang, L., Hong, Y., et al. (2014). The de novo biosynthesis of vitamin B6 Is required for disease resistance against *Botrytis cinerea* in tomato. *Mol. Plant Microbe Interact.* 27, 688–699. doi: 10.1094/MPMI-01-14-0020-R

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Ectopic Expression in *Arabidopsis thaliana* of an NB-ARC Encoding Putative Disease Resistance Gene from Wild Chinese *Vitis pseudoreticulata* Enhances Resistance to Phytopathogenic Fungi and Bacteria

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Plant resistance proteins mediate pathogen recognition and activate innate immune responses to restrict pathogen proliferation. One common feature of these proteins is an NB-ARC domain. In this study, we characterized a gene encoding a protein with an NB-ARC domain from wild Chinese grapevine *Vitis pseudoreticulata* accession “Baihe-35-1,” which was identified in a transcriptome analysis of the leaves following inoculation with *Erysiphe necator* (Schw.), a causal agent of powdery mildew. Transcript levels of this gene, designated *VpCN* (GenBank accession number KT265084), increased strongly after challenge of grapevine leaves with *E. necator*. The deduced amino acid sequence was predicted to contain an NB-ARC domain in the C-terminus and an RxCC-like domain similar to CC domain of Rx protein in the N-terminus. Ectopic expression of *VpCN* in *Arabidopsis thaliana* resulted in either a wild-type phenotype or a dwarf phenotype. The phenotypically normal transgenic *A. thaliana* showed enhanced resistance to *A. thaliana* powdery mildew *Golovinomyces cichoracearum*, as well as to a virulent bacterial pathogen *Pseudomonas syringae* pv. tomato DC3000. Moreover, promoter::GUS (β -glucuronidase) analysis revealed that powdery mildew infection induced the promoter activity of *VpCN* in grapevine leaves. Finally, a promoter deletion analysis showed that TC rich repeat elements likely play an important role in the response to *E. necator* infection. Taken together, our results suggest that *VpCN* contributes to powdery mildew disease resistance in grapevine.

Keywords: wild Chinese *Vitis*, *VpCN*, disease resistance, powdery mildew, promoter analysis

INTRODUCTION

Plants have evolved multiple mechanisms to protect themselves against pathogens (Jones and Dangl, 2006). The first line of defense is microbe-associated molecular pattern (MAMP)-triggered immunity (MTI) following MAMP perception by membrane-resident pattern recognition receptors (Maekawa et al., 2011). MTI is thought to limit the growth of invasive pathogens. The second line of defense is plant innate immunity, which is activated by the specific recognition of pathogen-derived effectors by intracellular host resistance (R) proteins, and is termed effector-triggered immunity (ETI) (Chisholm et al., 2006). ETI typically leads to a hypersensitive response (HR) and gives rise to a faster and stronger defensive response than MTI-triggered immunity (Cesari et al., 2013). Understanding the function of R proteins, and the mechanisms by which they recognize pathogen effectors, can potentially lead to the development of a long-term strategy for the control and prevention of pathogen invasion.

Over the past few decades, numerous R genes have been cloned from model plants and important crops (Pan et al., 2000b; Collier and Moffett, 2009; Sekine et al., 2012). Most R proteins contain a nucleotide binding (NB) domain and a C-terminal leucine-rich repeat (LRR) domain, and belong to the so-called NB-LRR protein family (Ooijen et al., 2008). The most conserved domain in NB-LRR proteins is an NB domain that is found in proteins such as human Apaf-1, plant R proteins and *Caenorhabditis elegans* Ced-4 (ARC), and as such is referred to as the NB-ARC domain (Ooijen et al., 2008; van der Biezen and Jones, 1998). As a consequence of determining its three-dimensional structure, Albrecht and Takken (2006) proposed that the NB-ARC domain can be further divided into three sub-domains (NB, ARC1, and ARC2). Several conserved motifs have been identified throughout the NB-ARC domain in R proteins, such as Walker B, GxP, hhGRExE, Walker A or P-loop, MHD, and RNBS-A-D (Meyers et al., 1999; Pan et al., 2000a; Ooijen et al., 2008). Crystal structure analysis of the NB-ARC domain has led to the suggestion that it may function as a molecular switch to regulate signaling pathways through conformational changes (Riedl et al., 2005; Takken et al., 2006). It has also been shown that the nucleotide binding of the NB-ARC domain in the R proteins, I-2, and Mi-1, requires a P loop, since a P-loop mutant abolished the binding capacity (Tamelink et al., 2010). Likewise, the oligomerization of an NB-ARC-LRR protein in the presence of its elicitor requires an intact P-loop in the NB-ARC domain (Mestre and Baulcombe, 2006).

Plant NB-LRR proteins can be divided into two distinct classes: the TNL and the CNL type, based on the domains present at their N terminus. Those that possess a Toll and human interleukin-1 receptor (TIR) domain are referred to as TIR-NB-ARC-LRR or TNL proteins, while those carrying a predicted coiled-coil (CC) domain are classified as CC-NB-ARC-LRR, or CNL proteins (Pan et al., 2000a; Lukasik-Shreepaathy et al., 2012). The potato (*Solanum tuberosum*) Rx protein is a typical CC-NB-ARC-LRR protein mediates resistance to potato virus X (PVX)(Kohm et al., 1993; Bendahmane et al., 1999), the CC domain of RX protein has a four bundle structure and forms a

heterodimer with RanGAP2 WPP domain (Hao et al., 2013). The N-termini of the CC and TIR domains are thought to mediate downstream immune responses. It has been reported that in CNL proteins, the CC domain of NRG1 is capable of independently inducing defense responses (Collier et al., 2011), and in TIR proteins the TIR domain plays a crucial role in the cell death signaling pathway (Zhang et al., 2004; Weaver et al., 2006).

The identification and functional characterization of NB-ARC domain R proteins is of considerable interest in developing novel sources of disease resistance in crop plants that are threatened by phytopathogens. For example, *Erysiphe necator* is a fungus that causes powdery mildew (PM) disease in grapevine worldwide, resulting in serious losses in both grape yield and quality. The most economically important cultivated grapevine is *V. vinifera*, which is highly susceptible to PM (Gadoury et al., 2012). To combat the pathogen, fungicides are widely used, which causes environmental and financial pressure on grape growers and reduces wine quality. Thus, developing new grape cultivars with enhanced disease resistance mechanisms is of considerable interest. The wild Chinese *Vitis*, “Baihe-35-1,” is an accession of wild Chinese *V. pseudoreticulata* W. T. Wang that possesses high resistance to multiple fungi, and particularly to *E. necator* (Wang et al., 1995; Lin et al., 2006; Yu et al., 2011). To elucidate the resistance mechanisms involved in the defense response to fungal infection in this species, we previously performed an RNA-seq based transcriptome analysis *V. pseudoreticulata* “Baihe-35-1” that had been inoculated with *E. necator* (Weng et al., 2014). Among the pathogen induced genes, one was predicted to encode an NB-ARC domain protein.

In this current study, we report the isolation of the full length cDNA of this gene, which we designated *VpCN*, and its functional characterization following ectopic expression in *Arabidopsis thaliana*. Conclusions regarding its role in conferring Chinese Wild *V. pseudoreticulata* “Baihe-35-1” with disease resistance to powdery mildew are presented.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Grapevines (Chinese wild *V. pseudoreticulata* accession Baihe-35-1 and *V. vinifera* cv. “Red globe”) were maintained in the grape germplasm resources orchard, Northwest A&F University, Yangling Shaanxi, China. *A. thaliana* (ecotype type, Columbia-0) was grown in a growth chamber under the following conditions: 22°C, 50% humidity, a 16/8 h day/night intensity of 125 $\mu\text{mol m}^{-2} \text{s}^{-1}$ provided by cool white fluorescent bulbs.

Cloning and Sequence Analysis

Total RNA was extracted from grapevine as previously described (Zhang et al., 2003). First strand cDNA was synthesized from 1 μg of total RNA with the PrimerScript™ II 1st Strand cDNA Synthesis kit (TaKaRa Bio Inc., Dalian, China), according to the manufacturer’s instructions. LA *Taq* (Takara Bio. Inc.) was used to amplify the ORF sequence of *VpCN*. The PCR products were cloned into the T-easy vector (Promega, USA), sequenced (Beijing Genomics Institute, Beijing, China) and

submitted to GenBank (accession number KT265084). The *VpCN* cDNA sequence was analyzed using BLAST (<http://Ncbi.nlm.nih.gov/blast>) in the NCBI database. Grapevine DNA extraction was conducted as previously described (Yu et al., 2013), primers for amplify promoter sequence were designed according to acquired sequence from Grape Genome Database (12 \times ; <http://www.genoscope.cns.fr>), after cloning into the T-easy vector and sequencing, the promoter sequence was analyzed using PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) (Lescot et al., 2002). The deduced amino acid sequence of *VpCN* was aligned with closely related proteins and a phylogenetic tree was generated using neighbor joining algorithm with 1000 bootstrapping with the ClustalW tool in the MegAlign program (Version 5.07, DNASTAR Inc.) (Figure 1D). A structural model of the NB-ARC domain of *VpCN* was constructed using the structure of PDB 4m9x.1.C (Huang et al., 2013) in SWISS-MODEL (Figure 1C). Real time PCR was conducted using SYBR® Premix EX Taq™II (Tli RNaseH Plus) (Takara Bio. Inc.) in a 20 μ L volume reaction following the manufacturer's instructions using the CFX96TM real-time system (Bio-Rad, Hercules, CA, USA). The amplification cycles were as follows: initial denaturation at 94°C for 30 s, 40 cycles at 95°C 5 s, 60°C for 30 s. For melting curve analysis: 40 cycles at 95°C for 15 s followed by a constant increase from 60–95°C. The grapevine *Actin 1* (GenBank Accession number AY680701) was used as reference gene.

Construction of Vectors for Ectopic Expression and *A. thaliana* Transformation

To generate 35S:*VpCN*, the open reading frame (ORF) region of *VpCN* was cloned into the binary vector, pCAMBIA 2300 (CAMBIA company), downstream of the CaMV 35S promoter. The construct was introduced into *Agrobacterium tumefaciens*, strain GV3101, via electroporation, and the transformed *A. tumefaciens* was used to transform *A. thaliana* using the floral dip method (Clough and Bent, 1998). Transgenic plants were screened on MS (Murshige and Skoog, 1962) medium containing 60 mg/mL kanamycin, PCR amplification was performed to identify transgenic plants with gene specific primers.

Construction of *VpCN* Promoter::GUS Gene Fusion Vectors and *A. tumefaciens* Mediated Transient Expression Assays

To generate the *VpCN* promoter:GUS vector, the *VpCN* promoter was cloned into the T-easy vector, digested with *Bam*H I and *Pst*I, and finally cloned into the binary vector pC0380GUS. 35S:GUS was used as a positive control (Xu et al., 2010). Four *pVpCN* promoter fragments with different 5' deletions were amplified (Supplement Table 1). All the constructs were introduced into *A. tumefaciens* strain GV3101 via electroporation. The *A. tumefaciens* mediated transient expression assays were performed as previously described (Guan et al., 2011). *A. tumefaciens* GV3101 lines harboring the different constructs were grown in liquid Yeast Extract Phosphate (YEP) (Smith and Goodman, 1975) medium (supplemented with 100 μ g/ml kanamycin, 60 μ g/ml gentamycin, and 30 μ g/ml

rifampicin) to an OD₆₀₀ of 0.6, and harvested by centrifugation at 5000 \times g for 10 min, before being resuspended in filtration solution (10 mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.7, 10 mM MgCl₂ and 15 μ M acetosyringone) and adjusted to an OD₆₀₀ of 0.6 for infiltration of young grapevine leaves using a vacuum infiltration method (Santos-Rosa et al., 2008). After infiltration, the leaves were kept in a chamber at 16/8 h day/night cycle at 23°C with 70% humidity for 48 h, before inoculation with *E. necator* (Guan et al., 2011; Yu et al., 2013).

Pathogen Inoculation Procedures

E. necator infected leaves were collected from a highly PM-susceptible wild Chinese wild *V. Adstricta*, Hance clone "Taishan-2." Leaves of the Chinese wild *V. pseudoreticulata* "Baihe-35-1" were inoculated by touching the adaxial epidermis of leaves with sporulating colonies on the surface of pathogen leaves, the inoculation were repeated three times (Guan et al., 2011). The samples were harvested 0, 6, 12, 24, 48, 72, 96, and 120 h after inoculation.

A. thaliana powdery mildew *G. cichoracearum* was maintained on highly susceptible *pad4* *A. thaliana* mutant plants. The infection was conducted as previously described (Tang and Innes, 2002). The susceptibility or resistance phenotypes were scored 8 days after infection (Nie et al., 2011). Analyses of pathogenesis-related 1 (PR1) gene expression were performed using qRT-PCR using the same PCR program as for the *VpCN* analysis. The *A. thaliana* tubulin gene (GenBank Accession number NM_179953) was used as a reference. Rosett leaves from 4 week old *Arabidopsis* were harvested at 0, 12, 24, 36, and 48 h after inoculation.

P. st DC3000 cells grown in King's B medium (supplemented with 100 μ g/ml kanamycin and 30 μ g/ml rifampicin) to an OD₆₀₀ of 0.6, harvested by centrifugation for 5000 \times g for 10 min and re-suspended in 10 mM MgSO₄, adjusted to optical density at OD₆₀₀ of 0.02. The bacterial suspension containing 0.025% Silwet-77, and the mixture were hand infiltrated into the abaxial side of the *A. thaliana* leaves using a needless 1 ml syringe (Fan et al., 2008). *P. st* DC3000 bacterial growth were assessed 3 and 5 days after infection as described (Ahn et al., 2007).

Trypan Blue Staining

For trypan blue staining, *A. thaliana* leaves were collected 12 hpi (hours post-inoculation) and boiled in alcoholic lactophenol trypan blue solution (20 mL of ethanol, 10 mL of phenol, 10 mL of water, 10 mL of lactic acid [83%], and 30 mg of trypan blue). Stained leaves were cleared in chloral hydrate (2.5 g dissolved in 1 mL of water) for 3 h, before placing under a coverslip in 50% glycerol (Koch and Slusarenko, 1990; Frye and Innes, 1998).

Peroxide Assay

Peroxide (H₂O₂) was assayed using a hydrogen peroxide kit, according to the manufacturer's instructions (Nanjing Bio Ins., Nanjing, China). Quantification of dead cells was performed 12 hpi by staining leaf discs (0.5 mm in diameter) with 0.2% Evans blue (Sigma) for 30 min, followed by several washes with water to remove excess stain (Mino et al., 2002; Ahn et al., 2007). One

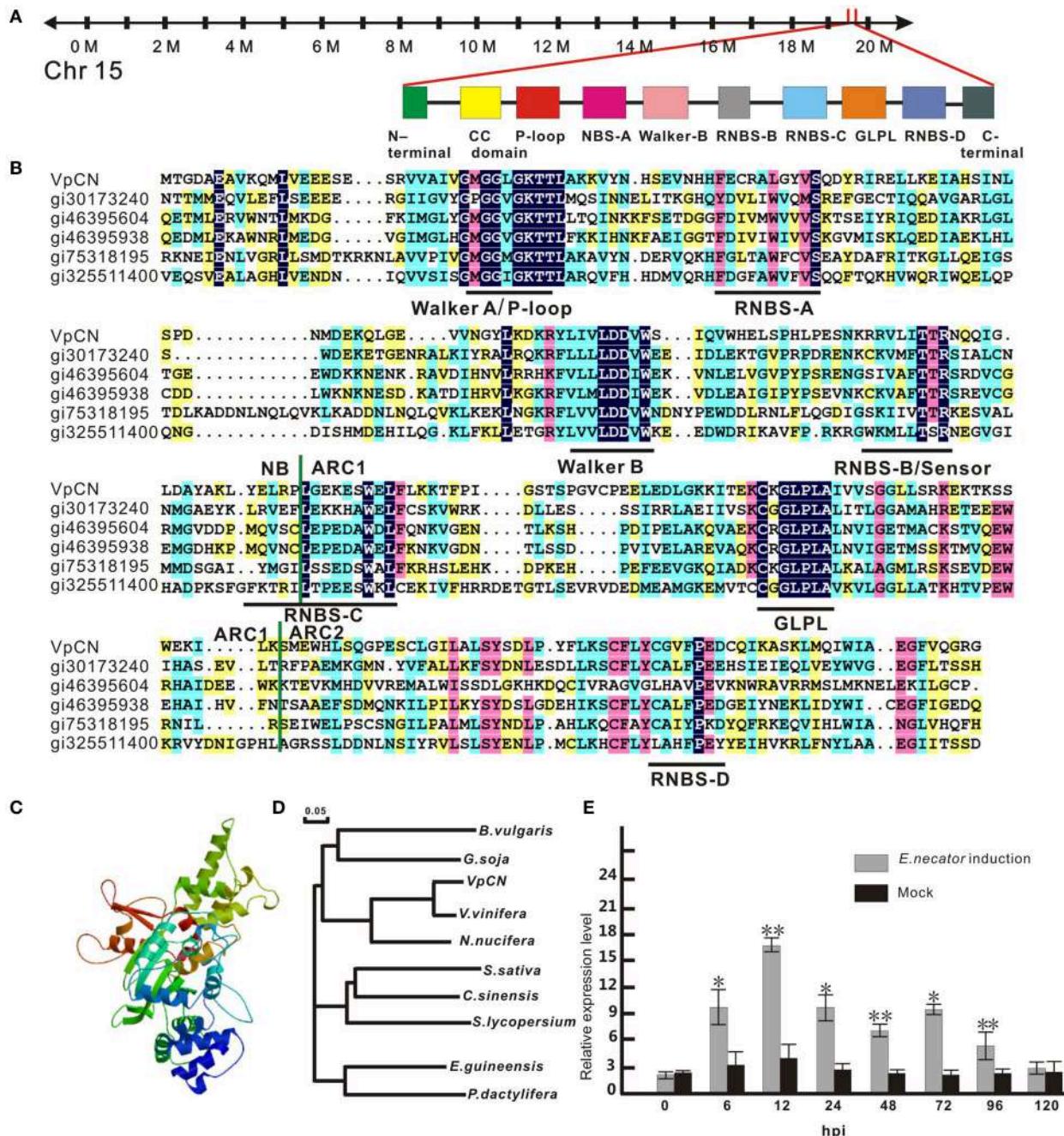


FIGURE 1 | Sequence analysis of VpCN and transcript level detection. (A) Schematic map of VpCN location and major motifs. **(B)** Multiple sequence alignment of the NB, ARC1 and ARC2 subdomains of NB-ARC in VpCN with closely related proteins. Domain borders are indicated by vertical green lines. Motifs are labeled by horizontal dark lines below the aligned sequences. gi30173240 (Bent et al., 1994), gi46395604 (Bevan et al., 1998), gi46395938 (Theologis et al., 2000), gi75318195 (Ori et al., 1997), gi325511400 (Theologis et al., 2000) **(C)** Structural model of the NB-ARC domain of VpCN. **(D)** Phylogenetic tree of VpCN and related proteins from other plant species. The tree was generated using the ClustalW function in the MegAlign program: *Vitis vinifera* (GenBank accession no. XP010661747), *Nelumbo nucifera* (GenBank accession no. XP012588251), *Glycine soja* (GenBank accession no. KHN19144), *Elaeis guineensis* (GenBank accession no. XP010913221), *Solanum lycopersicum* (GenBank accession no. XP010319316), *Beta vulgaris* subsp. *Vulgaris* (GenBank accession no. XPO10669409), *Phoenix dactylifera* (GenBank accession no. XPO08791188), *Camellia sinensis* (GenBank accession no. XPO10426119), *Citrus sinensis* (GenBank accession no. XP006470644). The scale bar represents 0.05 substitutions per site. **(E)** Structure model of NB-ARC in VpCN. **(E)** Analysis of VpCN expression in response to *E. necator* inoculation. The third to fifth fully expanded young grapevine leaves beneath the apex were selected for samples. The experiment encompass three independent biological replicates, for each biological replicate three leaves harvested from three plant and three technical replicates were performed. Data represent means of three biological replicates \pm SE, asterisks indicate statistical significance in comparison with control (Student's t-test, significance levels of * $P < 0.05$, ** $P < 0.01$ are indicated).

milliliter of 50% methanol supplemented with 1% SDS was added and the samples were incubated at 50°C for 1 h. Absorbance at OD₆₀₀ was determined by ultraviolet spectrophotometry after a 10-fold dilution of the extracts (Ahn et al., 2007). The nitro blue terazolium (NBT) staining was performed as described (Kim et al., 2011).

Callose Accumulation

To observe callose accumulation, leaves (3 dpi) were immersed in destaining solution (10 ml phenol, 10 ml glycerin, 10 ml lactic acid, 10 ml H₂O, and 80 ml ethanol) and kept in an oven at 60°C for 1 h to remove chlorophyll. The samples were washed to remove the destaining solution, and stained with 0.1% aniline. The fluorescence of callose was detected using an epifluorescence microscope (E800, Nikon) with a V-2A filter (Reuber et al., 1998; Ahn et al., 2007). For quantitative determination of callose, *A. thaliana*, leaves (3 dpi) were immersed in ethanol for 2–3 days to remove the chlorophyll, before centrifugation at 5000 × g for 10 min. The supernatant was discarded and the pellet resuspended in 0.4 ml DMSO. One hundred microliter of the supernatant was supplemented with loading mixture [400 µl 0.1% (w/v) aniline blue, 590 mL 1 M glycine/NaOH (pH 9.5), 210 mL 1 M HCl] and 200 µl 1 M NaOH. The control samples were not supplemented with aniline. The samples were incubated in a water bath 50°C for 20 min and cooled to room temperature before detection with a fluorescence spectrophotometer (F-4600, Hitachi, Tokyo, Japan) under 393 nm excitation, 479 nm emission and a voltage of 400 v. The fluorescence of the samples was determined by subtracting the fluorescence value of the control from those of the samples (Kohler et al., 2000).

GUS Staining, Histochemical and Fluorometric Assays for Determining GUS Activity

A histochemical β-glucuronidase (GUS) assay of leaves was carried out as previously described (Jefferson, 1987). Briefly, leaves were immersed in GUS staining solution at 37°C for 24 h, before washing in 70% ethanol at 37°C and viewing macroscopically (Guan et al., 2011; Yu et al., 2013). GUS fluorescence was determined quantitatively according to Jefferson (1987). Protein concentrations in grapevine extracts was normalized by dilution with extraction buffer according to Bradford (1976). GUS activity was expressed as pmol 4MU (Sigma-Aldrich China, Shanghai, China) per minute per mg of protein. Sample fluorescence was detected with an infinite 200® PRO (Tecan Trading AG, Switzerland). Three independent experiments were performed.

RESULTS

VpCN Expression during Powdery Mildew Infection

To identify potential resistance mechanisms and resistance related genes in the response of wild Chinese *V. pseudoreticulata* to powdery mildew, we previously performed a transcriptome analysis of the “Baihe-35-1” using RNA-seq (Weng et al., 2014).

We observed that the expression of *VpCN* (GenBank accession number KT265084) was strongly induced by inoculation with *E. necator*. To verify this, we performed quantitative real-time PCR (qPCR) analysis of *VpCN* expression in *V. pseudoreticulata* leaves that had been inoculated with *E. necator*, and observed 4.2-fold greater *VpCN* transcript levels than in leaves prior to inoculation. Subsequently, *VpCN* expression decreased but remained at a higher level than in mock inoculated plants (**Figure 1E**).

Cloning and Sequence Analysis of VpCN

To investigate the putative role of *VpCN* in providing resistance to pathogens, we first designed primers based on a cDNA sequence obtained from the Grape Genome Database (12x; <http://www.genoscope.cns.fr>), and isolated and designated the gene *VpCN* (GenBank accession number KT265084). The *VpCN* gene is located on chromosome 15 (**Figure 1A**), has an ORF of 1773 bp (**Supplement Figure 1**) and is predicted to encode a protein of 590 amino acids with a molecular mass of 67,390 Da and a theoretical pI value of 5.45. The amino acid sequence was further predicted to contain a RxCC-like domain in the N-terminus from residue 6–119, a Ran GTPase-acting protein 2 (RanGAP2) interaction site in the RxCC-like domain and an NB-ARC domain spanning residues 129–414. The NB-ARC sub-domains, NB, ARC1, and ARC2 were all present. Furthermore, several conserved motifs, such as a P-loop, RNBS A–D, and a GPLP (**Figure 1B**) were detected. In addition to a RxCC-like domain and an NB-ARC domain, we also found an AAA domain and a PLN03210 domain in the predicted amino acid sequence (picture not shown). A structure-based multiple amino acids sequence alignment was performed to compare the NB-ARC domain of *VpCN* with those of other closely related plant R proteins, including RPS2 (gi30173240) (Bent et al., 1994) and I-2 (gi75318159) (Ori et al., 1997). The amino acids sequence identity between the *VpCN* and the *A. thaliana* RPS2 NB-ARC domain was shown to be 33%, while the *VpCN* and I-2 NB-ARC domains had a 29% sequence identity, concentrated on the conserved motifs of the NB-ARC subdomains (**Figure 1B**).

Ectopic Expression VpCN in *A. thaliana* Enhance Resistance to Powdery Mildew

We next transformed the *VpCN* in *A. thaliana* under the control of the constitutive 35S promoter (**Figure 2A**). A total of 42 independent transgenic T1 lines were obtained and the presence of the transgene confirmed by PCR using *VpCN* specific primers. The T2 progeny segregated so that 39 lines displayed wild type morphology while three lines exhibited a dwarfed phenotype and morphological abnormalities, such as small yellow leaves, stunted growth, and chlorotic tissue (**Figure 2B**). These dwarf lines eventually died. The lines with a wild type phenotype were challenged with *G. cichoracearum*, and three transgenic lines with higher resistance were chosen for the generation of homozygous T3 generation lines. The transgenic lines displayed few visible white powdery areas on their leaves at 8 dpi, whereas the wild-type (Col-0) exhibited abundant powdery mildew development (**Figures 2C,D**). To determine whether the enhanced resistance to *G. cichoracearum* in the transgenic lines was related to

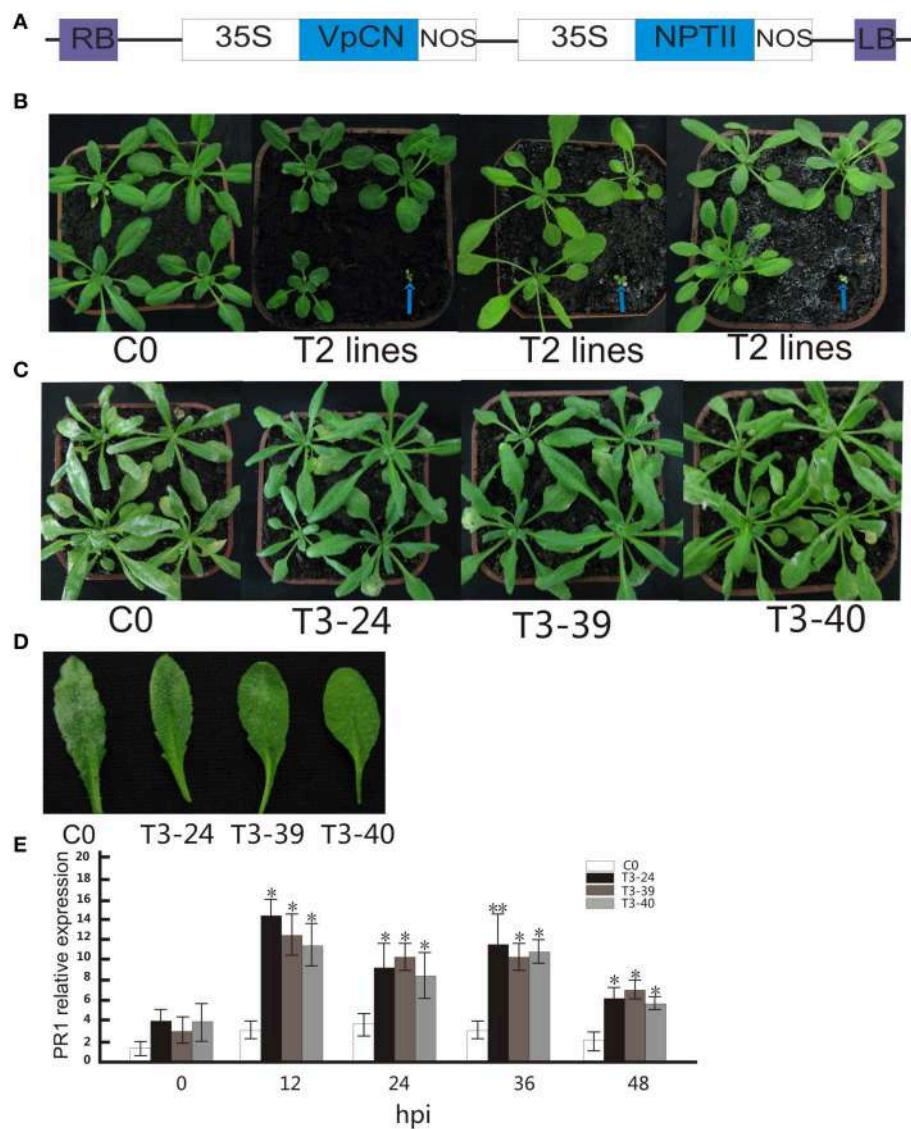


FIGURE 2 | Generation of CaMV 35S promoter-*VpCN* constructs used for transformation of *Arabidopsis thaliana*, morphology of wild type and transgenic *Arabidopsis thaliana* plants, with transgenic plants showing enhanced disease resistance to *G. cichoracearum* after ectopic expression of *VpCN*. **(A)** Structure of the CaMV 35S promoter-*VpCN* ectopic expression construct. LB, left border; RB, right border; 35S, CaMV 35S promoter; NOS, terminator; NPT II, aminoglycoside-3'- phosphotransferase. **(B)** Indicate T2 transgenic plants displayed either normal phenotypes or dwarfism. Blue arrows indicate the dwarf phenotype in 4 week old plants. **(C)** Transgenic *A. thaliana* leaves developed fungal spores 8 dpi with *G. cichoracearum*. **(D)** Disease symptoms developed on the leaves of transgenic lines and wild type plants 8 dpi with *G. cichoracearum*. **(E)** *A. thaliana* PR1 transcript levels in T3 lines and wild-type after inoculation with *G. cichoracearum*. Total RNA was extracted from *A. thaliana* leaves 0, 12, 24, 36, and 48 h post-inoculation (hpi) with *G. Cichoracearum*. The experiment encompass three independent biological replicates, for each biological replicate six rosette leaves were harvested from three plant and three technical replicates were performed. Data represent means of three biological replicates \pm SE, asterisks indicate statistical significance in comparison with WT (Student's t-test, significance levels of *P < 0.05, **P < 0.01 are indicated).

an increase in the expression of a known defense gene, we evaluated PR1 (Pathogenesis Related 1) (Friedrich et al., 1996) transcript levels at 0, 12, 24, 36, and 48 hpi. Three transgenic plants displayed higher PR1 transcript abundance after pathogen inoculation than wild type plants, reaching a maximum level at 12 hpi. The PR1 transcript levels of transgenic plants were ~4–5-fold higher after inoculation than in wild type at all time points (Figure 2E).

Ectopic Expression of *VpCN* Results In Enhanced Protection Against The Bacterial Pathogen, *P. st* DC3000

Since amino acid sequence of *VpCN* was predicted to contain a PLN03210 domain, which has been shown to be correlated with resistance to *Pseudomonas syringae* pv. *glycinea* race 6 (Kim et al., 2009), we hypothesized that it might function in providing

resistance to bacterial infection. To test this, transgenic and control plants were challenged with the bacterial *P. st* DC3000 pathogen by leaf infiltration (**Figure 3A**). Most infiltrated wild type leaves exhibited water-soaking at 1 dpi, turned yellow and finally wilted at 5 dpi. In contrast, the transgenic plants infected with the pathogen showed fewer symptoms (**Figure 3B**), and when the growth of *P. st* DC3000 in the inoculated plants was quantified, it was found that the bacterial number in the transgenic plants was significantly lower than in the wild type plants (**Figure 3F**). To observe the effect of *VpCN* expression on cell death, trypan blue staining was performed of leaves and we observed that cell death was more widespread in the transgenic lines than the wild type plants (**Figure 3C**). Additionally, cell death quantification by Evans blue staining followed by spectrophotometric analysis, showed a 5–6 fold higher level cell death in the transgenic plants (**Figure 3H**). Nitroblue tetrazolium (NBT) staining for the superoxide anion also showed higher accumulation in the transgenic plants (**Figure 3D**), as did quantitative measurements of H₂O₂ (**Figure 3G**). Finally, the accumulation of the (1,3)- β -glucan polymer callose, which is known to be involved in plant defense responses (Brown et al., 1998), was visualized by aniline blue staining of wild type and transgenic plants after treated with *P. st* DC3000 (**Figure 3E**). Greater accumulation of callose was observed in the transgenic plants than in wild, and when callose levels were quantified, it was confirmed that the transgenic lines contained significantly ($P < 0.05$) more callose (**Figure 3I**).

Isolation and Analysis of the *VpCN* Promoter Sequence

A 1440 bp upstream sequence was cloned using wild Chinese *V. pseudoreticulata* “Baihe-35-1” genomic DNA by PCR, regulatory *cis*-acting elements predicted showed that several putative regulatory elements involved in the activation of defense-related genes, including 72 predicted TATA boxes, 32 CAAT boxes, and two TC-repeat elements, which are known to be involved in defense and stress responses, a TCA element, which is involved in salicylic acid (SA) responses, a TGACG motif, which is associate with methyl jasmonate-response, an HSE element, which is involved in heat stress responses and two TATC elements, which are related to gibberellin responses (**Figure 4A**). Additional predicted *cis*-regulatory elements included light response elements (TCCC-motif, MRE, I-box, GT1-motif, GAG-motif, GA-motif, G-box, CATT motif, Box-I, AT1-motif, and Box-4), as well as others *cis*-elements (5UTR Py-rich stretch, circadian element and, TATC box). Several of the predicted *cis*-elements are known to be involved in responses to environmental stresses, further suggesting that the *VpCN* promoter may play a role in defense responses.

Promoter::GUS (Glucuronidase) Assays

To test the activity of the *VpCN* promoter, the 1440-bp promoter fragment was fused to a reporter gene encoding β -glucuronidase (GUS), generating the construct pCVpCNGUS. As a positive control, a CaMV35S::GUS (PC35SGUS) construct was used and a construct with no promoter was used as a negative control (pC0380GUS) (Xu et al., 2010; **Figure 4B**). All the constructs

were expressed transiently in grapevine leaves, which were subsequently subjected to GUS staining. Leaves transformed with the PC35SGUS construct showed strong GUS activity, while no activity was detected in wild type (WT) and very little in PC0380GUS. pCVpCNGUS transformed leaves showed GUS activity but at a lower level than leaves transformed with PC35SGUS (**Figure 4C**), and when leaves were infected with *E. necator* 2 dpi prior to GUS staining, the infected leaves exhibited stronger GUS activity than mock-inoculated control leaves. To further determine the location of the pathogen-responsive *cis*-regulatory region, we generated four promoter deletion fragments and fused them to GUS (−1360, −700, −400, and −240 bp) (**Figure 5A**). When the GUS activity was quantified fluorescently, the highest levels were measured in grapevines containing the −1440 bp fragment, where it was induced 1.57-fold after treatment with *E. necator* compared to mock controls. Leaves transformed with −1360, −700, and, −400 promoter fragments exhibited a relative low level of GUS activity; however, they showed increased GUS activity after being challenged with *E. necator* (**Figure 5B**). Since the leaves transformed with the −240 bp fragment showed no significant difference in GUS activity before and after treatment with *E. necator* (**Figure 5C**), the −400 bp promoter fragment was deduced to be the minimal promoter region required for the response to *E. necator* infection.

DISCUSSION

We previously reported the leaf transcriptome of wild Chinese grape (*V. Pseudoreticulata*, “Baihe-35-1”) that had been inoculated with *E. necator*, and showed that expression of a unigene corresponding to *VpCN* was strongly induced by the infection (Weng et al., 2014). Here, we isolated the ORF sequence of *VpCN* and ectopically expressed it in *A. thaliana*. This resulted in enhanced disease resistance to the pathogens *G. cichoracearum* and *P. st* DC3000. The deduced amino acid sequence of the corresponding protein is predicted to contain an RxCC-like and an NB-ARC domain. Most currently known R proteins have a NB-ARC domain and the CC domain is thought to initiate signaling (Radirdan et al., 2008). Given the rapid and strong up-regulation of *VpCN* transcript accumulation in wild Chinese *Vitis* after treatment with *E. necator*, we suggest that *VpCN* may play a role in the early defense signaling pathways in pathogen recognition. In addition to these two domains, the deduced amino acid sequence also contained a PLN03210 domain, which is thought to contribute to the identification of resistance signaling components and to convey resistance to *P. syringae* (Kim et al., 2009), suggesting that *VpCN* may also be associated with bacterial disease resistance.

Several studies have already demonstrated that over-expression of an R-gene can cause growth retardation, spontaneous cell death, and constitutive defense activation (Tao et al., 2000; Bendahmane et al., 2002; Stokes et al., 2002; Mohr et al., 2010; Nandety et al., 2013) due an over activation of the ETI system. In this study, three independent transgenic lines exhibited dwarfism and stunted growth, as well as other

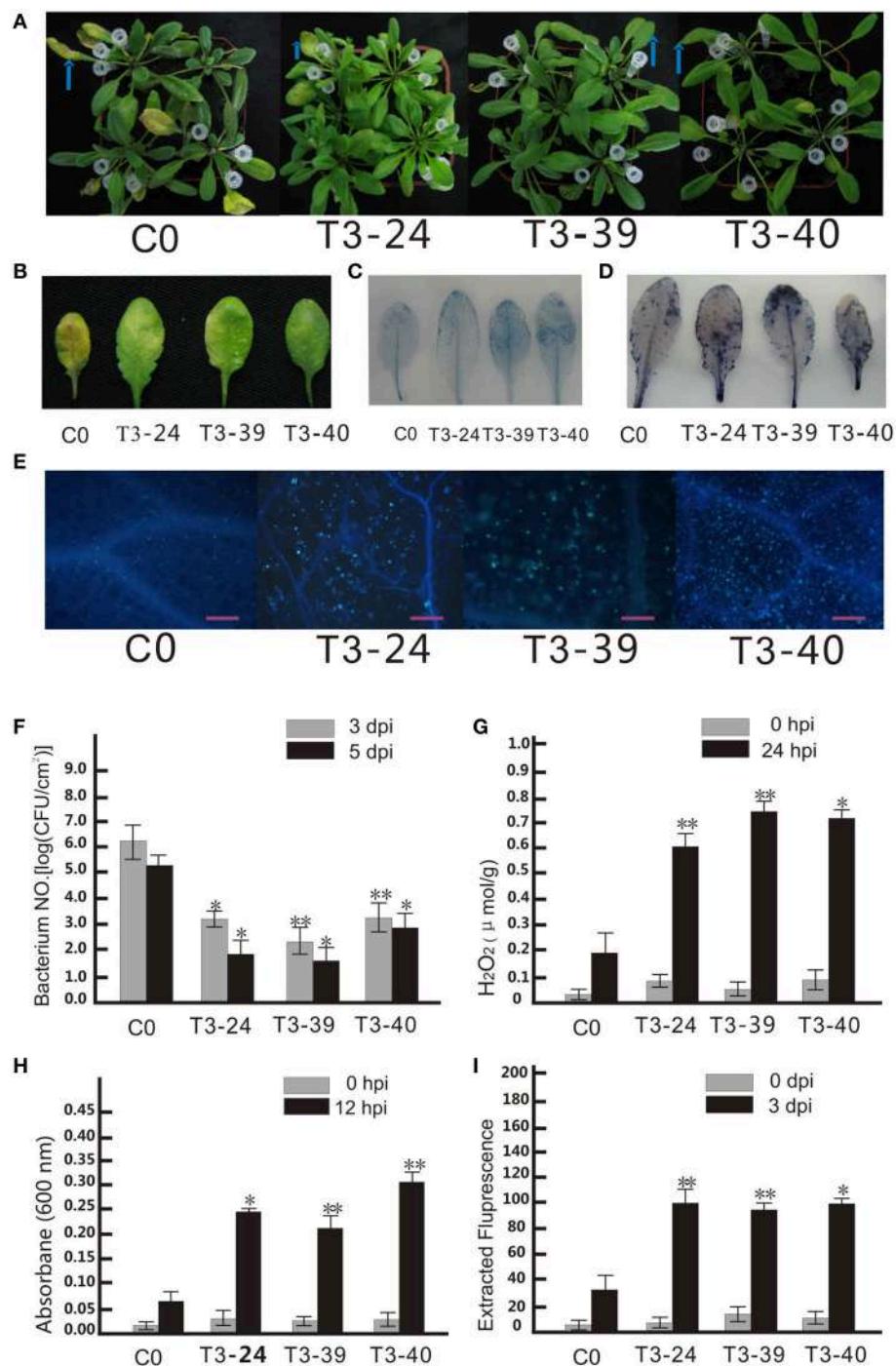


FIGURE 3 | Ectopic expression of *VpCN* in *Arabidopsis thaliana* enhanced disease resistance to *Pseudomonas syringae* pv. *tomato* DC3000. (A) *P. st* DC3000 was diluted to OD₆₀₀ 0.02 and injected into the middle of a leaf with needleless syringes. The injected leaves were marked with white pipette tips, and pictures taken 3 dpi. **(B)** Disease symptoms developed on the leaves of transgenic lines and wild type plants 3 dpi with *P.st* DC3000. **(C)** Transgenic plants and wild type leaves were stained with trypan blue 12 hpi with *P.st* DC3000. **(D)** Transgenic plants and wild type leaves were stained with nitro blue terazolium (NBT). **(E)** Microscopic observation of callose deposition after 3 dpi. Bars = 50 μm . **(F)** The numbers of bacterial cells in the leaves were determined at 3 and 5 dpi. **(G)** Detection of H₂O₂ concentration in *Arabidopsis* leaf samples harvested at 24 hpi. **(H)** Quantification of dead cells at 12 hpi. **(I)** Quantification of callose from *A. thaliana* leaves at 3 dpi. The experiment encompass three independent biological replicates, for each biological replicate six rosette leaves were harvested from three plant and three technical replicates were performed. Data represent means of three biological replicates \pm SE, asterisks indicate statistical significance in comparison with WT (Student's *t*-test, significance levels of *P < 0.05, **P < 0.01 are indicated).

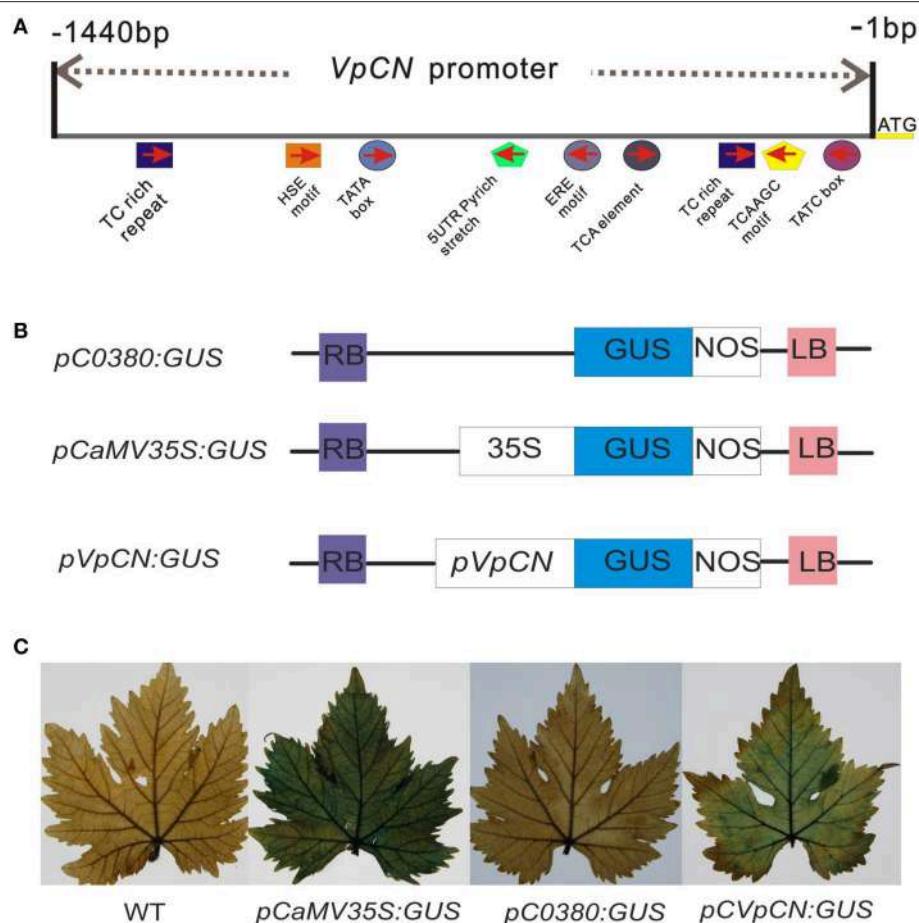


FIGURE 4 | The main predicted *cis*-acting elements in the *pVpCN* promoter sequence, structure of the *VpCN* promoter fused to the *GUS* reporter gene and *GUS* staining of the transient constructs in transformed grapevine leaves. **(A)** Schematic diagram of the main predicted *cis*-acting elements in the *VpCN* promoter sequence of Chinese wild *V. pseudoreticulata*. **(B)** The *pVpCN* promoter was fused to the *GUS* gene. The plasmid pCaMV35S:GUS was used as a positive control and pC0380:GUS was used as a negative control. **(C)** The fully expanded grapevine leaves of *V. vinifera* “Red globe” were collected from a grape germplasm resources orchard and used for agroinfiltration.

morphological defects, although since these plants eventually died, we were unable to investigate whether they also exhibited enhanced resistance to *G. Cichoracearum*. In agree with these results we suggest that *VpCN* ectopic expression may active ETI system and cause constitutive defense in three transgenic plants and cause growth retardation, spontaneous cell death. Further studies will investigate whether the three dwarf and lethal phenotypes is caused by toxic effects of high level of *VpCN* expression or the co-suppression between *VpCN* and *Arabidopsis* endogenous genes with *VpCN*-homologous sequences.

There have been several reports suggesting that over-expression of R genes enhances disease resistance due to constitutive SA accumulation, PR gene expression and active defense responses (Keller et al., 1999; Tang et al., 1999; Kim et al., 2001; Shirano et al., 2002; Stokes et al., 2002). In this study, ectopic expression of *VpCN* in *A. thaliana* enhanced disease resistance to *G. cichoracearum*, and when the PR1 transcript

levels was assessed, a 4-5 fold increase in expression was observed in 12 hpi in transgenic plants compared to WT, and these levels remained higher over the time course. These results suggest that ectopic expression of *VpCN* in *A. thaliana* activate defense responses after pathogen inoculation.

The production of reactive oxygen species (ROS), mainly in the form of a superoxide burst and H₂O₂ accumulation, is thought to enhance plant defense responses and to be essential for the establishment of plant immunity (Alvarez et al., 1998; Grant and Loake, 2000; Punja, 2004; Choi and Hwang, 2011; Kim and Hwang, 2014). In agreement with these results, we found that higher levels of O₂⁻ anions and H₂O₂ in the transgenic plants than in WT after challenging with *P. st* DC3000. This suggests that ectopic expression of *VpCN* triggers an oxidative burst to induce plant immunity to *P. st* DC3000; however, further studies are needed to investigate how oxidative burst and H₂O₂ accumulation is mediated by *VpCN*. High concentrations of ROS can result in HR-like cell death (Kovtun et al., 2000;

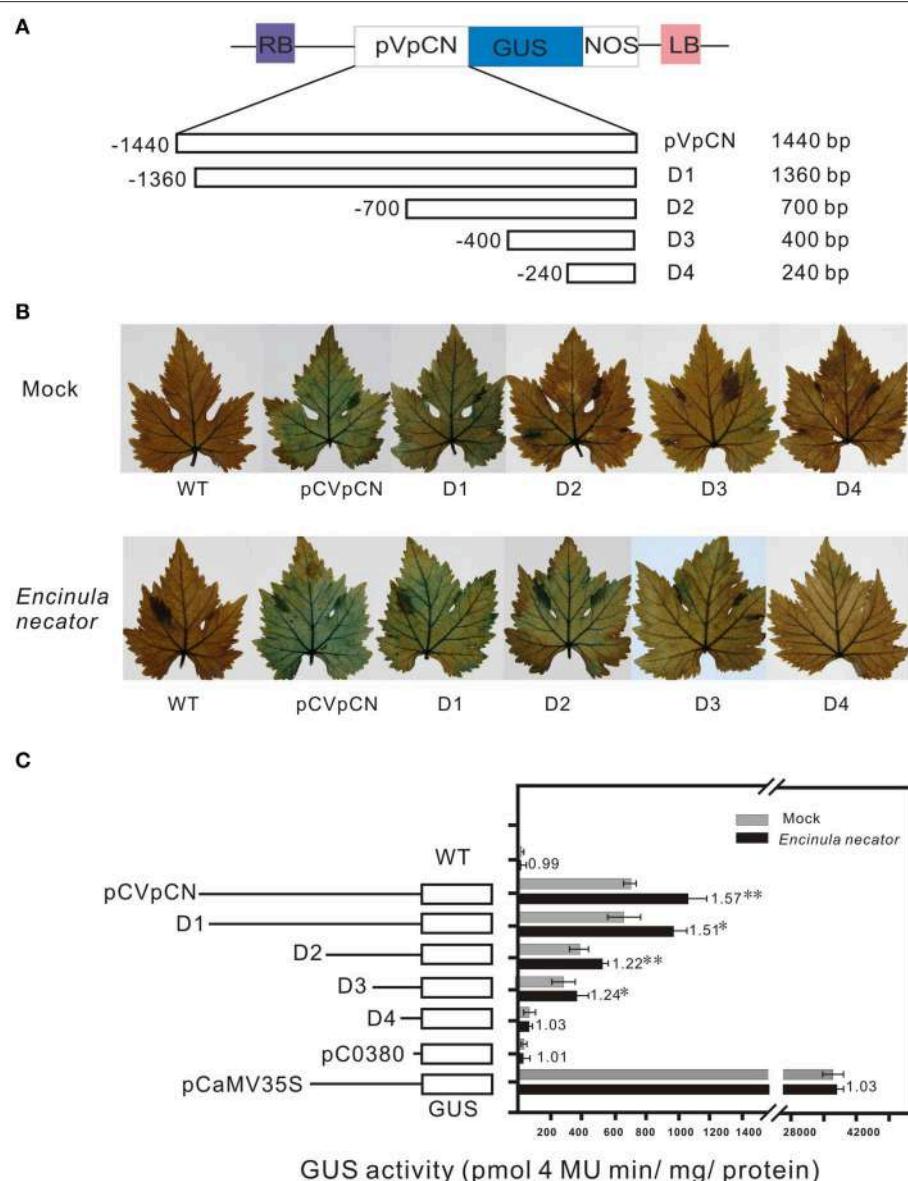


FIGURE 5 | Schematic map of the *pVpCN* promoter-*GUS* gene fusion deletion constructs, histochemical analysis of *GUS* expression in transiently transformed *V. vinifera* “Red globe” leaves after inoculation with *E. necator*, and fluorometric analysis of *GUS* activity in the transiently transformed grapevine leaves. (A) The *GUS* gene was driven by the *VpCN* promoter deletions, the exact locations of the promoter fragments are shown in **Supplement Figure 2**. The deletion size is indicated at the far right. **(B)** *GUS* staining was carried out 2 days after treatment with sterile water (upper panel) or *E. necator* (lower panel). **(C)** The various deletion fragments of the *VpCN* promoter fused to *GUS* and relative *GUS* activity driven in the transiently transformed grapevine leaves. The dark bars indicate the average *GUS* activity for deletion constructs in transiently transformed grapevine leaves treated with *E. necator*, the gray bars indicate the mock treatment (sterile water). Numbers adjacent to the bars indicate the fold difference in *GUS* activity leaves harboring the various constructs challenged with *E. necator* relative to the mock samples. The mean *GUS* activity (\pm SD) is averaged from three independent experiments ($n = 3$), the error bars indicate the stand deviation. Significant difference between treatment and mock conditions was analyzed using one sided paired *t*-test (**and * meaning $P < 0.01$ or $P < 0.05$, respectively).

Wang et al., 2007; Zhang et al., 2012), and over-expression of a TIR-NB-LRR gene from wild north American grapevine in *V. vinifera* wine grape cultivars was reported to lead to HR-like cell death after inoculation with *E. necator* (Feechan et al., 2013). Moreover, over-expression of a RPP1A truncation in *A. thaliana* induced elicitor-independent HR-like cell death (Weaver et al., 2006). In this study, an increase in ROS (O_2^- and

H_2O_2) accumulation followed by H_2O_2 induced HR-like cell death was observed after ectopic expression of *VpCN* in *A. thaliana*, when the transgenic plants were inoculated with *P. st* DC3000.

Callose-containing cell-wall appositions, called papillae, provide a physical barrier that slows pathogen invasion at the site of pathogen attack (Luna et al., 2011). Callose deposition is

typically triggered by conserved pathogen-associated molecular patterns (PAMPs) and contributes to the innate immunity (Brown et al., 1998; Luna et al., 2011). Ellinger et al. (2013) demonstrated that over-expression of *PMR4* in transgenic plants promoted early callose accumulation at attempted fungal penetration sites, which provided complete resistance to *G. cichoracearum*, and the non-adapted PM agent, *B. graminis*. In this study, transgenic plants displayed more callose deposition than WT plants in response to treatment with *P. st* DC3000, suggesting that callose deposition may contribute to the enhanced disease resistance to the pathogen displayed by the transgenic plants.

To elucidate the molecular basis of *VpCN* transcript induction after inoculation with *E. necator*, the *VpCN* promoter was isolated and its activation investigated using *A. tumefaciens*-mediated transient expression of *VpCN* in *V. vinifera* leaves. Bioinformatic analysis of the promoter sequence revealed two TC-rich repeats ('5'-ATTCTCTAAC-3'), which are thought to be involved in defense and stress responses (Diaz-De-Leon et al., 1993). We hypothesized that these might be involved in the response to *E. necator*, and generated four promoter deletion constructs to test this idea. Plants harboring a -1360, -700, or -400 bp region of the promoter sequence, all of which contain two or one TC rich repeat elements (**Supplement Figure 2**), showed increased GUS activity after challenge with *E. necator*. However, plants containing only a -240 bp region sequence, which has no TC-rich repeat elements (**Supplement Figure 2**), showed no significant change in GUS activity after inoculation with *E. necator*. Thus, we propose that the TC-rich repeat elements may play a role in the *VpCN* promoter activity in response to *E. necator* infection. This study suggests that *VpCN* is a disease resistance gene, and we will investigate that whether the *VpCN* is interact with AVR protein (effector) from *Erysiphe necator*. Further functional studies to the *VpCN* with other proteins and downstream defense signaling involved in the powdery mildew

disease resistance will be helpful in understanding the molecular mechanisms of powdery mildew disease resistance in Chinese wild *V. pseudoreticulata*.

AUTHOR CONTRIBUTIONS

XW and ZW designed the experiments. ZW, LY, RW, and ZL performed the experiments. XW, ZW, and CL analyzed the results and wrote the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01087>

Supplement Table 1 | List of primer sequence used in this study. F, Forward primer; R, Reverse primer.

Supplement Figure 1 | Sequence analysis *VpCN* from Chinese wild *V. pseudoreticulata* W. T. Wang "Baixe-35-1." The ORF sequence of *VpCN* is 1773 bp and encodes a polypeptide of 590 amino acids. The Rx-CC-like domain is labeled by single underline and the NB-ARC domain by a double underline.

Supplement Figure 2 | Sequence analysis of the *VpCN* promoter. Motifs with significant similarity to previously identified *cis*-acting elements are shaded and the names are given under each element. Sequences labeled in yellow correspond to primer design positions. Arrow heads represent the start point of the 5-deleted promoter derivatives.

REFERENCES

- Ahn, L. P., Kim, S., Lee, Y. H., and Suh, S. C. (2007). Vitamin B1-induced priming is dependent on hydrogen peroxide and the NPRL gene in *Arabidopsis*. *Plant Physiol.* 143, 838–848. doi: 10.1104/pp.106.092627
- Albrecht, M., and Takken, F. L. W. (2006). Update on the domain architectures of NLRs and R proteins. *Biochem. Biophys. Res. Commun.* 339, 459–462. doi: 10.1016/j.bbrc.2005.10.074
- Alvarez, M. E., Pennell, R. I., Meijer, P. J., Ishikawa, A., Dixon, R. A., and Lamb, C. (1998). Reactive oxygen intermediates mediate a systemic signal network in the establishment of plant immunity. *Cell* 92, 773–784. doi: 10.1016/S0092-8674(00)81405-1
- Bendahmane, A., Farnham, G., Moffett, P., and Baulcombe, D. C. (2002). Constitutive gain-of-function mutants in a nucleotide binding site-leucine rich repeat protein encoded at the Rx locus of potato. *Plant J.* 32, 195–204. doi: 10.1046/j.1365-313X.2002.01413.x
- Bendahmane, A., Kanyuka, K., and Baulcombe, D. C. (1999). The Rx gene from potato controls separate virus resistance and cell death responses. *Plant Cell* 11, 781–792. doi: 10.1105/tpc.11.5.781
- Bent, A. F., Kunkel, B. N., Dahlbeck, D., Brown, K. L., Schmidt, R., Giraudat, J., et al. (1994). RPS2 of *Arabidopsis thaliana*: a leucine-rich repeat class of plant disease resistance genes. *Science* 265, 1856–1860. doi: 10.1126/science.8091210
- Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., et al. (1998). Analysis of 1.9Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* 391, 485–493. doi: 10.1038/35140
- Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dyebinding. *Anal. Biochem.* 72, 248–254. doi: 10.1016/0003-2697(76)90527-3
- Brown, I., Trethowan, J., Kerry, M., Mansfield, J., and Bolwell, G. P. (1998). Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. *Plant J.* 15, 333–343. doi: 10.1046/j.1365-313X.1998.00215.x
- Cesari, S., Thilliez, G., Ribot, C., Chalvon, V., Michwl, C., Jauneau, A., et al. (2013). The rice resistance protein RGA4/RGA5 recognizes the *Magnaporthe oryzae* effectors AVR1-CO39 by direct binding. *Plant Cell* 25, 1463–1481. doi: 10.1105/tpc.112.107201
- Chisholm, S. T., Coaker, G., Day, B., and Staskawicz, B. J. (2006). Host-microbe interactions: shaping the evolution of plant immune response. *Cell* 124, 803–814. doi: 10.1016/j.cell.2006.02.008
- Choi, D. S., and Hwang, B. K. (2011). Proteomics and functional analyses of pepper abscisic acid-responsive 1 (ABR1), which is involved in cell death and defense signaling. *Plant Cell* 23, 823–842. doi: 10.1105/tpc.110.082081

- Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* 16, 735–743. doi: 10.1046/j.1365-313x.1998.00343.x
- Collier, S. M., Hamel, L. P., and Moffett, P. (2011). Cell death mediated by the N-terminal domains of a unique and highly conserved class of NB-LRR Protein. *Mol. Plant Microbe Interact.* 24, 918–931. doi: 10.1094/MPMI-03-11-0050
- Collier, S. M., and Moffett, P. (2009). NB-LRRs works a “bait and switch” on pathogens. *Trends Plant Sci.* 14, 521–529. doi: 10.1016/j.tplants.2009.08.001
- Diaz-De-Leon, F., Klotz, K. L., and Lagrimini, M. (1993). Nucleotide sequence of the tobacco (*Nicotiana tabacum*) anionic peroxides gene. *Plant Physiol.* 101, 1117–1118. doi: 10.1104/pp.101.3.1117
- Ellinger, D., Naumann, M., Falter, C., Zwirkowics, C., Jamrow, T., Manisseri, C., et al. (2013). Elevated early callose deposition results in complete penetration to powdery mildew in *Arabidopsis*. *Plant Physiol.* 161, 1433–1444. doi: 10.1104/pp.112.211011
- Fan, J., Crooks, C., and Lamb, C. (2008). High-throughput quantitative luminescence assay of the grow thin planta of *Pseudomonas syringae* chromosomally tagged with *Photobacterium luminescens* lux CDABE. *Plant J.* 53, 393–399. doi: 10.1111/j.1365-313X.2007.03303.x
- Feechan, A., Anderson, C., Torregrosa, L., Jermakow, A., Mestre, P., Wiedemann, M. S., et al. (2013). Genetic dissection of a TIR-NB-LRR locus from the wild North American grapevine species *Muscadina rotundifolia* identifies paralogous genes conferring resistance to major fungal and oomycete pathogens in cultivated grapevine. *Plant J.* 76, 661–674. doi: 10.1111/tpj.12327
- Friedrich, L., Lawton, K., Ruess, W., Masnet, P., Specker, N., Rella, M. G., et al. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant J.* 10, 61–70. doi: 10.1046/j.1365-313X.1996.10010061.x
- Frye, C. A., and Innes, W. (1998). An *Arabidopsis* mutant with enhanced resistance to powdery mildew. *Plant Cell* 10, 947–956. doi: 10.1105/tpc.10.6.947
- Gadoury, D. M., Cadle-Davidson, L., Wilcox, W. F., Dry, I. B., Seem, R. C., and Milgroom, M. G. (2012). Grapevine powdery mildew (*Erysiphe necator*): a fascinating system for the study of the biology, ecology and epidemiology of an obligate biotroph. *Mol. Plant Pathol.* 13, 1–16. doi: 10.1111/j.1364-3703.2011.00728.x
- Grant, J. J., and Loake, G. J. (2000). The Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. *Plant Physiol.* 124, 21–29. doi: 10.1104/pp.124.1.21
- Guan, X., Zhao, H. Q., Xu, Y., and Wang, Y. J. (2011). Transient expression of glyoxal oxidase from the Chinese wild grape *Vitis pseudoreticulata* can suppress powdery mildew in a susceptible genotype. *Protoplasma* 248, 415–423. doi: 10.1007/s00709-010-0162-4
- Hao, W., Collier, S. M., Moffett, P., and Chai, J. J. (2013). Structural basis for the interaction between the potato virus X resistance protein (Rx) and its cofactor Ran GTPase-activating protein 2 (RanGAP2). *J. Biol. Chem.* 288, 35868–35876. doi: 10.1074/jbc.M113.517417
- Huang, W. J., Jiang, T. Y., Choi, W., Pang, Y. X., Hu, Q., Xu, Y. H., et al. (2013). Mechanistic insights into CED-4-mediated activation of CED-3. *Genes Dev.* 27, 2039–2048. doi: 10.1101/gad.224428.113
- Jefferson, R. (1987). Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep.* 5, 387–405. doi: 10.1007/BF02667740
- Jones, J. D., and Dangl, K. L. (2006). The plant immune systems. *Nature* 444, 323–329. doi: 10.1038/nature05286
- Keller, H., Pamboukdjian, N., Ponchet, M., Poupet, A., Delon, R., Verrier, J. L., et al. (1999). Pathogen-induced elicitor production in transgenic tobacco generates a hypersensitive response and nonspecific disease resistance. *Plant Cell* 11, 223–235. doi: 10.1105/tpc.11.2.223
- Kim, D. S., and Hwang, B. K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signaling of the defense response to microbial pathogens. *J. Exp. Bot.* 65, 2295–2306. doi: 10.1093/jxb/eru109
- Kim, S., Ahn, I. P., Park, C., Park, S. G., Park, S. Y., Jwa, N. S., et al. (2001). Molecular characterization of the cDNA encoding an acidic isoform of PR-1 gene protein in rice. *Mol. Cells* 11, 115–121. Available online at: <http://europepmc.org/abstract/MED/11266113>
- Kim, S. H., Kwon, S. I., Saha, D., Anyanwu, N. C., and Gassmann, W. (2009). Resistance to the *Pseudomonas syringae* effector HopA1 is governed by the TIR-NBS-LRR protein RPS6 and is enhanced by mutations in SRFR1. *Plant Physiol.* 150, 1723–1732. doi: 10.1104/pp.109.139238
- Kim, S. H., Woo, D. H., Kim, J. M., Lee, S. Y., Chung, W. S., and Moon, Y. H. (2011). *Arabidopsis* MKK4 mediates osmotic-stress response via its regulation of MPK3 activity. *Biochem. Biophys. Res. Commun.* 412, 150–154. doi: 10.1016/j.bbrc.2011.07.064
- Koch, E., and Slusarenko, A. (1990). *Arabidopsis* is susceptible to infection by a downy mildew fungus. *Plant Cell* 2, 437–445. doi: 10.1105/tpc.2.5.437
- Kohler, A., Schwindling, S., and Conrath, U. (2000). Extraction and quantitative determination of callose from *Arabidopsis* leaves. *Biotechniques* 28, 1084–1086.
- Kohm, B. A., Goulden, M. G., Gilbert, J. E., Kavanagh, T. A., and Baulcombe, D. C. (1993). A potato virus X resistance gene mediates an induced, nonspecific resistance in protoplasts. *Plant Cell* 5, 913–920. doi: 10.1105/tpc.5.8.913
- Kovtun, Y., Chiu, W. L., Tena, G., and Sheen, J. (2000). Functional analysis of oxidative stress-activated mitogen-activated protein kinase cascade in plants. *Proc. Natl. Acad. Sci. U.S.A.* 97, 2940–2945. doi: 10.1073/pnas.97.6.2940
- Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., et al. (2002). PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. *Nucleic Acids Res.* 30:325. doi: 10.1093/nar/30.1.325
- Lin, L., Wang, X. P., and Wang, Y. J. (2006). cDNA clone, fusion expression and purification of the novel gene related to ascorbate peroxidase from Chinese wild *Vitis pseudoreticulata* in *E.coli*. *Mol. Biol. Rep.* 33, 197–206. doi: 10.1007/s11033-006-0008-5
- Lukasik-Shreepaathy, W., Slootweg, E., Richter, H., Goverse, A., Cornelissen, B. J. C., and Takken, F. L. W. (2012). Dual regulatory roles of the extended N terminus for activation of the tomato Mi-1.2 resistance protein. *Mol. Plant Microbe Interact.* 25, 1045–1057. doi: 10.1094/MPMI-11-11-0302
- Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., and Ton, J. (2011). Callose deposition: a multifaceted plant defense response. *Mol. Plant Microbe Interact.* 24, 183–193. doi: 10.1094/MPMI-07-10-0149
- Maekawa, T., Cheng, W., Spiridon, L. N., Töller, A., Lukasik, E., and Sajio, Y. (2011). Coiled-coil domain-dependent homo-dimerization of intracellular barley immune receptors defines a minimal functional module for triggering cell death. *Cell Host Microbe* 9, 187–199. doi: 10.1016/j.chom.2011.02.008
- Mestre, P., and Baulcombe, D. C. (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. *Plant Cell* 18, 491–501. doi: 10.1105/tpc.105.037234
- Meyers, B. C., Dickerman, A. W., Michelmore, R. W., Sivaramakrishnan, S., Sobral, B. W., and Young, N. D. (1999). Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. *Plant J.* 20, 317–333. doi: 10.1046/j.1365-313X.1999.t01-1-00606.x
- Mino, M., Maekawa, K., Ogawa, K., Yamagishi, H., and Inoue, M. (2002). Cell death processes during expression of hybrid lethality in interspecific F1 hybrid between *Nicotiana gossei* domin and *Nicotiana tabacum*. *Plant Physiol.* 130, 1776–1787. doi: 10.1104/pp.006023
- Mohr, T. J., Mammarella, N. D., Hoff, T., Woffenden, B. J., Jelesko, J. G., and McDowell, J. M. (2010). The *Arabidopsis* downy mildew resistance gene RPP8 is induced by pathogens and salicylic acid and is regulated by W Box cis elements. *Mol. Plant Microbe Interact.* 23, 1303–1315. doi: 10.1094/MPMI-01-10-0022
- Murshige, T., and Skoog, F. (1962). A revised medium for rapid growth bioassays with tobacco tissue cultures. *Physiol. Plant.* 15, 473–497. doi: 10.1111/j.1399-3054.1962.tb08052.x
- Nandety, R. S., Caplan, J. L., Cavanaugh, K., Perroud, B., Wroblewski, T., Michelmore, R. W., et al. (2013). The role of TIR-NBS and TIR-X proteins in plant basal defense response. *Plant Physiol.* 162, 1459–1472. doi: 10.1104/pp.113.219162
- Nie, H. Z., Wu, X. Y., Yao, C. P., and Tang, D. Z. (2011). Suppression of edr2-mediated powdery mildew resistance, cell death and ethylene-induced senescence by mutations in ALD1 in *Arabidopsis*. *J. Genet. Genomics* 38, 137–148. doi: 10.1016/j.jgg.2011.03.001
- Oijen, G. V., Mayr, G., Albrecht, M., Cornelissen, B. J. C., and Takken, F. L. W. (2008). Transcomplementation, but not physical association of the CC-NB-ARC and LRR domains of tomato R protein Mi-1.2 is altered by mutations in the ARC2 sub-domain. *Mol. Plant* 1, 401–410. doi: 10.1093/mp/ssp009
- Ori, N., Eshed, Y., Paran, I., Presting, G., Aviv, D., Tanksley, S., et al. (1997). The I2C family from the wilt disease resistance locus I2 belongs to the nucleotide binding, leucine-rich repeat superfamily of plant resistance genes. *Plant Cell* 9, 521–532. doi: 10.1105/tpc.9.4.521

- Pan, Q., Liu, Y. S., Budai-Hadrian, O., Sela, M., Carmel-Goren, L., Zamir, D., et al. (2000b). Comparative genetics of nucleotide binding site-leucine rich repeat resistance gene homologues in the genomes of two dicotyledons: tomato and *Arabidopsis*. *Genetics* 155, 309–322. Available online at: <http://www.genetics.org/content/155/1/309.long>
- Pan, Q., Wendel, J., and Fluhr, R. (2000a). Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. *J. Mol. Evol.* 50, 203–213. doi: 10.1007/s002399910023
- Punja, Z. K. (2004). *Fungal Disease Resistance in Plants*. New York, NY; London; Oxford: Food Products Press® An important of the Haworth Press, Inc.
- Radirdan, G. J., Collier, S. M., Sacco, M. A., Baldwin, T. T., Boettcher, T., and Moffett, P. (2008). The coiled-coil and nucleotide binding domains of the potato Rx disease resistance protein function in pathogen recognition and signaling. *Plant Cell* 20, 739–751. doi: 10.1105/tpc.107.056036
- Reuber, T. L., Plotnikova, J. M., Dewdney, J., Rogers, E. E., Wood, W., and Ausubel, F. M. (1998). Correlation of defense gene induction defects with powder mildew susceptibility in *Arabidopsis* enhanced disease susceptibility mutants. *Plant J.* 16, 473–485. doi: 10.1046/j.1365-313x.1998.00319.x
- Riedl, S. J., Li, W., Chao, Y., Schwarzenbacher, R., and Shi, Y. (2005). Structure of the apoptotic protease-activating factor 1 bound to ADP. *Nature* 434, 926–933. doi: 10.1038/nature03465
- Santos-Rosa, M., Poutaraud, A., Merdinoglu, D., and Mestre, P. (2008). Development of a transient expression system in grapevine via agroinfiltration. *Plant Cell Rep.* 27, 1053–1063. doi: 10.1007/s00299-008-0531-z
- Sekine, K. T., Tomita, R., Takeuchi, S., Atsumi, G., Saitoh, H., Miroyuki, H., et al. (2012). Functional differentiation in the leucine-rich repeat domains of closely related plant virus-resistance proteins that recognize common avr proteins. *Mol. Plant Microbe Interact.* 25, 1219–1229. doi: 10.1094/MPMI-11-11-0289
- Shirano, Y., Kachroo, P., Shah, J., and Klessig, D. F. (2002). A gain-of-function mutation in an *Arabidopsis* Toll Interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. *Plant Cell* 14, 3149–3162. doi: 10.1105/tpc.005348
- Smith, L. D., and Goodman, N. L. (1975). Improved culture method for the isolation of *Histoplasma capsulatum* and *Blastomyces dermatitidis* from contaminated specimens. *Am. J. Clin. Pathol.* 63, 276–280. Available online at: <http://europepmc.org/abstract/med/1115035>
- Stokes, T. L., Kunkel, B. L., and Richards, E. J. (2002). Epigenetic variation in *Arabidopsis* disease resistance. *Genes Dev.* 16, 171–182. doi: 10.1101/gad.952102
- Takken, F. L. W., Albrecht, M., and Tameling, W. I. L. (2006). Resistance proteins: molecular switches of plant defence. *Curr. Opin. Plant Biol.* 9, 383–390. doi: 10.1016/j.pbi.2006.05.009
- Tameling, W. I. L., Noojien, C., Ludwig, N., Boter, E., Goverse, A., Shirasu, K., et al. (2010). RanGAP2 mediates nucleocytoplasmic partitioning of the NB-LRR immune receptor Rx in the solanaceae, There by dictating Rx function. *Plant Cell* 22, 4176–4194. doi: 10.1105/tpc.110.077461
- Tang, D. Z., and Innes, R. W. (2002). Over-expression of a kinase-deficient form of the EDR1 gene enhances powdery mildew resistance and ethylene-induced senescence in *Arabidopsis*. *Plant J.* 32, 975–983. doi: 10.1046/j.1365-313X.2002.01482.x
- Tang, X. Y., Xie, M. T., Kim, Y. J., Zhou, J. M., Klessig, D. F., and Martin, G. B. (1999). Over expression of Pto activates defense responses and confers broad resistance. *Plant Cell* 11, 15–29.
- Tao, Y., Yuan, F. H., Leister, R. T., Ausubel, F. M., and Katagiri, F. (2000). Mutational analysis of the *Arabidopsis* nucleotide binding site-leucine-rich repeat resistance gene RPS2. *Plant Cell* 12, 2541–2554.
- Theologis, A., Ecker, J. R., Palm, C. J., Federspiel, N. A., Kaul, S., White, O., et al. (2000). Sequence and analysis of chromosome 1 of the plant *Arabidopsis thaliana*. *Nature* 408, 816–820. doi: 10.1038/35048500
- van der Biezen, E. A., and Jones, J. D. G. (1998). The NB-ARC domain: a novel signaling motif shared by plant resistance gene products and regulators of cell death in animals. *Curr. Biol.* 8, 226–227. doi: 10.1016/S0960-9822(98)70145-9
- Wang, W. M., Devoto, A., Turner, J. G., and Xiao, S. Y. (2007). Expression of the membrane-associated resistance protein RPW8 enhances basal defense against biotrophic pathogens. *Mol. Plant Microbe Interact.* 20, 966–976. doi: 10.1094/MPMI-20-8-0966
- Wang, Y., Liu, Y., He, P., Chen, J., Lamikanra, O., and Lu, J. (1995). Evaluation of foliar resistance to *Uncinula necator* in Chinese wild *Vitis* species. *Vitis* 3, 159–164.
- Weaver, L. M., Swiderski, M. R., Li, Y., and Jones, J. D. G. (2006). The *Arabidopsis thaliana* TIR-NB-LRR R proteins, RPP1A; Protein localization and constitutive activation of defense by truncated alleles in tobacco and *Arabidopsis*. *Plant J.* 47, 829–840. doi: 10.1111/j.1365-313X.2006.02834.x
- Weng, K., Li, Z. Q., Liu, Q. R., Wang, L., Wang, Y. J., and Xu, Y. (2014). Transcriptome of *Erysiphe necator*-infected *Vitis pseudoreticulata* leaves provides insight into grapevine resistance to powdery mildew. *Hortic. Res.* 1:14049. doi: 10.1038/hortres.2014.49
- Xu, W. R., Yu, Y. H., Ding, J. H., Hua, Z. H., and Wang, Y. J. (2010). Characterization of a novel stilbene synthase promoter involved in pathogen- and stress-inducible expression from Chinese wild *Vitis pseudoreticulata*. *Planta* 231, 475–487. doi: 10.1007/s00425-009-1062-8
- Yu, Y. H., Xu, R. W., Wang, J., Wang, L., Yao, W. K., Xu, Y., et al. (2013). A core functional region of the *RFP1* promoter from Chinese wild grapevine is activated by powdery mildew pathogen and heat stress. *Planta* 237, 293–303. doi: 10.1007/s00425-012-1769-9
- Yu, Y. H., Xu, W. R., Wang, S. Y., Xu, Y., Li, H. E., Wang, Y. J., et al. (2011). *VpRFP1*, a novel C4C4-type RING finger protein gene from Chinese wild *Vitis pseudoreticulata*, functions as a transcriptional activator in defence response of grapevine. *J. Exp. Bot.* 62, 5671–5682. doi: 10.1093/jxb/err253
- Zhang, J. J., Wang, Y. J., Wang, X. P., Yang, K. Q., and Yang, J. X. (2003). An improved method for rapidly extracting total RNA from *Vitis*. *J. Fruit Sci.* 53, 771–787. Available online at: <http://www.gskk.cbpt.cnki.net/WKA/WebPublication/paperDigest.aspx?>
- Zhang, L., Li, Y. Z., Lu, W., Meng, F., Wu, C. A., and Guo, X. Q. (2012). Cotton GhMKK5 affects disease resistance, induces HR-like cell death, and reduces the tolerance to salt and drought stress in transgenic *Nicotiana benthamiana*. *J. Exp. Bot.* 63, 3935–3951. doi: 10.1093/jxb/ers086
- Zhang, Y., Dorey, S., Swiderski, M., and Jones, J. D. (2004). Expression of RPS4 in tobacco induced an AvrRPS4-independent HR that requires EDS1, SGT1 and HSP90. *Plant J.* 40, 213–224. doi: 10.1111/j.1365-313X.2004.02201.x
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Variation in Host and Pathogen in the *Neonectria/Malus* Interaction; toward an Understanding of the Genetic Basis of Resistance to European Canker

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Apple canker caused by the phytopathogenic fungus *Neonectria ditissima* is an economically important disease, which has spread in recent years to almost all pome-producing regions of the world. *N. ditissima* is able to cross-infect a wide range of apple varieties and causes branch and trunk lesions, known as cankers. Most modern apple varieties are susceptible and in extreme cases suffer from high mortality (up to 50%) in the early phase of orchard establishment. There is no known race structure of the pathogen and the global level of genetic diversity of the pathogen population is unknown. Resistance breeding is underway in many global breeding programmes, but nevertheless, a total resistance to canker has not yet been demonstrated. Here we present preliminary data from a survey of the phylogenetic relationships between global isolates of *N. ditissima* which reveals only slight evidence for population structure. In addition we report the results of four rapid screening tests to assess the response to *N. ditissima* in different apple scion and rootstock varieties, which reveals abundant variation in resistance responses in both cultivar and rootstock material. Further seedling tests show that the segregation patterns of resistance and susceptibility vary widely between crosses. We discuss inconsistencies in test performance with field observations and discuss future research opportunities in this area.

Keywords: European canker, pathogenicity test, *Neonectria ditissima*, phylogenetics, disease resistance

INTRODUCTION

European canker (caused by *Neonectria ditissima*) is one of the most destructive diseases of apple and pear. The fungus attacks trees in the orchard, causing cankers and dieback of young shoots, resulting in loss of fruiting wood and increased pruning costs (Swinburne, 1975). Apple canker can be particularly damaging in young orchards where, in some years, up to 10% of trees can be lost annually in the first few years of orchard establishment as a result of trunk cankers (Angela Berrie,

personal communication). In some regions of the world (i.e., Northern Europe) *N. ditissima* can also cause a fruit rot in stored fruit. The rot, which is often found at the fruit stalk end, is difficult to spot on the grading line, but becomes obvious during marketing leading to rejection of fruit consignments (Xu and Robinson, 2010).

The taxonomic history of the pathogen is somewhat complex, having altered in name repeatedly over the past 150 years. Studies based largely on host range and morphology have at various times divided the original pathogen (named *Neonectria ditissima* Tul. and C. Tul, Tulasne and Tulasne, 1865) into two separate species, *Nectria ditissima* and *Nectria galligena* (Bres.) (Cayley, 1921) and later renamed *Neonectria galligena* (Bres.) before returning to its original name some 10 years ago (Castlebury et al., 2006). The anamorphic state is *Cylindrocarpon heteronema* (Berk. and Broome) Wollenw. 1916.

The host range of *N. ditissima* encompasses multiple hardwood tree species such as *Fagus*, *Populus*, *Acer*, *Salix*, and *Betula* species (Castlebury et al., 2006; Walter et al., 2015). Phylogenetic studies have revealed that European and American populations appear to have a significant level of nucleotide divergence at β -tubulin and RPB2 loci (Castlebury et al., 2006), indicating that the populations may be allopatrically isolated. American populations of *N. distissima* have been shown to contain abundant within-population diversity (Plante et al., 2002), which led to the hypothesis that America is the center of origin of *N. ditissima*. However, as stated by Castlebury, without further sampling in Europe (despite recent work) this cannot yet be confirmed (Ghasemkhani et al., 2016).

Much is known about the epidemiology of the disease in the orchard (see **Figure 1** for a graphical depiction of the lifecycle). The fungus produces two spore types, conidia (imperfect/aseexual spores) and ascospores (sexual/perfect spores). Conidia are generally produced within the first year of canker formation when the temperature increases in the spring and summer and are spread throughout the season by rain splash. By contrast, ascospores are mainly produced by old canker lesions during the autumn, winter and spring and are discharged during rain, and wind- or splash-dispersed. Both spore types enter through wounds, either natural such as bud-scale scars, leaf scars, fruit scars or artificial such as pruning wounds. Thus, inoculum and points of entry on the tree are available all year round (Amponsah et al., 2015) and the only limiting factor is rain, which is essential for spore production, spread, germination and infection (Xu et al., 1998). The disease is most destructive in young trees infected with canker, as latent infections appear as systemic infection and trunk cankers several years after planting (McCracken et al., 2003). Factors that affect canker expression are not understood but possibly relate to stress (cold, drought, water-logging and herbicide applications), or fertilizer applications. For example, post-harvest foliar nitrogen applications increased leaf scar infections six- to nine-fold in New Zealand orchards (Dryden et al., 2016). New cultivars being planted in the UK such as "Scifresh," "Cameo," "Kanzi," "Zari," "Rubens," and older cultivars such as "Gala" and "Braeburn" are all very susceptible to *N. ditissima* and the development of systemic canker in young orchards leading to tree loss is a significant problem, with severe

financial loss particularly in modern intensive planting systems (Weber, 2014). In contrast to the orchard, the epidemiology of *N. ditissima* in the nursery is not understood and infected trees are rarely seen in nursery production so it is assumed that the disease is present as a latent infection (McCracken et al., 2003).

Currently canker is controlled in the orchard by a combination of cultural methods to remove canker lesions and the use of protectant fungicides. However, Cooke showed that even the most stringent fungicide programmes only reduce the increase of canker incidence but canker incidence continued to increase (Cooke, 1999). Therefore, this approach does not seem to prevent the fungus from invading the trees, causing cankers.

For the apple—*N. ditissima* pathosystem, very little is known about the pathogenicity factors of the pathogen or the resistance mechanisms of the host. Recent work using the cultivar "Royal Gala" has demonstrated that there are strains of *N. ditissima* that are almost non-pathogenic and others that are pathogenic, though it is not yet known whether the nearly non-pathogenic isolates are more pathogenic on other cultivars (Scheper et al., 2015). It is also unknown how resistance may be expressed in different tissues of the host, e.g., wood vs. fruit. It may be that resistance mechanisms are localized at the leaf scar, an area that is vulnerable to pathogen attack, as many reports have shown variation in susceptibility of leaf scar infections (Alston, 1970; Amponsah et al., 2015).

Malus species and apple cultivars show variation in susceptibility to *N. ditissima* (Alston, 1970; Van De Weg, 1987; van de Weg, 1989; Ghasemkhani et al., 2015) though most modern varieties are susceptible. Variations in disease susceptibility may partly be a result of disease escape, e.g., the speed of wound healing in relation to *N. ditissima* infection has been shown to differ between cultivars (Xu et al., 1998). Other studies have shown that variation in colonization rate is important for resistance responses (Van De Weg, 1987; van de Weg, 1989). There is clear evidence in some breeding material of a genetic based resistance with resistance controlled predominantly by additive gene action (Gelvonauskiene et al., 2007). This offers the potential to map quantitative trait loci (QTL) in progeny segregating for disease resistance (i.e., derived from parents which have high resistance and susceptibility). Once discovered, QTLs may be cloned and the underlying resistance mechanism determined through functional genomics. Ultimately, molecular markers designed close to the QTLs, or in the causal resistance genes can be utilized in breeding programmes. At Plant and Food Research (PFR), evaluation of a germplasm sub-set showed that "Robusta 5," "Golden Delicious," "Priscilla," and "Close" have good levels of resistance and are good candidates for future QTL studies (Bus et al., in press).

No specific molecular resistance mechanisms have yet been reported to *N. ditissima*. It is therefore unknown whether basal defenses are constitutively higher in resistant cultivars, or whether the strength or breadth of downstream induced resistance responses contributes to quantitative variation in resistance to *N. ditissima*. Variation in loci implicated in basal resistance, for example allelic variation in clusters of germin-like proteins, have been implicated in quantitative resistance in

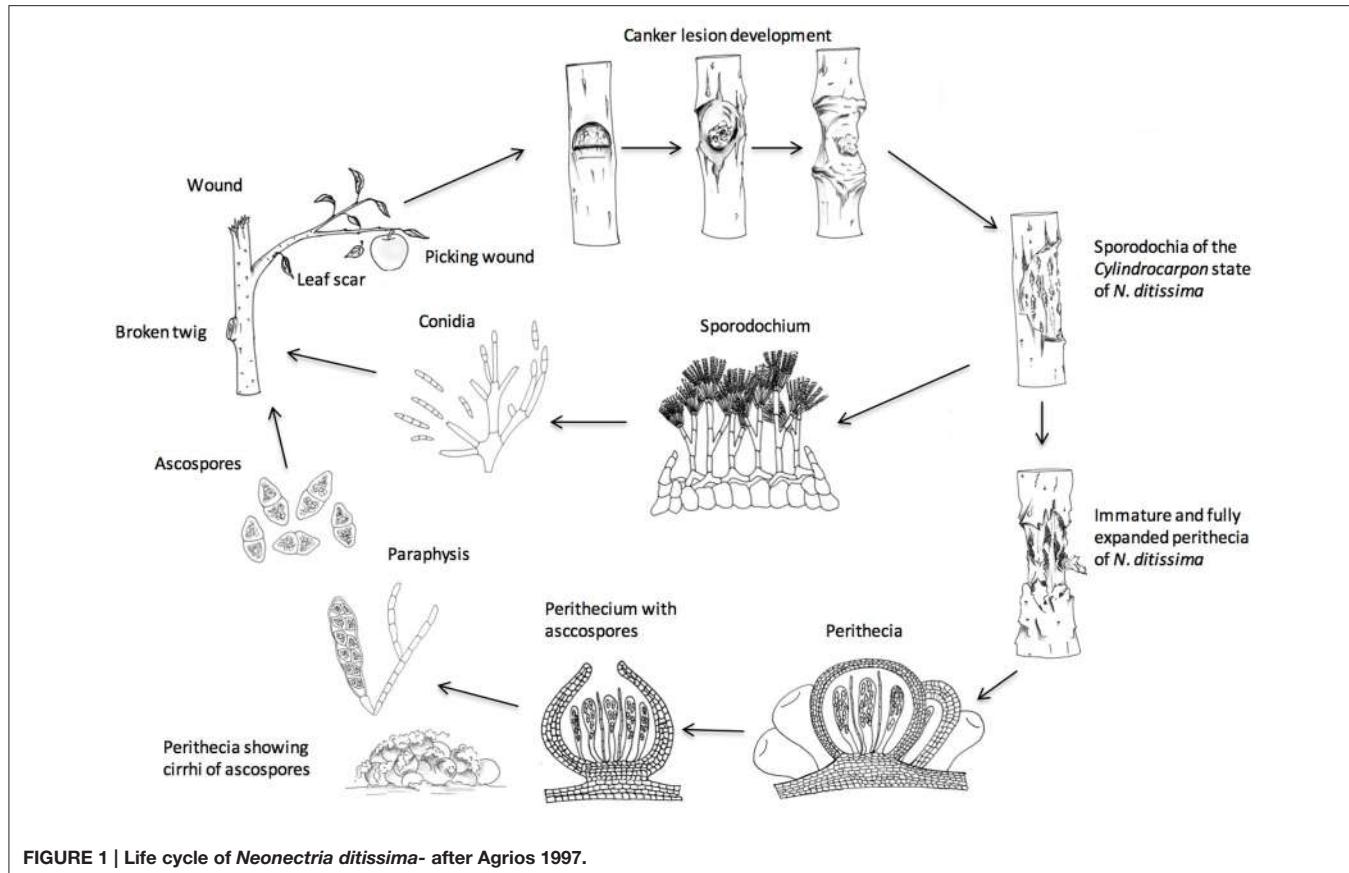


FIGURE 1 | Life cycle of *Neonectria ditissima*- after Agrios 1997.

other systems, indicating that both the complement of basal defense genes and the strength of the induced responses are important (Manosalva et al., 2009). It is important to understand not only the genetic architecture of resistance (and the tissues in which it is expressed), but also the mechanism by which the pathogen is detected by the host. In a classical gene-for-gene system, loss or mutation of genes or motifs within proteins in the pathogen, that the plant uses to recognize and activate defenses (*R*-gene mediated resistance), results in a loss of resistance (Jones and Dangl, 2006). This model applies equally to major gene resistance or a quantitative gene-for-gene model. Work done with *Phytophthora infestans* late-blight and the cultivated potato, *Solanum tuberosum* is a current example of quantitative gene-for-gene resistance which is dependent upon recognition of multiple RxLR-containing pathogen effector genes (Rietman et al., 2012). Similar examples of quantitative *R*-gene mediated resistance have been reported in *Oryza sativa*-*Magnaporthe* interactions (Liu et al., 2011) and non-host resistance in pepper against *P. infestans* (Lee et al., 2014). It is likely that *R* genes may also underpin resistance to *N. ditissima*, coupled to a MAMP-triggered immune response, i.e., basal defense below the level required to activate the hypersensitive response (HR) as reported for the SCFE1/RLP30 interaction with the necrotrophic pathogen *Sclerotinia sclerotiorum* and *Arabidopsis thaliana* (Zhang et al., 2013). Alternatively resistance could also follow an inverse gene-for-gene model (Fenton et al., 2009), whereby resistance

genes act as factors that the pathogen may exploit to activate HR deliberately, as in the case of *Botrytis cinerea* and other necrotrophic pathogens, in order to provide a nutrient source for the pathogen (Govrin and Levine, 2000). In this case, loss of recognition by *R* genes would lead to lack of HR and therefore a loss of susceptibility. Either of these is a possibility and is one of the fundamental questions that remains to be addressed in this pathosystem. In order to assess the durability of resistance (Vleeshouwers et al., 2011) it is important to understand what evolutionary constraints pathogen genes that a host may recognize are under. By assessing the likelihood that these genes can be lost, i.e., whether they are dispensable or indispensable for pathogen virulence (polymorphic for presence/absence in a pathogenic population), under relaxed selective constraints, or able to rapidly adapt (elevated non-synonymous polymorphisms or substitutions), an assessment can be made about whether these factors are likely to rapidly evolve to evade recognition.

The objective of this study was to identify patterns of nucleotide diversity in a global sample of *N. ditissima* to understand whether there are significant differences between geographically distinct populations and to develop methods to study pathogen variability and host responses using a range of inoculation tests with apple material at different developmental stages. Understanding the patterns of nucleotide diversity of different populations of *N. ditissima* along with key population genetic parameters, such as the level of recombination within

populations, gene flow between populations and the likely origin of different subpopulations is key when considering how to deploy resistance in a globally grown commodity crop such as apple. Due to the lack of regionally adapted ideotypes in apple, it is likely that the same cultivar could be grown in all areas of the world. It is therefore important to understand the level of pathogen variability and whether there are significant differences between the levels of standing genetic variation, sexual reproduction (and hence efficacy of selection) and pathogenicity of locally prevalent pathogenic isolates.

MATERIALS AND METHODS

Locus Identification for Phylogenetic Analysis

Existing primer sets from Marra and Corwin (2009), Shivas and Tan (2009), Gräfenhan et al. (2011) and Armitage et al. (2015) were BLASTed to the R09/05 genome sequence in order to identify loci (Gómez-Cortecero et al., 2015). Extracted regions were then BLASTed to the N305S21 and N324S12 *N. ditissima* genomes (Deng et al., 2015). Hits to the three genomes were examined for polymorphism and primers that contained no polymorphic sites between isolates with an amplicon length of approximately 500 bp were designed. Primers were BLASTed back to the R09/05 genome to ensure they only hit a single gene region. In short, primers to a CDP-diacylglycerol-3-phosphate-phosphatidyltransferase (CDP) (Armitage et al., 2015) spanning the second and third exons were designed, along with primers to an intergenic region upstream of NdCAA4, named NDCAA4_prox (Marra and Corwin, 2009), primers within the same gene in which NdCAA11 primers mapped (spanning the first intron, named NDCAA11_sub) and primers from a putative ATP-citrate synthase subunit 2 gene which multi-species ACL1 primers hit (again spanning the first intron; Shivas and Tan, 2009; Gräfenhan et al., 2011). Primer sequences are shown in Supplementary Table 1.

DNA Extraction and PCR Amplification

N. ditissima mycelium was grown in YPD liquid media (20 g Bacto peptone, 10 g yeast extract, 950 mL of water, 50 mL of 40% w/v glucose). A sterile toothpick was used to scrape young mycelia of *N. ditissima* from an agar plate and to inoculate a flask with 20 ml of YPD. The flask was closed with a cotton gauze and covered with aluminum foil. The culture flask was incubated in a shaker at a constant 20°C at 120 rpm for 1 week. Cultures were then centrifuged at 5000 g and the supernatant removed. The mycelium was washed with 10 ml of sterile water and the supernatant removed after centrifugation. Liquid nitrogen was used to freeze the mycelium and 100 mg of wet weight was homogenized using ball bearings and a tissue lyser for 2 min at 15 Hz. For DNA extraction the Macherey-Nagel NucleoSpin Plant II kit was used following a modified manufacturer's protocol.

The PCR reaction mixture contained 1 µl of gDNA (5 ng/µl), 0.5 µM of each primer, 0.625U of Taq polymerase, 0.2 mM of dNTPs, 1X PCR buffer and water to a final volume of 25 µl. The thermal profile used for the amplifications was

slightly different depending upon the primers used. For the polymorphic microsatellite loci primer pairs NDCAA4_prox and NDCAA11_sub the following amplification programme was used: 95°C for 2 min followed by 30 cycles of 95°C for 30 s and then 55°C for 1 min and 72°C for 1 min. The ACL1 and the CDP loci were amplified following the thermal profile: 95°C for 2 min followed by 25 cycles of 95°C for 30 s and then 62°C for 1 min and 72°C for 30 s.

The resulting PCR products were purified using the Macherey-Nagel NucleoSpin Gel and PCR clean-up kit following the manufacturer's protocol.

Alignment and Population Analysis

Sequenced ABI reads were imported into Geneious 9.0.4 software (www.geneious.com) and forward and reverse reads were aligned and consensus called. Each gene was aligned individually using the MAFFT alignment tool within Geneious (Katoh et al., 2002) and end regions trimmed so that all isolates had complete sequence information. Alignments were exported in nexus file format and diversity statistics (π , θ_W , Tajima's D and the 4 gamete test) calculated in DNAsp v5 (Librado and Rozas, 2009). Construction of combined SNP and microsatellite haplotypes was carried out manually.

Inoculum Preparation

Inoculum of *N. ditissima* used for all pathogenicity experiments was obtained from single ascospore cultures. Three isolates were used in the pathogenicity experiments; R09/05, Hg199, and R28/15 (Table 1).

The isolates were sub-cultured onto SNAY media (1 g potassium dihydrogen phosphate, 1 g potassium nitrate, 0.5 g magnesium sulfate, 0.5 g potassium chloride, 0.2 g glucose, 0.2 g sucrose, 1 g yeast extract, 20 g agar made up to 1 liter with distilled water). Plates were incubated in 16/8 h light/dark regime at 22°C for 13–15 days. On the day of inoculation, each plate was flooded with 3 ml of sterile water and conidia were released from sporodochia using a plastic spreader. Mixed spore (macro and microconidia) suspension was prepared from each isolate.

Macroconidia and microconidia in the suspension were counted using a haemocytometer. Two isolates, R09/05 and Hg199, were used for the cultivar cut-shoot test with concentrations of 5×10^4 and 3×10^3 conidia ml^{-1} , respectively. When this test was repeated, three isolates, R28/15, Hg199 and R45/15 were used at a concentration of 1×10^5 conidia ml^{-1} . Isolate R28/15 was used in the rootstock potted tree test at 1.1×10^5 conidia ml^{-1} . For the apple seedling test, isolate R09/05 was used at 2.7×10^5 conidia ml^{-1} . For the leaf scar inoculation test, isolate R09/05 was used at 6×10^5 conidia ml^{-1} .

Cultivar Cut Shoot Test

Shoots were inoculated with two *N. ditissima* isolates, Hg199 and R09/05 along with a water control. Dormant 1-year old shoots with a length of approximately 5 cm were collected from mature trees of "Aroma," "Beauty of Bath," "Cox's Orange Pippin," "Gala," "Gloster 69," "Golden Delicious," "Grenadier," "Idared," "M9," "Robusta 5," and "Wolf River" at the beginning of February 2015 (in the UK). Shoots were wrapped in moist paper and kept at 4°C

in darkness for 12 weeks. Four days before inoculation, shoots were placed into a controlled environment cabinet on a 20/4 h light/dark cycle with a corresponding day/night temperature of 22/18°C at a constant humidity of 80% relative humidity (RH). Shoots were immobilized at their base in Oasis floral foam, which was placed into a tray containing water, adapted from van de Weg (1989). Three axillary buds on each shoot were inoculated. Buds were prepared by cutting just below the bud, a little below the second abscission layer (but without removing the bud). The width of the incision was approximately 2–3 mm. The chosen buds were the sixth, eighth, and tenth counting basipetally (from the apex to the base of the shoot). An inoculum volume of 10 µl of spore suspension was applied to the wound within 5 min of making the wound. Following inoculation, wounds were covered with white petroleum jelly, which was removed after 4 days with a paper towel. This step is necessary to ensure that the wound does not dry out during early establishment of infection. During the first 4 days after inoculation RH was increased to 100%, again to ensure that sufficient humidity was maintained for successful infection. The experiment was divided between two growth cabinets, within which were trays containing cut shoots of cultivars, inoculated at three points (pseudo-replicates) with one of two *N. ditissima* isolates or a water control (not included in this analysis). Within each tray a single replicate of the experiment was randomly arranged in a 6 × 2 grid (eleven cultivars were analyzed in this test); there were six biological replicates per treatment. Trays of inoculated and control material were randomized between cabinets. Lesion length was recorded using digital calipers at 12, 16, 22, 27, 31, and 35 days post-inoculation. The Area Under Disease Progress Curve (AUDPC) was calculated using the agricolae package (de Mendiburu, 2015), using R version 3.2.2 (Team, 2015). AUDPC values were analyzed using a linear mixed effects model. The fixed effects followed a three-way factorial treatment structure, which was isolate × cultivar × pseudo-rep. The random effect model was cultivar, nested within trays within growth cabinets. The REML command was used within Genstat (VSN International). Wald tests were carried out in order to assess the effect of the different fixed effects and any higher order interactions that may have occurred.

This experiment was repeated in January 2016, using a subset of cultivars inoculated with isolates R28/15, Hg199, and R45/15. The protocol differed slightly since instead of three inoculations per shoot, a single inoculation was carried out to allow lesion expansion in highly susceptible cultivars to be accurately recorded at the later stages of the experiment (14, 18, 21, 27, 34, 39, 45, 49, and 54 days post-inoculation). The data are presented from day 34, to facilitate comparison with the 2015 experiment.

Apple Seedling Test

Apple seeds from biparental crosses (see Results) were washed in a weak (2%) bleach solution, sown by family in trays, with 45 seeds per tray in standard horticultural compost (peat based) and stratified for 12 weeks at 2°C. Trays were then moved to a warm glasshouse 25/16°C (day/night temperature) and 16/8 h day/night length (achieved using supplementary lighting). Seedlings were grown for 6 months under these conditions and

then potted into two liter pots and moved to a chilled glasshouse in early UK summer (July) 2015, at a maximum day temperature of 20°C with no additional lighting. Misting lines were hung under benches (with 360° misting units at approximately 60 cm intervals along the underside of the bench). These were placed on a timer, spraying for 10 min at 6 h intervals to ensure a minimum humidity level of 80% RH.

Three leaves from each plant were removed; either the fifth, seventh, and ninth (or fourth, sixth, and eighth) leaves depending upon the size of the plant. The corresponding axillary bud was also removed. Inoculation points were prepared by cutting just below the bud wound, a little below the second abscission layer; the width of the incision was approximately 2–3 mm. Within 5 min of cutting, 3 µl of a conidial suspension of a single *N. ditissima* isolate was placed onto the wound with an automatic micropipette. The order of inoculation was randomized into eight different sets of seedlings for logistical reasons and eight different inoculum tubes were used, prepared from a common source. This was done to avoid prolonged use of a single tube of inoculum, or to confound position in the glasshouse of the seedlings with inoculation time. Inoculated wounds were covered with white petroleum jelly within 5 min of the droplet being absorbed which was removed 7 days later with a tissue. Lesion size was recorded with digital calipers every 3 days after the first signs of infection, in this case 11 days after infection. In total seven assessments were carried out, up to 31 days post-inoculation. Seedlings were fully randomized and divided into sets of 88 and placed on two benches either side of the glasshouse. A subset of 16 seedlings (all genetically non-identical) from eleven bi-parental crosses (total 176 seedlings) were used in this test.

Rootstock Potted Tree Test

The experiment was carried out in a single glasshouse compartment within which were fifteen randomized blocks of five rootstock types (all 2-year old trees) with temperature, light and humidity conditions identical to the apple seedling test. Rootstocks were inoculated at three points (pseudo-replicates) with a single *N. ditissima* isolate, however this time at nodes 5, 10, and 15 to allow room for lesion expansion; this allowed the experiment to be run for much longer than the cut shoot or seedling scion experiments. Lesion length was measured at 25, 34, 49, 74, and 96 days post-inoculation. As before, AUDPC values were analyzed using a linear mixed effects model. The fixed effects followed a two-way factorial treatment structure, which was cultivar × pseudo-rep. The random effect model was cultivar, nested within blocks. REML analysis was carried out as described.

Leaf Scar Inoculation Potted Tree Test

Dormant 1-year-old shoots from mature trees of “Aroma,” “Golden Delicious,” “Gala,” “Gloster 69,” “Grenadier,” “Robusta 5,” “M9,” “E93-79,” “E202-6,” and “Idared” were grafted onto M9 rootstocks in February 2015 (UK). Trees were moved to a glasshouse 1 day before inoculation, at the end of October 2015. Temperature varied in the glasshouse from 10°C to 25°C and no additional lights were used during the experiment. To ensure a minimum humidity level of 80% RH, misting lines were

hung over the trees spraying for 30 min at 6 h intervals. On each tree, five leaves were removed randomly along the tree leaving approximately the same distance among them. An inoculum volume of 10 µl of spore suspension or water control was applied to each leaf scar. The position of the trees in the glasshouse and the order of inoculation was randomized in five different sets with one tree per cultivar, inoculating four sets with a single *N. ditissima* isolate and one with water. After 5 weeks, trees were moved outside keeping the same randomized design. The first symptoms of infection appeared 70 days post-inoculation and lesion length was recorded using digital calipers at approximately fortnightly intervals. The experiment was ended at 115 days post-inoculation.

RESULTS

Population Analysis of *N. ditissima* Reveals Only Slight Evidence for Geographically Structured Populations

Little is known about the extent or patterns of nucleotide diversity of *N. ditissima*, or whether there are any patterns of isolation by distance on a local or a global scale. In order to study this isolates of *N. ditissima* gathered from the UK, Netherlands, Belgium, New Zealand, and Brazil were evaluated at four single copy loci found to be polymorphic in the three recently published *N. ditissima* reference genomes (Deng et al., 2015; Gómez-Cortecero

et al., 2015). These loci span the introns of two conserved genes (ACL1 and CDP) and two microsatellite-containing loci, one (CAA4_prox) within an intergenic region and another (CAA11_sub) within a hypothetical protein-encoding region (**Supplementary Table 1**). The latter two loci were developed based on the earlier work of Marra and Corwin (2009). For each locus, between 20 and 22 isolates were evaluated originating from the UK (8 isolate), Belgium (3 isolates) the Netherlands (9 isolates), Brazil (2 isolates), and New Zealand (2 isolates) (**Table 1**).

The number of segregating SNP sites varied between 2 and 12 and estimates of π , a measure of nucleotide diversity, ranged by approximately an order of magnitude (0.002–0.018), depending upon the locus (**Table 2**). In all but one case, Tajima's *D* (a comparison of the scaled mean number of pairwise differences and the number of segregating sites) revealed no evidence for selective or demographic processes acting on the chosen loci. However, in the case of the CDP gene, where a clear haplotype containing 11/12 SNPs can be seen, there is a significantly positive measure of Tajima's *D*, indicative of non-neutral patterns of nucleotide polymorphism.

The number of SNP haplotypes was the same (3) in each locus under study and only two private SNP haplotypes were found (SNPs found only in a single subpopulation), one in the Netherlands, in a single individual, for the CAA11_sub locus and one in a Belgian isolate for the ACL1 locus, indicating that most polymorphism is shared between populations (**Table 2**).

TABLE 1 | Isolate name, origin and contribution.

Isolate accession	SYN	CV	Origin	Year of isolation	Contributor
R09/05	–	Cox	Kent, UK	2005	Angela Berrie, EMR, UK
HG199	–	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
HG23	–	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
HG187/B	–	Gala	Kent, UK	1999	Angela Berrie, EMR, UK
TL109	–	Cox	Kent, UK	1999	Angela Berrie, EMR, UK
TL88	–	Gala	Kent. UK	1999	Angela Berrie, EMR, UK
M46/A	–	Various	Kent, UK	1990's	Angela Berrie, EMR, UK
R28/15	–	Gala	Hampshire, UK	2015	Angela Berrie, EMR, UK
R36/15	PCF171	Jonagold	Belgium	2006	Tom Smets, PCF, B
R37/15	PCF191	Jonagold	Belgium	1999	Tom Smets, PCF, B
R38/15	PCF188	Golden Delicious	Belgium	2006	Tom Smets, PCF, B
R40/15	–	Kanzi	The Netherlands	2015	Marcel Wenneker, WUR, NL
R41/15	–	Wellant	The Netherlands	2015	Marcel Wenneker, WUR, NL
R42/15	–	Elstar	The Netherlands	2015	Marcel Wenneker, WUR, NL
R43/15	–	Junami	The Netherlands	2015	Marcel Wenneker, WUR, NL
R44/15	–	Rubens	The Netherlands	2015	Marcel Wenneker, WUR, NL
R45/15	–	Elstar	The Netherlands	2015	Marcel Wenneker, WUR, NL
R46/15	–	Jonagold	The Netherlands	2015	Marcel Wenneker, WUR, NL
R47/15	–	Delcorf	The Netherlands	2015	Marcel Wenneker, WUR, NL
R48/15	–	Natyra	The Netherlands	2015	Marcel Wenneker, WUR, NL
NB8/15	–	Royal Gala	Santa Catarina, Brazil	2015	Hugo Medeiros, EPAGRI, BR
NB9/15	–	Royal Gala	Santa Catarina, Brazil	2015	Hugo Medeiros, EPAGRI, BR
LDPL01	RS324p	Golden Delicious	Taranaki, New Zealand	2009	Reiny Schepers, PFR, NZ
LDPK01	RS305p	Brookfield Gala	Lower Moutere, New Zealand	2009	Reiny Schepers, PFR, NZ

Gene	Samples	Sites	Sites for SNP	Segregating	π (Average pairwise differences per site)	$\hat{\theta}_W$ (Matheron's theta- segregating sites)	Tajima's D (SNP)	Haplotypes (SNP)	Private SNP (origins)	Haplotypes incl Microsat	Private SNP and microsatellite haplotypes (origins)	Evidence for recombination
ACL1	21	409	409	4	0.00447	0.00272	1.84 (ns)	3	Belgium	NA	Belgium	No
CDP	22	339	339	12	0.01794	0.00971	2.97 ($p < 0.01$)	3	NA	NA	NA	No
NDCAA4_prox	22	321	297	2	0.00155	0.00185	-0.37 (ns)	3	Brazil, Netherlands, New Zealand, UK	7	Brazil, Netherlands, New Zealand, UK	No
NDCAA11_sub	20	362	278	9	0.00841	0.00913	-0.027 (ns)	3	Netherlands	7	Brazil, UK and Netherlands	No

Including both SNP and microsatellite variation (for which the mutation rate per cell division may be over twice as high 7×10^{-8}) in the analysis of private haplotypes reveals that despite the small sample size, distinct private haplotypes could also be detected in UK, Netherlands, Brazilian, and New Zealand samples (Table 2, Supplementary Table 2). Across all samples no evidence for recombination within loci could be detected using the four-gamete test (Hudson and Kaplan, 1985), however segregation could be detected between loci.

Differences in Partial Resistance to Canker among Cut Shoots of Apple Cultivars

It is widely known that cultivars vary in their susceptibility to canker, though the exact molecular mechanism is unknown. In order to further study the response of cultivars to different inocula, different infection methods and at different physiological conditions, a pathogenicity screen using two UK isolates (R09/05 and Hg199) was carried out first, using dormant cut shoot material (van de Weg, 1989). This test allows colonization rate to be calculated and compared between isolates and cultivars. After inoculation, lesions progressed vertically along the shoots. The symptoms consisted of a sunken and necrotic bark area around the inoculation point, the progress of which was measured in a non-destructive manner with calipers. These symptoms were noticeable after 12 days after inoculation in the cut-shoot test. Using REML analysis followed by tests for fixed effects, no effect of the growth cabinet could be seen (the experimental design explicitly controlled for this eventuality). Cut shoot tests revealed abundant variation in resistance and susceptibility to *N. ditissima*, but little variation in isolate pathogenicity (Table 3). This variation in the response among the cultivars was consistent regardless of the differences in the inoculation pressure between the isolates (see also Supplementary Figure 1). There was a significant effect of pseudo-replicate position (three inoculation points per scion were used).

For apple scion material, it was found that the species *Malus × robusta* c.v. "Robusta 5" had the highest level of resistance in the cut shoot tests (Figure 2), followed by the known resistant cultivar "Golden Delicious." At the other end of the resistance spectrum, the known susceptible cultivars "EMLA-'M9" (a rootstock) and "Cox" were highly susceptible

TABLE 3 | Wald tests for fixed effects- sequentially adding terms to fixed model.

Fixed effect	Wald statistic	d.F.	Chi pR
Cabinet	0.01	1	0.91
Isolate	0.9	1	0.34
Cultivar	108.24	11	2×10^{-16}
Pseudo-replicate	6.50	2	0.04
Isolate:Cultivar	16.72	11	0.12
Isolate:Pseudo-replicate	0.67	2	0.71
Cultivar:Pseudo-replicate	17.64	22	0.73
Isolate:Cultivar:Pseudo-replicate	28.30	22	0.17

Significant differences are highlighted in bold.

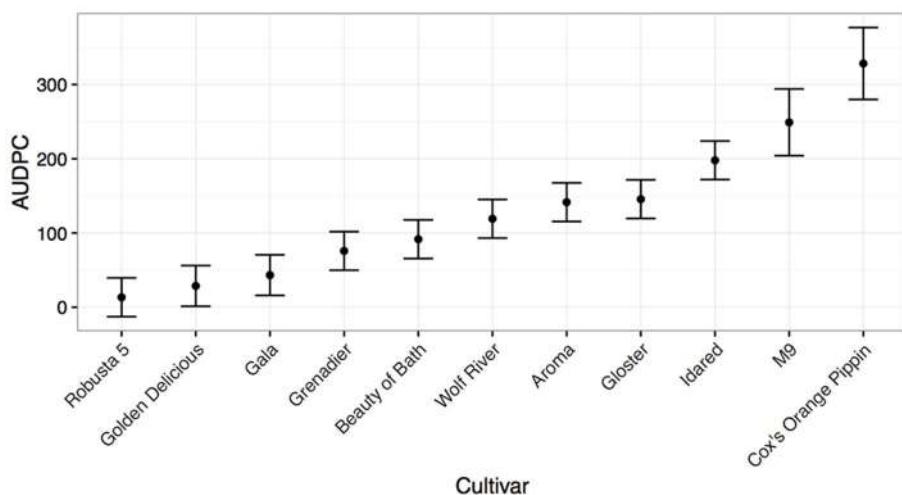


FIGURE 2 | Mean Area under disease progress for inoculated cut shoots of common apple scion material calculated 35 days post-infection (shown with standard errors). The rootstock M9 is also included as a qualitative comparison.

(Figure 2). Intermediate levels of resistance were seen for other reported field-resistant or tolerant material, including “Aroma,” “Beauty of Bath,” and “Grenadier.” Somewhat surprisingly the field-susceptible cultivar, “Gala” was found to be more resistant than expected to *N. ditissima* infection using this method. Based on its reported parentage (“Golden Delicious” × “Kidd’s Orange Red”- the latter reported to be a “Delicious” × “Cox” cross), it has both resistant and presumed susceptible material in its pedigree indicating the potential for at least partial resistance, consistent with the performance of “Golden Delicious” and “Cox” in this test). Repetition of this experiment in 2016 with three isolates of *N. ditissima* revealed similar results, with “Gala” and its offspring “Scifresh” and “Scilate” (“Gala” × “Braeburn”) all showing low levels of lesion spread (Supplementary Figure 1) and no cultivar by isolate interaction (data not shown).

Differences in Partial Resistance to Canker among Apple Rootstocks

As with the cultivar test, significant effects of both rootstock cultivar and pseudo-replicate were detected, though this time a significant two-way interaction between cultivar and pseudo-replicate was detected (Table 4). In this experiment, five rootstocks were tested (including two clonal variants of “M9”). “MM106” (“M2” × “Northern Spy”) was the most resistant, while the “M9” clone (337) was the most susceptible (Figure 3).

Differences in Partial Resistance to Canker Determined by Leaf Scar Inoculation

Alongside cut shoot tests, leaf scar infection tests were carried out (Alston, 1970; Amponsah et al., 2015; Schepers et al., 2015). Again, the species level accession “Robusta 5” demonstrated high levels of resistance (Figure 4). As with previous reports, “Gala” was extremely susceptible in this pathogenicity test, with high levels of colonization after inoculation with the same isolate of *N. ditissima* as used in the cut shoot test (Schepers et al.,

TABLE 4 | Wald tests for fixed effects- sequentially adding terms to fixed model.

Fixed effect	Wald statistic	d.F.	Chi pR
Cultivar	64.82	4	2.8×10^{-13}
Pseudo-replicate	52.57	2	3.5×10^{-12}
Cultivar.Pseudo-replicate	25.24	8	0.0014

Significant differences are highlighted in bold.

2010). “Gloster 69” and “E202-6” also showed high levels of susceptibility. Intermediate levels of resistance were seen in “Golden Delicious,” “Idared,” “Aroma,” “M9,” “Grenadier,” and “E93-79.”

Seedling Tests Indicate a Complex Genetic Basis for Resistance

In order to further test the resistance responses of different parental material with respect to variation in colonization rate following wound inoculation, and the manner in which resistance is transmitted, crosses were made between parents, many of which were tested in a cut shoot test. The experiment was run for a total of 31 days; significant symptom development was seen in some progenies 11 days after inoculation. Examination of the AUDPC values after 31 days revealed that segregation patterns varied and crosses with both highly resistant offspring (MDX053 and MDX051 having the lowest median AUDPC values) and highly susceptible offspring (MDX057, MDX068) were observed (Figure 5 and Table 5).

The segregation patterns that were observed were complex and some resistant parents showed poor transmission of resistance into the progeny. For example, crosses involving “Golden Delicious,” even when crossed with other moderately resistant parental lines (e.g., “Aroma,” MDX054 and “Grenadier,” MDX068) showed higher median levels of disease progress

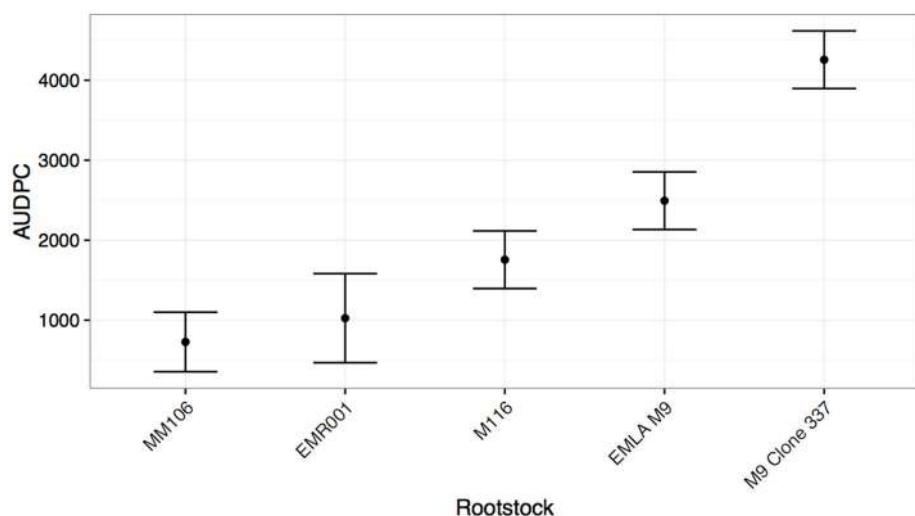


FIGURE 3 | Mean Area under disease progress for inoculated shoots of common apple rootstocks (shown with standard errors), calculated 75 days post-infection.

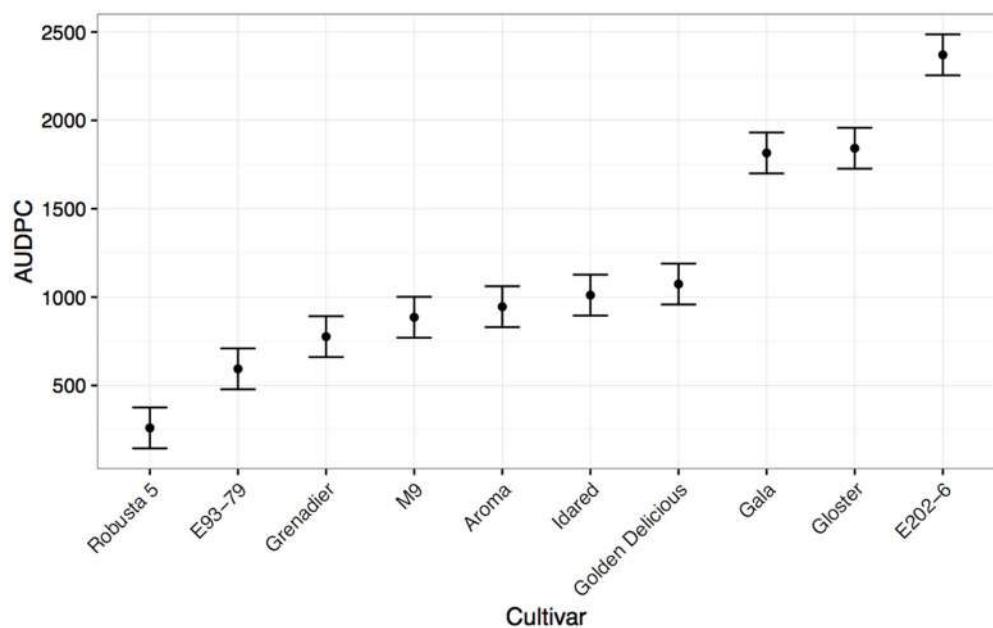


FIGURE 4 | Mean Area under disease progress for inoculated leaf scars of common apple scion material calculated 153 days post-infection (shown with standard errors). The rootstock M9 is also included as a qualitative comparison.

(Median AUDPC 181.42) than crosses involving the same parental material (e.g., ‘Aroma’ crossed to more susceptible material (e.g., ‘Gala,’ MDX052- Median AUDPC 60.41), though significant differences were only observed in a single pairwise non-parametric Kruskal Wallis test between MDX052 and MDX068, but not MDX052 × MDX054 and MDX054 × MDX068 (see Supplementary Table 3).

The four most resistant crosses involved ‘Gala,’ ‘Santana,’ ‘Aroma,’ ‘Fuji,’ and ‘3760’- the latter an open pollinated line

derived from *M. × robusta* (see Supplementary Table 4 for pedigree details).

DISCUSSION

Our data, although incomplete, present a pattern of SNP diversity consistent with the notion that there is broad similarity between geographically isolated populations and that much of the genetic

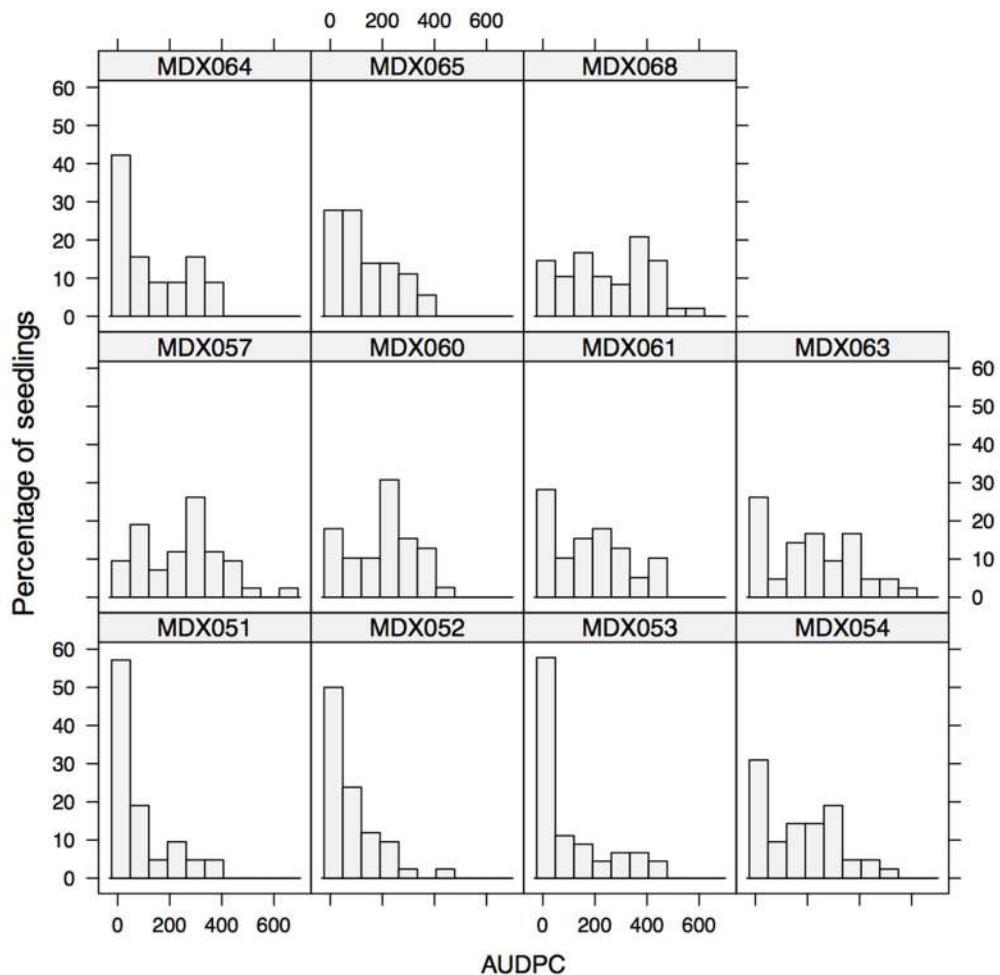


FIGURE 5 | Histogram of AUDPC values for inoculated shoots of eleven seedling families of *M. x domestica*. Segregation patterns vary considerably between families and unexpected segregation patterns occur between crosses of highly resistant varieties.

TABLE 5 | Cross combinations tested in the seedling test and population median and inter-quartile range of the AUDPC values.

Cross number	Female parent	Male parent	Population median (AUDPC)	Population IQR (AUDPC)	Cross combination resistance based on cut-shoot R- resistant, I- intermediate, S- susceptible, U- unknown (F × M)
MDX051	Gala	Santana	28.58	101.5	R × U
MDX052	Aroma	Gala	60.41	108.8	I × S
MDX053	Aroma	Fuji	25.92	148.41	I × U
MDX054	Aroma	Golden Delicious	181.42	248.95	I × R
MDX057	Gloster 69	Idared	266.61	227.03	I × S
MDX060	E248-2	E616-57	234.06	185.85	U × U
MDX061	E93-79	Gala	180.53	251.23	U × S
MDX063	E202-6	Golden Delicious	244.59	292.96	U × S
MDX064	Gala	3760	85.8	242.69	U × R
MDX065	Gala	3762	105.05	158.53	S × U
MDX068	Grenadier	Golden Delicious	253.39	251.96	I × R

diversity seen in the European population of *N. ditissima* is also seen in South American and Oceanian populations. At the SNP level (with an approximate mutation rate of the order of

3×10^{-8} for nuclear base substitutions per cell division, Lynch et al., 2008) there is little information about recent geographic isolation, as there is no clear pattern of private allelic variation

with geographic origin. These data are consistent with the idea that *N. ditissima* spread from Europe to other regions of the world on imported apple plant material. Further study will be needed with much larger sample sizes to provide estimates of local levels of diversity in these populations. Despite the small sample size, it was possible to detect with the aid of more rapidly evolving microsatellite loci evidence for some distinct patterns of polymorphism in UK, Netherlands, Brazilian, and New Zealand populations of *N. ditissima* (**Table 2** and **Supplementary Table 2**). Again, further sampling will be needed to confirm this, but again the pattern of differentiation at microsatellites is suggestive that local populations of *N. ditissima* with unique allelic variation may be detectable with more polymorphic loci.

Interestingly, despite *N. ditissima* being a sexually reproducing species, with a year-round reproductive potential no evidence for recombination within loci could be detected in our sequenced isolates, though segregation between loci can be seen. Other recent work has proven that recombination does occur in the field by SSR tests of ascospores (Ghasemkhani et al., 2016). Further data are needed to study the genome-wide patterns of recombination to determine whether the patterns observed from the loci used in this study are general to the whole genome. The levels of nucleotide diversity vary widely and there are signatures of non-neutral processes (evidenced by significantly positive Tajima's D values). A key question to address in the future is whether these patterns are present across the whole genome; if so this would be indicative of demographic processes influencing patterns of nucleotide diversity. In the model system *Saccharomyces cerevisiae* it was shown that "modern" wine and baking yeast strains were admixed individuals with contributions from multiple different subpopulations, previously allopatrically isolated (Liti et al., 2009). This pattern was postulated to be driven by human influences, bringing geographically isolated strains together by human migration. It is conceivable that *N. ditissima* has also experienced similar human-driven secondary contact in the recent evolutionary past, which has contributed to the extant patterns of nucleotide diversity. In order to test this hypothesis, multiple samples from across Europe, the Americas and Oceania, as well as other areas of the world where *N. ditissima* is established must be sequenced and subjected to population genomics analysis. Alternatively, it could be that different populations of *N. ditissima* have expanded their host-ranges onto apple to create hybrid recombining populations. It is important to undertake population-level analyses as genome-wide association studies of pathogenicity may be confounded by high levels of ancestral population structure.

The finding from both preliminary cultivar cut shoot tests, that there is no isolate by cultivar interaction, suggests that the host response is consistent, regardless of the isolate that is inoculated. To confirm these initial observations, a further study of more isolates is required. All higher order interactions were non-significant, indicating that there may be a relatively simple pattern of host response which is not influenced by an isolate race-structure, consistent with previous reports (van de Weg, 1989). The finding that there was a positional effect of pseudo-replicate can be explained by the fact that in some cultivars lesion

growth was so rapid, that after a point it became impossible to distinguish between lesion leading edges, at which point the experiment was ended.

While our results do not support the existence of distinct pathogen races, this has no bearing on whether the resistance that has been identified may be durable or not (as this is primarily determined by the capability of the pathogen to overcome specific defense or recognition mechanisms). However, it may suggest that resistance is targeting conserved factors in the pathogen and therefore the resistance present in the tested cultivars may be broad spectrum and thus has the potential to be durable. It is interesting to note that the most resistant cultivar "Robusta 5" is a representative of a species that is distinct from *M. × domestica*. Little is known about natural *M. × robusta* species, since much of the material that is present in Europe was collected in Northern China. It is described as a hybrid species, though this is only by morphology (Forsline et al., 2002). What is interesting to note is that *N. ditissima* is not reported as a significant pathogen of apple in China, indicating that *M. × robusta* may be a non-host and therefore that the mode of resistance in *M. × robusta* vs. the cultivated apple *M. × domestica* may be of distinct evolutionary origin. It is therefore important to study multiple origins of resistance, as some may be more durable than others, or pyramiding combinations of different alleles may offer greater resistance by combining multiple mechanisms of resistance.

The finding that rootstock material also has resistance to *N. ditissima* is encouraging. It has been shown that during nursery propagation, infected rootstock material may be one of the primary mechanisms by which the disease is spread and therefore rootstock material with high levels of resistance may reduce the subsequent emergence of latent *N. ditissima* in the orchard (McCracken et al., 2003). The surprising finding that M9 clones exhibit differing levels of susceptibility merits further investigation. The mechanism for this is unknown, but it could be that during the selection for clonal variants with improved propagation or yield characteristics other somatic mutations that alter the resistance response may have been inadvertently selected. This may also explain differences in field susceptibility of supposed identical cultivars. Many of the commercially grown "Gala" clones are in fact sports selected for skin color or ripening date. It may also be a risk, when considering clonally propagated crops, that resistance gene pyramids may be disrupted by somatic mutations in clonal material, which could lead to loss of resistance durability. In order to identify whether clonal propagation and selection of material leads to differences in susceptibility a more comprehensive study must be undertaken, evaluating clones produced under the same propagation conditions.

The seedling test that was carried out revealed that resistance sources differed in their transmission characteristics. Most striking was the observation that crosses involving "Golden Delicious," found to be highly resistant in cut shoot tests, had a greater level of susceptibility when crossed to resistant material, than supposed resistant × intermediate/susceptible crosses. This could be explained if the nature of the resistance sources differed among cultivars, i.e., if the resistance from "Golden Delicious" was recessive, or if susceptibility factors in some

cross combinations lead to resistance that is non-additive. These preliminary results suggest that the likelihood of transmission of resistance varies between resistant parental material and that some parental material appears to be superior to others in ability to donate resistance, despite slightly lower overall resistance in the cut shoot tests (i.e., moderately resistant "Aroma" vs. highly resistant "Golden Delicious"). This part of the study highlights the importance of trial evaluation of seedling populations prior to embarking upon QTL studies and the importance of considering the mode and mechanism of resistance and the way in which it is phenotyped in breeding programmes.

It is still unclear whether the methods that have been tested in this paper are of direct relevance to the orchard situation. The methods used in this study have not been compared with orchard inoculations and therefore it is entirely possible that a newly developed cultivar that is resistant according to these tests turns out to be susceptible in the field. It is clear that both resistance to colonization and initial infection are important components of field resistance. Some cultivars that we have studied, such as "Gala" appear to have consistently high levels of resistance to colonization in cut shoot tests (**Figure 2**, **Supplementary Figure 1**) and yet are often considered to be field susceptible and indeed in whole-tree leaf scar tests (**Figure 4**) are much more susceptible. Conversely, some material (see E93-79, **Figure 4** and **Supplementary Figure 1**) exhibits rapid colonization in wound inoculated cut shoot tests, but low susceptibility to leaf scar infection. It should be noted that in both types of pathogenicity test "Robusta 5" displays low levels of infection and subsequent colonization. This suggests that the cut shoot and leaf scar tests are querying different components of resistance and that for strong resistance, low levels of colonization and lesion expansion in both tests are required. In order to be considered to be field resistant, trees must have low disease incidence when several wound types are inoculated; a small lesion size when infection does occur; low spore production from lesions; negligible internal (latent) growth of the pathogen. Future work needs to be carried out to compare the results presented in this study with trees grown outside in an orchard setting, inoculated using several different wounds (leaf scars, pruning cuts, picking wounds) to determine whether the methods developed in this paper can be considered to be sufficient for rapid selection in breeding programmes.

It is also important to consider the role of abiotic stresses in modulating plant resistance. It is unclear at present, when issues with drainage in the orchard occur or other changes in tree health, or nitrogen applications, whether the resistance status of some trees may alter more than others. It is rare that multifactorial experiments are carried out on a field scale that address biotic stress responses in relation to abiotic stress tolerance. However, a study by Dryden et al. (2016), as well as

anecdotal evidence suggest that this is an important topic of future study (Dryden et al., 2016).

With the recent publication of three *N. ditissima* genome sequences (Deng et al., 2015; Gómez-Cortecero et al., 2015) and the increasing amount of genomic information available for apple (Velasco et al., 2010; Antanaviciute et al., 2012; Bianco et al., 2014; Bink et al., 2014) it is likely that rapid progress can be made in identifying the genetic basis of resistance to *N. ditissima* from multiple resistance sources and the corresponding pathogenicity factors that may be manipulating host defenses.

AUTHOR CONTRIBUTIONS

RH conceived the study and led the writing of the manuscript, AG, JK, and RJS carried out experimental work, HA, RWAS, and JB contributed to the phylogenetic analysis. AG, RH, and XX analyzed the pathogenicity test data. All authors contributed to writing and editing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01365>

Supplementary Figure 1 | Mean Area under disease progress for inoculated cut shoots of common apple scion material calculated 34 days post-inoculation (shown with standard errors). The rootstock M9 is also included as a qualitative comparison.

Supplementary Table 1 | Primer sequences used for population analysis of *N. ditissima*.

Supplementary Table 2 | Combined SSR and SNP haplotypes reported by population of origin.

Supplementary Table 3 | Multiple comparison of seedling populations using Kruskal-Wallis tests.

Supplementary Table 4 | Pedigree relationships between seedling material evaluated for canker resistance. Color coding indicates generation- black (F1), red (F0), blue (F-1) orange (F-3), green (F-4), and purple (F-5) generations. Inter-generational crosses are highlighted by the color of the generation in which they first appear.

REFERENCES

- Alston, F. (1970). Response of apple cultivars to canker, *Nectria galligena*. *Annu. Rep. East Malling Res. Stn.* 1969 A53, 147–148.

- Amponsah, N. T., Walter, M., Beresford, R. M., and Schepers, R. W. A. (2015). Seasonal wound presence and susceptibility to *Neonectria ditissima* infection in New Zealand apple trees. *N.Z. Plant Prot.* 68, 250–256.

- Antanaviciute, L., Fernández Fernández, F., Jansen, J., Banchi, E., Evans, K. M., Viola, R., et al. (2012). Development of a dense SNP-based linkage map of an apple rootstock progeny using the *Malus* Infinium whole genome genotyping array. *BMC Genomics* 13:203. doi: 10.1186/1471-2164-13-203
- Armitage, A. D., Barbara, D. J., Harrison, R. J., Lane, C. R., Sreenivasaprasad, S., Woodhall, J. W., et al. (2015). Discrete lineages within *Alternaria alternata* species group: identification using new highly variable loci and support from morphological characters. *Fungal Biol.* 119, 994–1006. doi: 10.1016/j.funbio.2015.06.012
- Bianco, L., Cestaro, A., Sargent, D. J., Banchi, E., Derdak, S., Di Guardo, M., et al. (2014). Development and validation of a 20K single nucleotide polymorphism (SNP) whole genome genotyping array for apple (*Malus × domestica* Borkh.). *PLoS ONE* 9:e110377. doi: 10.1371/journal.pone.0110377
- Bink, M. C. A. M., Jansen, J., Madduri, M., Voorrips, R. E., Durel, C.-E., Kouassi, A. B., et al. (2014). Bayesian QTL analyses using pedigree families of an outcrossing species, with application to fruit firmness in apple. *Theor. Appl. Genet.* 127, 1073–1090. doi: 10.1007/s00122-014-2281-3
- Bus, V., Singla, G., Ward, S., Brewer, L., Morgan, C., Bowatte, D., et al. (in press). Progress in pipfruit resistance breeding and research at Plant & Food Research. *Acta Hortic.*
- Castlebury, L. A., Rossman, A. Y., and Hyten, A. S. (2006). Phylogenetic relationships of *Neonectria/Cylindrocarpon* on *Fagus* in North America. *Can. J. Bot.* 84, 1417–1433. doi: 10.1139/b06-105
- Cayley, D. M. (1921). Some observations on the life-history of *Nectria galligena*, Bres. *Ann. Bot.* 35, 79–92.
- Cooke, L. R. (1999). The influence of fungicide sprays on infection of apple cv. Bramley's seedling by *Nectria galligena*. *Eur. J. Plant Pathol.* 105, 783–790. doi: 10.1023/A:1008778900607
- de Mendiburu, F. (2015). *agricolae: Statistical Procedures for Agricultural Research*. R Package Version 1.2-3. Available online at: <http://CRAN.R-project.org/package=agricolae>
- Deng, C. H., Schepers, R. W. A., Thrimawithana, A. H., and Bowen, J. K. (2015). Draft genome sequences of two isolates of the plant-pathogenic fungus *Neonectria ditissima* that differ in virulence. *Genome Announc.* 3:e01348-15. doi: 10.1128/genomeA.01348-15
- Dryden, G. H., Nelson, M. A., Smith, J. T., and Walter, M. (2016). Postharvest foliar nitrogen applications increase *Neonectria ditissima* leaf scar infection in apple trees. *N.Z. Plant Prot.* 69, 230–237.
- Fenton, A., Antonovics, J., and Brockhurst, M. A. (2009). Inverse-gene-for-gene infection genetics and coevolutionary dynamics. *Am. Nat.* 174, E230–E242. doi: 10.1086/645078
- Forsline, P. L., Aldwinckle, H. S., Dickson, E. E., Luby, J. J., and Hokanson, S. C. (2002). "Collection, maintenance, characterization, and utilization of wild apples of Central Asia," in *Horticultural Reviews: Wild Apple and Fruit Trees of Central Asia*, Vol. 29, ed J. Janick (Oxford, UK: John Wiley & Sons, Inc.), 1–62. doi: 10.1002/9780470650868.ch1
- Gelvonauskienė, D., Sasnauskas, A., Gelvonauskis, B., Gelvonauskienė, D., Sasnauskas, A., and Gelvonauskis, B. (2007). The breeding of apple tree resistant to European Canker (*Nectria galligena* Bres.). *Sci. Work. Lith. Inst. Hortic. Lith. Univ. Agric.* 26, 174–178.
- Ghasemkhani, M., Garkava-Gustavsson, L., Liljeroth, E., Nybom, H., Bernier, L., Hubbes, M., et al. (2016). Assessment of diversity and genetic relationships of *Neonectria ditissima*: the causal agent of fruit tree canker. *Hereditas* 153, 7. doi: 10.1186/s41065-016-0011-3
- Ghasemkhani, M., Liljeroth, E., Sehic, J., Zborowska, A., and Nybom, H. (2015). Cut-off shoots method for estimation of partial resistance in apple cultivars to fruit tree canker caused by *Neonectria ditissima*. *Acta Agric. Scand. Sect. B Soil Plant Sci.* 65, 412–421. doi: 10.1080/09064710.2015.1016101
- Gómez-Cortecero, A., Harrison, R. J., and Armitage, A. D. (2015). Draft genome sequence of a European isolate of the apple canker pathogen *Neonectria ditissima*. *Genome Announc.* 3, 10–11. doi: 10.1128/genomeA.01243-15
- Govrin, E. M., and Levine, A. (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. *Curr. Biol.* 10, 751–757. doi: 10.1016/S0960-9822(00)00560-1
- Gräfenhan, T., Schroers, H.-J., Nirenberg, H. I., and Seifert, K. A. (2011). An overview of the taxonomy, phylogeny, and typification of nectriaceous fungi in *Cosmospora*, *Acremonium*, *Fusarium*, *Stilbellia*, and *Volutella*. *Stud. Mycol.* 68, 79–113. doi: 10.3114/sim.2011.68.04
- Hudson, R. R., and Kaplan, N. L. (1985). Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics* 111, 147–164.
- Jones, J. D. G., and Dangl, J. L. (2006). The plant immune system. *Nature* 444, 323–329. doi: 10.1038/nature05286
- Katoh, K., Misawa, K., Kuma, K., and Miyata, T. (2002). MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066. doi: 10.1093/nar/gkf436
- Lee, H.-A., Kim, S.-Y., Oh, S.-K., Yeom, S.-I., Kim, S.-B., Kim, M.-S., et al. (2014). Multiple recognition of RXLR effectors is associated with nonhost resistance of pepper against *Phytophthora infestans*. *New Phytol.* 203, 926–938. doi: 10.1111/nph.12861
- Librado, P., and Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics* 25, 1451–1452. doi: 10.1093/bioinformatics/btp187
- Liti, G., Carter, D. M., Moses, A. M., Warringer, J., Parts, L., James, S. A., et al. (2009). Population genomics of domestic and wild yeasts. *Nature* 458, 337–341. doi: 10.1038/nature07743
- Liu, Y., Zhu, X. Y., Zhang, S., Bernardo, M., Edwards, J., Galbraith, D. W., et al. (2011). Dissecting quantitative resistance against blast disease using heterogeneous inbred family lines in rice. *Theor. Appl. Genet.* 122, 341–353. doi: 10.1007/s00122-010-1450-2
- Lynch, M., Sung, W., Morris, K., Coffey, N., Landry, C. R., Dopman, E. B., et al. (2008). A genome-wide view of the spectrum of spontaneous mutations in yeast. *Proc. Natl. Acad. Sci. U.S.A.* 105, 9272–9277. doi: 10.1073/pnas.0803466105
- Manosalva, P. M., Davidson, R. M., Liu, B., Zhu, X., Hulbert, S. H., Leung, H., et al. (2009). A germin-like protein gene family functions as a complex quantitative trait locus conferring broad-spectrum disease resistance in rice. *Plant Physiol.* 149, 286–296. doi: 10.1104/pp.108.128348
- Marra, R. E., and Corwin, J. A. (2009). Isolation and characterization of codominant markers for the perennial canker fungal pathogen *Neonectria ditissima*. *Mol. Ecol. Resour.* 9, 906–909. doi: 10.1111/j.1755-0998.2008.02438.x
- McCracken, A. R., Berrie, A., Barbara, D. J., Locke, T., Cooke, L. R., Phelps, K., et al. (2003). Relative significance of nursery infections and orchard inoculum in the development and spread of apple canker (*Nectria galligena*) in young orchards. *Plant Pathol.* 52, 553–566. doi: 10.1046/j.1365-3059.2003.00924.x
- Plante, F., Hamelin, R. C., and Bernier, L. (2002). A comparative study of genetic diversity of populations of *Nectria galligena* and *N. coccinea* var. *faginata* in North America. *Mycol. Res.* 106, 183–193. doi: 10.1017/S095375620105329
- Rietman, H., Bijsterbosch, G., Cano, L. M., Lee, H.-R., Vossen, J. H., Jacobsen, E., et al. (2012). Qualitative and quantitative late blight resistance in the potato cultivar Sarpo Mira is determined by the perception of five distinct RXLR effectors. *Mol. Plant Microbe Interact.* 25, 910–919. doi: 10.1094/MPMI-01-12-0010-R
- Schepers, R. W. A., Fisher, B. M., and Wood, P. N. (2010). Pathogenicity of field and laboratory-grown inoculum of *Neonectria galligena* on potted apple trees. *N.Z. Plant Protect.* 63:280.
- Schepers, R. W. A., Frijters, L., Fisher, B. M., and Hedderley, D. I. (2015). Effect of freezing of *Neonectria ditissima* inoculum on its pathogenicity. *N.Z. Plant Prot.* 68, 257–263.
- Shivas, R., and Tan, Y. (2009). A taxonomic re-assessment of *Colletotrichum acutatum*, introducing *C. florianiae* comb. et stat. nov. and *C. simmondsii* sp. nov. *Fungal Divers.* 39, 111–112.
- Swinburne, T. R. (1975). European canker of apple (*Nectria galligena*). *Rev. Plant Pathol.* 54, 787–799.
- Team, R. C. (2015). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing. Available online at: <https://www.R-project.org/>
- Tulasne, L., and Tulasne, C. (1865). *Selecta Fungorum Carpologica III Nectriei-Phacidie-Pezizei*. Paris: Imperial: English Translation by WB Grove 1931; Oxford: Clarendon.
- Van De Weg, W. E. (1987). Note on an inoculation method to infect young apple seedlings with *Nectria galligena* Bres. *Euphytica* 36, 853–854. doi: 10.1007/BF00051869

- van de Weg, W. E. (1989). Screening for resistance to *Nectria galligena* Bres. in cut shoots of apple. *Euphytica* 42, 233–240. doi: 10.1007/BF00034459
- Velasco, R., Zharkikh, A., Affourtit, J., Dhingra, A., Cestaro, A., Kalyanaraman, A., et al. (2010). The genome of the domesticated apple (*Malus × domestica* Borkh.). *Nat. Genet.* 42, 833–839. doi: 10.1038/ng.654
- Vleeshouwers, V. G. A. A., Raffaele, S., Vossen, J. H., Champouret, N., Oliva, R., Segretin, M. E., et al. (2011). Understanding and exploiting late blight resistance in the age of effectors. *Annu. Rev. Phytopathol.* 49, 507–531. doi: 10.1146/annurev-phyto-072910-095326
- Walter, M., Glaister, M. K., Clarke, N. R., Von Lutz, H., Eld, Z., Amponsah, N. T., et al. (2015). Are shelter belts potential inoculum sources for *Neonectria ditissima* apple tree infections? *N.Z. Plant Prot.* 68, 227–240.
- Weber, R. W. S. (2014). Biology and control of the apple canker fungus *Neonectria ditissima* (syn. *N. galligena*) from a Northwestern European perspective. *Erwerbs Obstbau* 56, 95–107. doi: 10.1007/s10341-014-0210-x
- Xu, X., Butt, D. J., and Ridout, M. S. (1998). The effects of inoculum dose, duration of wet period, temperature and wound age on infection by *Nectria galligena* of pruning wounds on apple. *Eur. J. Plant Pathol.* 104, 511–519.
- Xu, X.-M., and Robinson, J. D. (2010). Effects of fruit maturity and wetness on the infection of apple fruit by *Neonectria galligena*. *Plant Pathol.* 59, 542–547. doi: 10.1111/j.1365-3059.2009.02232.x
- Zhang, W., Fraiture, M., Kolb, D., Löffelhardt, B., Desaki, Y., Freddy, F. G., et al. (2013). Arabidopsis RECEPTOR-LIKE PROTEIN30 and receptor-like kinase SUPPRESSOR OF BIR1-1/EVERSHELD mediate innate immunity to necrotrophic fungi. *Plant Cell* 25, 4227–4241. doi: 10.1105/tpc.113.117010

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Aromatic Glucosinolate Biosynthesis Pathway in *Barbarea vulgaris* and its Response to *Plutella xylostella* Infestation

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The inducibility of the glucosinolate resistance mechanism is an energy-saving strategy for plants, but whether induction would still be triggered by glucosinolate-tolerant *Plutella xylostella* (diamondback moth, DBM) after a plant had evolved a new resistance mechanism (e.g., saponins in *Barbara vulgaris*) was unknown. In *B. vulgaris*, aromatic glucosinolates derived from homo-phenylalanine are the dominant glucosinolates, but their biosynthesis pathway was unclear. In this study, we used G-type (pest-resistant) and P-type (pest-susceptible) *B. vulgaris* to compare glucosinolate levels and the expression profiles of their biosynthesis genes before and after infestation by DBM larvae. Two different stereoisomers of hydroxylated aromatic glucosinolates are dominant in G- and P-type *B. vulgaris*, respectively, and are induced by DBM. The transcripts of genes in the glucosinolate biosynthesis pathway and their corresponding transcription factors were identified from an Illumina dataset of G- and P-type *B. vulgaris*. Many genes involved or potentially involved in glucosinolate biosynthesis were induced in both plant types. The expression patterns of six DBM induced genes were validated by quantitative PCR (qPCR), while six long-fragment genes were validated by molecular cloning. The core structure biosynthetic genes showed high sequence similarities between the two genotypes. In contrast, the sequence identity of two apparent side chain modification genes, the *SHO* gene in the G-type and the *RHO* in P-type plants, showed only 77.50% identity in coding DNA sequences and 65.48% identity in deduced amino acid sequences. The homology to GS-OH in *Arabidopsis*, DBM induction of the transcript and a series of qPCR and glucosinolate analyses of G-type, P-type and F₁ plants indicated that these genes control the production of S and R isomers of 2-hydroxy-2-phenylethyl glucosinolate. These glucosinolates were significantly induced by *P. xylostella* larvae in both the susceptible P-type and the resistant G-type, even though saponins are the main DBM-resistance causing metabolites in G-type plants. Indol-3-ylmethylglucosinolate was induced in the G-type only. These data will aid our understanding of the biosynthesis and induction of aromatic glucosinolates at the molecular level and also increase our knowledge of the complex mechanisms underpinning defense induction in plants.

Keywords: *Barbarea vulgaris*, diamondback moth, glucosinolate, gene expression profile, induced defenses, plant-herbivore interaction, side chain modification

INTRODUCTION

Plants have evolved constitutive and inducible resistance against herbivores, which compete for the same resources in the plant (Rasmann et al., 2015). The classic theory presumed that inducible defenses is a cost-saving strategy, because resources can divert from defense to growth under suitable growth conditions (Rasmann et al., 2015). Hence, induction of defenses is potentially advantageous in crops, where resources allocated to defense should be minimized.

Barbarea vulgaris is a wild crucifer, growing in temperate regions (Badenes-Pérez et al., 2010; Toneatto et al., 2010). It is a model plant for studying saponin and glucosinolate biosynthesis, insect resistance and plant-insect co-evolution (Kuzina et al., 2011). A long term goal of this research is identification of genes, metabolites and regulatory mechanisms that could confer resistance traits to cultivated crucifers. In addition, this research aims at a deeper understanding of insect counter-resistance development. There are two morphologically distinct types of *B. vulgaris*: G-type and P-type, which are named from their glabrous and pubescent leaves, respectively (Nielsen, 1997; Christensen et al., 2014). These types also have contrasting resistance phenotypes and secondary metabolite profiles (Dalby-Brown et al., 2011). The G-type is strongly resistant to some crucifer-specific insect species, including the diamondback moth (DBM, *Plutella xylostella*) and some kinds of flea beetles (*Phyllotreta nemorum*), while the P-type is completely susceptible to them. The G-type DBM and flea beetle resistance is attributed to biosynthesis of triterpenoid saponins, a unique feature among crucifers (Nielsen, 1997; Shinoda et al., 2002; Kuzina et al., 2009, 2011; Dalby-Brown et al., 2011; Khakimov et al., 2015; Liu et al., 2015a; Zhang et al., 2015). P- and G-type *B. vulgaris* also differ in the type and content of glucosinolates, which are secondary metabolites known as effectors in plant defenses against other insects and some diseases.

Glucosinolates (Figure 1) are thioglucosides derived from amino acids and are the distinctive secondary metabolites in crucifers (Agerbirk and Olsen, 2012). They provide an activated defense system because their hydrolysis catalyzed by endogenous thioglucosidases, also known as myrosinases (E.C. 3.2.1.147), produce toxic isothiocyanates and other products (Kuchernig et al., 2012) as well as signal molecules important for resistance against microbes (Bednarek et al., 2009; Clay et al., 2009). In contrast to antimicrobial phytoalexins, which are only biosynthesized upon induction (Pedras et al., 2015), glucosinolates are classified as phytoanticipins because they are pre-formed defenses. However, additional induction of some biosynthetic groups of glucosinolates, in particular the tryptophan derived indole glucosinolates, is well-known (Bodnaryk, 1992; Hopkins et al., 1998; Bartlet et al., 1999). In this way, the glucosinolate-myrosinase defense system is available immediately upon tissue damage, while supplementary induction serves to allocate additional resources only when needed.

The G-type of *B. vulgaris* is resistant to DBM larvae due to its saponin content, while DBM larvae are known to be insensitive to glucosinolates (Ratzka et al., 2002). The major glucosinolates in both types are phenethyl glucosinolates

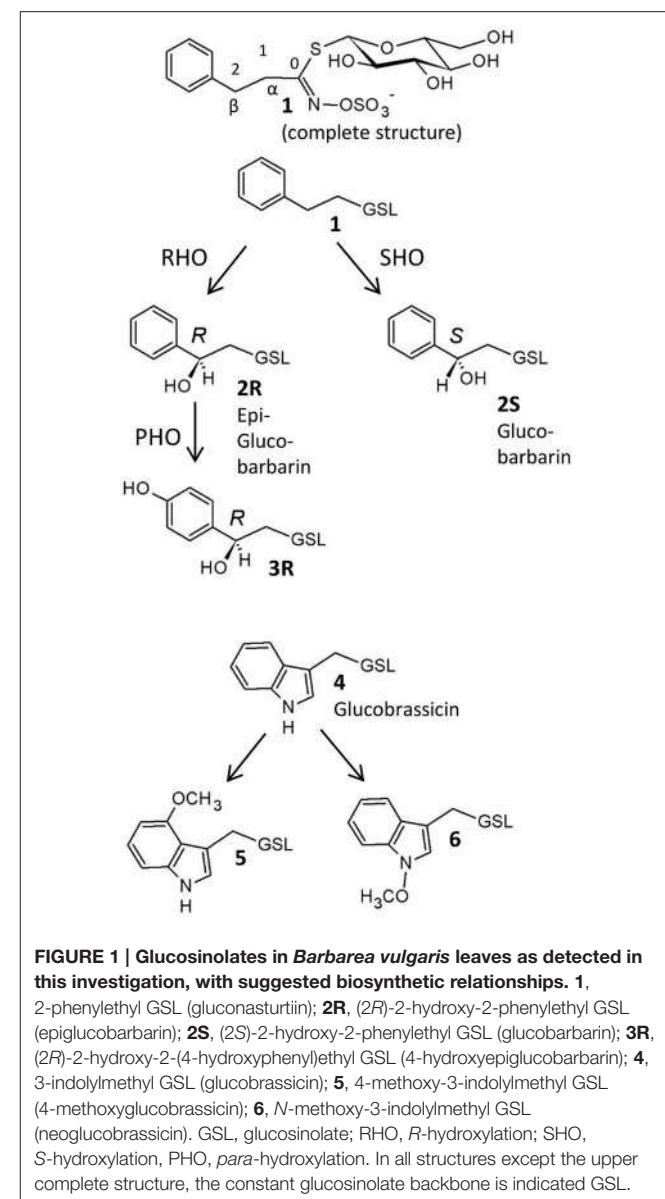


FIGURE 1 | Glucosinolates in *Barbarea vulgaris* leaves as detected in this investigation, with suggested biosynthetic relationships. 1,

2-phenylethyl GSL (gluconasturtiin); **2R**, (2*R*)-2-hydroxy-2-phenylethyl GSL (epiglucobarbin); **2S**, (2*S*)-2-hydroxy-2-phenylethyl GSL (glucobarbin); **3R**, (2*R*)-2-hydroxy-2-(4-hydroxyphenyl)ethyl GSL (4-hydroxyepiglucobarbin); **4**, 3-indolylmethyl GSL (glucobrassicin); **5**, 4-methoxy-3-indolylmethyl GSL (4-methoxyglucobrassicin); **6**, N-methoxy-3-indolylmethyl GSL (neoglucobrassicin). GSL, glucosinolate; RHO, *R*-hydroxylation; SHO, S-hydroxylation, PHO, *para*-hydroxylation. In all structures except the upper complete structure, the constant glucosinolate backbone is indicated GSL.

with a β-hydroxy group (Figure 1), which are hydrolyzed to non-isothiocyanate metabolites (oxazolidine-2-thiones and thiazolidine-2-ones). These products were recently suggested to be intermediates in phytoalexin biosynthesis in *B. vulgaris* (Agerbirk and Olsen, 2015; Pedras et al., 2015). In addition to the defensive function, glucosinolates also stimulate feeding and oviposition of many glucosinolate-adapted insects such as the DBM. Indeed, gravid DBM prefer oviposition on *B. vulgaris* because of the glucosinolate content (Badenes-Pérez et al., 2011); however, no larvae could survive on G-type plants. Thus, this plant could be used as a “dead-end” trap crop (Lu et al., 2004; Badenes-Pérez et al., 2005). A quantitative increase of glucosinolates in *B. vulgaris* was accomplished by sulfur fertilization and found to improve the effectiveness of the insect trap (Badenes-Pérez et al., 2010, 2011). For all of these reasons,

it was interesting to test the induction of glucosinolates and compare their magnitude in both types of *B. vulgaris*.

In contrast to the dominance of methionine derived glucosinolates in *Arabidopsis thaliana* (Gigolashvili et al., 2008), the major glucosinolates in *B. vulgaris* are derived from homo-phenylalanine, and their biosynthesis is largely unknown. Collectively, these glucosinolates are best named phenethyl glucosinolates. A number of genetic variants contain various phenethyl glucosinolates and yield different hydrolysis products upon damage (Agerbirk et al., 2014; Agerbirk and Olsen, 2015), conferring differential effects on insect herbivores (van Leur et al., 2008). The G- and P-type *B. vulgaris* differ in their content of stereochemical isomers (diastereomers with respect to hydroxyl groups and hence termed epimers) of 2-hydroxy-2-phenylethylglucosinolate (**Figure 1**; Kuzina et al., 2011). The G-type mainly contains the (2S)-epimer (glucobarbarin, **2S**) and the P-type mainly contains the (2R)-epimer (epiglucobarbarin, **2R**), which are both assumed to be biosynthesized by hydroxylation of a common precursor, 2-phenylethylglucosinolate (**1**) (Kuzina et al., 2011). Two previous reports have investigated the genetics of this hydroxylation. In the first report, the gene coding for biosynthesis of **2S** was found to be a single dominant gene, while a rare phenotype dominated by **1**, devoid of a hydroxyl group, was controlled by a recessive allele of the same locus (van Leur et al., 2006). In the second report, quantitative trait locus mapping was applied to identify the genes involved in G- and P-type *B. vulgaris* glucosinolate polymorphism using a P × G-type derived F₂ population (Kuzina et al., 2011). The genes determining the **2S/2R** difference between G-type and P-type were mapped on two chromosome regions spanning 20–60 cM (Kuzina et al., 2011). Also in this study, recombinant plants dominated by **1** rather than **2R** or **2S** were reported, in accordance with separate loci for biosynthesis of **2R** and **2S**, respectively, from **1**.

Despite these pioneering genetic investigations, the glucosinolate biosynthesis pathway has not been compared in molecular detail between the two plant types, neither has their DBM-feeding-responses been studied. RNA-seq allows simultaneous acquisition of sequences for gene discovery, as well as transcript identification involved in specific biological processes (Wang et al., 2013). In recent papers, using Illumina paired-end sequencing, we reported the transcriptome profile of G-type *B. vulgaris* at 0, 1, 4, 8, 12, 24, and 48 h (Wei et al., 2013) and P-type at 0 and 4 h after infestation by DBM (Zhang et al., 2015). These data offered sufficient information to study glucosinolate synthesis related genes and their regulation in different types of *B. vulgaris* in response to DBM.

In the present work, we evaluated the impact of constitutive and DBM-induced changes in glucosinolates of *B. vulgaris*, using G-type and P-type plants, which contain resistant and non-resistant saponins, respectively. Consequently, we identified genes that were likely to be involved in phenethyl glucosinolate biosynthesis in G- and P-type *B. vulgaris*, based on RNA-seq data by comparison with the known pathway of methionine derived glucosinolates in *A. thaliana* (Wittstock and Halkier, 2002). We then characterized the gene expression patterns in response to DBM. The sequences and expression of some genes in the pathway were additionally validated by molecular

cloning and quantitative PCR (qPCR). Two genes controlling the S and R epimers of glucosinolates in the two genotypes were identified. These data revealed a correlation between glucosinolate production and gene expression, and provide a better understanding of the defensive strategy of DBM resistant and susceptible *B. vulgaris* in terms of glucosinolates. At the same time, these results will deepen our understanding of the biosynthesis of phenethyl glucosinolates at the molecular level.

MATERIALS AND METHODS

Plant Material and Cultivation

Seeds of *B. vulgaris* accessions B4 (P-type) and B44 (G-type) were obtained from the University of Copenhagen, Denmark (Agerbirk and Olsen, 2015). Accession B4 is identical to accession NGB23547 publicly available at www.nordgen.org. The F₁ hybrid was generated by a G-type (male) × P-type (female) cross in the Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences, China. Plant growth conditions were the same as in previous pyrosequencing studies (Wei et al., 2013; Zhang et al., 2015). *Barbarea vulgaris* seeds were surface-sterilized in 1% NaClO and sown into 10 × 10 cm pots filled with a mixture of peat soil. Plants were kept in a growth chamber at 25°C/20°C (light/dark), and 60% relative humidity, at 225 μmol·m⁻²·s⁻¹ light intensity, and on a 16 h:8 h (light:dark) photoperiod. Plants were watered as needed and fertilized with half-strength Hoagland's nutrient solution. Plants at 10 weeks old were used in this study.

Insect Feeding Treatment

Diamondback moth larvae were originally obtained from a cabbage field in Taigu, Shanxi, China in the autumn of 2010 and reared on cabbage at 25°C, 12 h:12 h photoperiod and 60% relative humidity. Three DBM third-instar larvae were inoculated on each fully extended leaf of the 10 weeks old *B. vulgaris* plants from time zero until the time of sampling (8 or 48 h); seven leaves per plant were inoculated. The transcriptome results showed that most genes were significantly induced after 4 h of infestation by DBM; therefore, this time point was set for qPCR. To obtain optimal glucosinolate induction, 8 and 48 h of infestation by DBM were used for glucosinolate analysis. Plants with DBM and DBM-free control plants were kept in a growth chamber under the same condition. After 48 h, the leaves of control plants (not including the petiole) were cut and immediately flash frozen in liquid nitrogen. For DBM-treated plants, the DBM larvae were removed with a brush and the leaves were harvested using the same method used to harvest the control. Five plants were used as biological replicates. All material was stored at -80°C.

Glucosinolate Extraction and Analysis

Glucosinolates were extracted according to the method of La et al. (2009). Two hundred milligrams of freeze-dried leaf powder was weighed in a 15 mL tube and 5 mL of boiling 100% methanol was added. Glucotropaeolin (benzylglucosinolate) was added as an internal standard.

Samples were then incubated at 80°C for 15 min in a water bath. The mixture was centrifuged at 7000 × g for 10 min at 4°C and the supernatant was decanted into another tube. The extraction was repeated twice from residues using the same procedure with 70% methanol (v/v). The three supernatants were combined and 2 mL of each glucosinolate extract was added to a mini-column filled with diethylaminoethanol (DEAE) Sephadex A-25 (Amersham Bio-sciences, Uppsala, Sweden) activated with 0.02 M NaAc (Sinopharm Chemical Reagent, Beijing, China), and desulfated by sulfatase (Dikma Technologies, CA, USA). After reaction at room temperature overnight (16 h), the desulfated glucosinolates were eluted with 2 mL de-ionized water and stored at -20°C before to high performance liquid chromatography (HPLC) analysis.

Samples were analyzed by HPLC on an Agilent HP 1100 Series instrument equipped with a C-18 reversed-phase column (Nova-PakR, 3.9 × 150 mm, 5 μm particle size) using 0.5 g·L⁻¹ ammonium acetate (solvent A)-a mixture of 1 L 0.1 mol·L⁻¹ ammonium acetate and 300 mL methanol (solvent B) gradient at a flow rate of 1 mL·min⁻¹ (injection volume 20 μL). The gradient was as follows: constant 100% (A) at 0–6 min, a linear gradient from 100 to 30% (A) at 6–21 min, a linear gradient from 30% to 0 (A) at 21–24 min, constant 100% (B) at 24–28 min, a linear gradient from 0 to 100% (A) at 28–30 min, and constant 100% (A) at 30–35 min. The eluent was monitored by diode array detection between 200 and 400 nm. Desulfoglucosinolates were identified by comparing retention times and UV absorption spectra with those of known standards. Results are given as μmol·g⁻¹ dry weight, calculated using peak areas and generally agreed relative response factors for UV detection at 229 nm: 0.95 in case of **1**, **2R**, **2S** and the internal standard benzylglucosinolate, 0.50 in case of the phenolic **3R**, 0.29 in case of **4**, 0.25 in case of **5**, and 0.20 in case of **6** (Agerbirk et al., 2015).

Database for Glucosinolate Biosynthetic Genes Identification in *B. vulgaris*

Sequences representing the complete set of glucosinolate biosynthetic genes in *A. thaliana* were acquired from the TAIR database (www.arabidopsis.org). Data for G- and P-type *B. vulgaris* transcriptome sequence were obtained from previous pyrosequencing studies [<ftp://shanjie:shanjie123@brassicadb.org> and EMBL/NCBI/SRA (accession numbers SRR1582492 and SRR1583630); Wei et al., 2013; Zhang et al., 2015]. We identified candidate genes related to glucosinolate biosynthesis and transcription factors of *B. vulgaris* using BLASTN with a cutoff *E*-value ≤ 1E-10.

RNA Extraction and First-Strand cDNA Synthesis

Total RNA of the samples was isolated using an *EasyPure®* Plant RNA Kit (TransGen Biotech, China), according to the manufacturer's instructions. 800 ng of total RNA was reverse transcribed to synthesize first-strand cDNA using oligo dT primers and *EasyScript®* One-Step gDNA Removal and cDNA

Synthesis SuperMix (TransGen Biotech, China) and diluted 20-fold as templates for molecular cloning and qPCR.

Quantitative Real-Time PCR (qPCR)

Quantitative real-time PCR was performed on a StepOne™ Real-Time PCR System (Applied Biosystems), using the *TransStart®* Green qPCR SuperMix (TransGen Biotech), following the manufacturer's instructions. Primers were designed using Primer3web (version 4.0.0, <http://primer3.ut.ee/>; Untergasser et al., 2012). A list of genes and primers is shown in Table S1. The reaction volume was 20 μL, including 0.4 μL of 10 mM Forward and Reverse primer respectively, 10 μL of 2 × *TransStart* Green qPCR SuperMix, 2.0 μL of the cDNA sample, 0.4 μL of Passive Reference Dye I, and 6.8 μL of ddH₂O. The thermal cycling profile was: 95°C for 10 min; 40 cycles of 95°C for 15 s, 59°C for 15 s, 72°C for 10 s; then 95°C for 15 s, 60°C for 1 min, ramping to 95°C for 15 s. Three independent biological and technical replicates were performed. Data were analyzed using StepOne™ Software v.2.0 (Applied Biosystems). *Tubulin* was used as an internal control. The relative expression level were estimated by the 2^{-ΔΔCT} method (Livak and Schmittgen, 2001).

Gene Cloning, Sequencing, and Sequence Analysis

Reverse transcription (RT)-PCR cloning was performed to confirm the assembly quality of genes involved in glucosinolate biosynthesis. Specific PCR primers for the six selected genes were designed corresponding to the ends of longer unigenes of G-type and P-type *B. vulgaris*, using Primer3web (version 4.0.0, <http://primer3.ut.ee/>; Untergasser et al., 2012). The list of genes and primers is shown in Table S1. PCR was performed in a total volume of 50 μL, including 5 μL of 10 × PCR buffer, 5 μL of 25 mM MgSO₄, 3 μL of 2 mM dNTPs, 1.5 μL of each 10 mM primer, 1 μL of 1.0 U/μL KOD-Plus-Neo polymerase (Toyobo, Osaka, Japan), 2 μL of cDNA and 31 μL of ddH₂O, with the following reactions: an initial denaturation step at 94°C for 2 min; followed by 35 cycles of 98°C for 10 s and 68°C for 60 s. The PCR products were separated on 1% (w/v) agarose gel and isolated using a MaxiGel Extraction Kit (CoWin Biotech, Beijing, China), ligated into the *pEASY®*-Blunt Cloning vector (TransGen Biotech, Beijing, China), and then transformed into *Escherichia coli* DH 5α. Positive clones were confirmed by PCR and sequenced using an ABI 3730 instrument (Applied Biosystems, CA, USA). Sequence data alignment and amino acid deduce and alignment were performed using DNAMAN version 8 (Lynnon, Quebec, Canada) with the default parameters.

Statistical Analysis

The SPSS 17.0 software package for Windows was used for all statistical analyses. The data were analyzed for significant differences using Tukey's HSD test at a significance threshold of *p* = 0.05.

RESULTS

A Pair of Phenethyl Glucosinolate Epimers is Dominant in G- and P-Type *B. vulgaris* and Inducible by DBM

We examined leaf damage, glucosinolate types and their quantities of G- and P-type *B. vulgaris* over 0, 8, and 48 h after DBM infestation. The DBM-resistance ability differed significantly between the G- and P-type *B. vulgaris*, as evaluated by the fraction of leaf area damaged. G-type plants were more resistant and only suffered minor injuries, while about a quarter of P-type leaves were damaged at 48 h after DBM infestation (Figure 2). Glucosinolate profiling indicated that seven types of major glucosinolates were present in the leaves of *B. vulgaris* (Table 1 and Figure 3). The dominant class was aromatic glucosinolates with a phenethyl backbone, of which the most abundant compound was glucobarbarin (2S) in G-type and epiglucobarbarin (2R) in the P-type (Figures 1, 3); both of them were induced by DBM infestation (Table 1). As previously reported for the P-type, the phenolic glucosinolate 3R, apparently a ring-oxidized derivative of 2R, was also detected but at moderate levels. Neither 3R nor its S-epimer has ever been detected in the G-type by specific HPLC-MS. However, for purely statistical reasons a trace signal with similar retention time was quantitated for the G-type (Table 1, Figure 3). Glucobrassicin (4) was the most abundant indole glucosinolate in both types, accompanied by the 4-methoxy derivative (5) and traces of the N-methoxy derivative (6). Indole glucosinolates accumulated more abundant in the G-type. Only levels of three individual glucosinolates could be induced by DBM infestation: 2S (only in G-type), 2R (only in P-type) and 4 (only in G-type). Because the dominating glucosinolate in each plant type was induced, total glucosinolate levels were also “induced” in both types, but only due to changes in the three mentioned individual glucosinolates. The contents of both 2S and 4 in the G-type were gradually induced at 8 and 48 h, while the P-type’s response to DBM infestation was more rapid: significant induction of epiglucobarbarin was observed at 8 h, with no significant difference with 48 h (Table 1). In relative terms, the induction of the glucobarbarins (2R and 2S) was similar, around 1.4-fold in 48 h. This extend of induction was comparable to the mean induction of the indole glucosinolate glucobrassicin in the G-type (around 1.5-fold). In absolute terms the induction of 2R in the P-type was moderately higher than the induction of 2S in the G-type, reflecting a higher glucosinolate level in this type at our growth conditions (Table 1).

Prediction of Glucosinolate Metabolic Pathway in *B. vulgaris* and its Candidate Genes Response to DBM Infestation

To investigate the molecular basis of the glucosinolate biosynthesis in *B. vulgaris*, a glucosinolates metabolic pathway was deduced according to the KEGG pathway (PATHWAY: map00966) and previous reports (Sønderby et al., 2010; Figure 4A). The pathway is generally characterized into four stages: (i) chain elongation of selected precursor amino

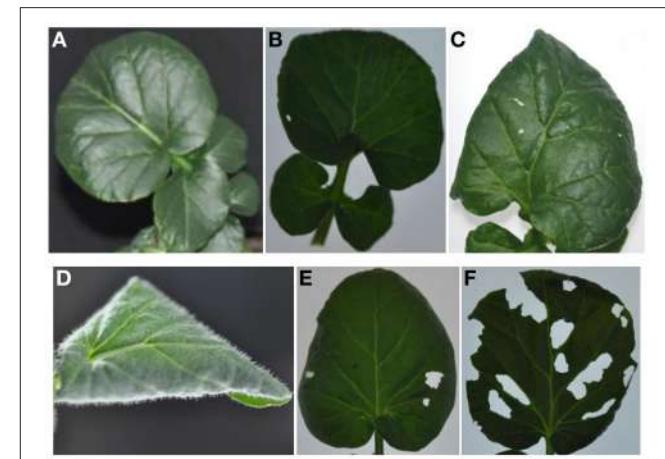


FIGURE 2 | G-type (A–C) and P-type (D–F) *B. vulgaris* leaves exposed to larvae of the diamondback moth (*Plutella xylostella*) over 8 and 48 h. (A,D), control leaves; (B,E), leaves treated with three DBM third-instar larvae for 8 h; (C,F), leaves treated with three DBM third-instar larvae for 48 h.

acids (e.g., Met and Phe), (ii) core structure formation, (iii) secondary modification of the side chain, and (iv) degradation of glucosinolate. Forty-two G-type and 33 P-type unigenes were identified as candidates of the 30 enzymes in the pathway, by homology searching from our former reported *B. vulgaris* transcriptome dataset with the glucosinolate metabolic genes from *A. thaliana* as baits. The gene list and their corresponding *Arabidopsis* homolog AGI codes, as well as the sequence similarities with G- or P-type, are shown in Tables S2 and S4.

The biosynthesis pathway of methionine derived glucosinolates starts from the side-chain elongation process catalyzing the parent amino acid deamination to form 2-oxo acid by a branched-chain amino acid aminotransferase (BCAT4; Figure 4A and Table S3). In our annotated *B. vulgaris* transcriptome unigene dataset, a unigene corresponding to BCAT4 was identified in both G- and P-type plants. It was significantly induced by DBM in the P-type, but not significantly in the G-type plant (Figure 4B and Table S3). The 2-oxo acid then enters a cycle of three successive transformations (Sønderby et al., 2010): (1) condensation with acetyl-CoA by MAM (methylthioalkylmalate synthase); (2) isomerization by IPMI LSU (isopropylmalate isomerase large subunit) and IPMI SSU (isopropylmalate isomerase small subunit); and (3) oxidative decarboxylation by IPMDH (isopropylmalate dehydrogenase). A similar chain elongation is also needed for biosynthesis of phenethyl glucosinolates (Figure 1, 1–3), but would not be relevant for the tryptophan derived (Figure 1, 4–6). One MAM, two IPMI LSU, two IPMI SSU, four IPMDH transcripts were identified in G-type while one MAM, one IPMI LSU, two IPMI SSU, and two IPMDH transcripts were identified in P-type plants. All of these genes were upregulated by DBM infestation in P-type plants but not in the G-type (Figure 4B and Table S3). Thereafter, the molecules are transaminated by BCAT3. Two unigenes encoding BCAT3 were identified in both G- and P-type plants, amongst, the genes in the P-type were induced by

TABLE 1 | Glucosinolate contents in rosette leaves of G-type and P-type *Barbarea vulgaris* under diamondback moth larvae infestation.

<i>B. vulgaris</i> type	Time after DBM infestation	1 (μmol/g Dw)	2S (μmol/g Dw)	2R (μmol/g Dw)	3R (μmol/g Dw)	4 (μmol/g Dw)	5 (μmol/g Dw)	6 (μmol/g Dw)	Total (μmol/g Dw)
G-type	0 h	0.262 ± 0.104 b	29.572 ± 0.122 c	0.893 ± 0.009 d	0.008 ± 0.002 b	1.197 ± 0.018 c	0.344 ± 0.035 a	0.001 ± 0.001 a	32.277 ± 0.286 e
	8 h	0.324 ± 0.012 a	32.121 ± 0.114 b	1.079 ± 0.086 d	0.017 ± 0.004 b	1.576 ± 0.019 b	0.346 ± 0.003 a	0.001 ± 0.001 a	35.464 ± 0.230 d
	48 h	0.123 ± 0.040 b	42.122 ± 0.407 a	1.088 ± 0.017 d	0.013 ± 0.002 b	1.819 ± 0.034 a	0.312 ± 0.033 a	0.001 ± 0.001 a	45.478 ± 0.579 c
P-type	0 h	0.388 ± 0.035 a	51.585 ± 0.120 c	4.486 ± 0.024 a	1.144 ± 0.015 d	0.034 ± 0.002 b	0.002 ± 0.001 a	0.002 ± 0.001 a	58.527 ± 0.112 b
	8 h	0.322 ± 0.031 a	0.933 ± 0.007 d	67.329 ± 0.165 b	4.709 ± 0.018 a	0.976 ± 0.001 e	0.032 ± 0.002 b	0.002 ± 0.001 a	74.303 ± 0.054 a
	48 h	0.310 ± 0.008 a	1.068 ± 0.034 d	69.273 ± 0.496 a	4.148 ± 0.022 a	0.988 ± 0.010 e	0.029 ± 0.002 b	0.002 ± 0.001 a	75.798 ± 0.494 a

The data were presented as mean ± standard error ($N = 5$ plants of each type and for each infestation type). Different letters in the same column indicate significant differences at 0.05 level (Tukey's HSD test). Trace levels of **3R** in G-type represents maximum estimates based on integration of a trace signal at the same retention time, and is not proof of presence of this glucosinolate in the G-type. Glucosinolate numbers according to **Figure 1**.

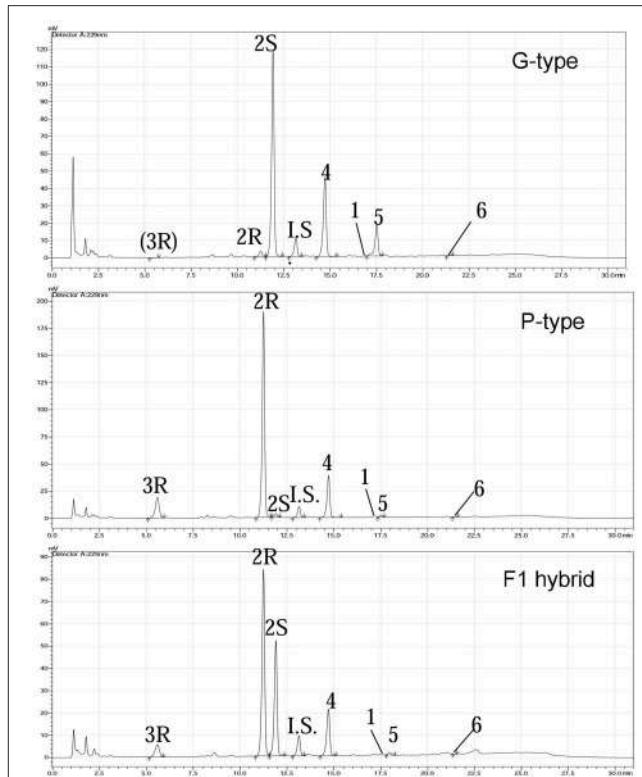


FIGURE 3 | Quantification of glucosinolates from G-type, P-type, and hybrid F₁ plants by HPLC after desulfation of the native metabolites and an added standard. The desulfated glucosinolates, in order of elution, are: **3R**, (2R)-2-hydroxy-2-(4-hydroxyphenylethyl) GSL (4-hydroxyepiglucobarbin); **2R**, (2R)-2-hydroxy-2-phenylethyl GSL (epiglucobarbin); **2S**, (2S)-2-hydroxy-2-phenylethyl GSL (glucobarbin); **LS**, Glucotropaeolin (internal standard); **4**, 3-indolylmethyl GSL (glucobrassicin); **1**, 2-phenylethyl GSL (gluconasturtiin); **5**, 4-methoxy-3-indolylmethyl GSL (4-methoxyglucobrassicin); **6**, N-methoxy-3-indolylmethyl. Trace levels of **3R** in G-type represents maximum estimates based on integration of a trace signal at the same retention time, and is not proof of presence of this glucosinolate in the G-type.

DBM. The products of the above reactions then enter the core glucosinolate structure pathway.

The formation of the glucosinolate core structure is accomplished by 13 enzymes catalyzing five biochemical steps (glutathione S-transferase (GSTF), glutathione S-transferase tau (GSTU) are predicted and gamma-glutamyl peptidase (GGP) is a partially characterized enzyme; they are considered as one step along with CYP83 in previous and current reports; Grubb and Abel, 2006; Sønderby et al., 2010). It begins with the oxidation of the side-chain elongated amino acids to convert them to aldoximes by cytochromes P450 of the CYP79 family. There are seven CYP79 genes in the *Arabidopsis* genome and one of them (CYP79A2) uses Phe as substrate. A high similarity homolog of CYP79A2 was not found in *B. vulgaris*. However, three and two CYP79s were identified in G- and P-type *B. vulgaris* respectively. Among them, CL10668.Contig1_All in G-type and T_Unigene_BMK.15233 in P-type had higher expression levels and were induced by DBM. They are most

likely the candidate genes for the enzyme involved in phenethyl glucosinolate biosynthesis. Next step is catalyzed by cytochrome P450 of the CYP83 family. Two unigenes in each G- and P-type *B. vulgaris* were identified as homologs to the two *Arabidopsis* CYP83 genes (*CYP83A1* and *CYP83B1*). The *CYP83B1* were classified and named *CYP83B1v1* (G-type) and *CYP83B1v2* (P-type) based on sequence comparisons. This was also done for additional *B. vulgaris* CYPs mentioned below. The next step involves conjugation with a sulfur donor to form a GSH conjugate. Two predicted enzymes, encoded by *GSTF* and *GSTU*, may be involved in this reaction, but this reaction can also happen non-enzymatically (Sønderby et al., 2010). Three homolog unigenes of *GSTF*, two homolog unigenes of *GSTU* were identified in G- and P-type *B. vulgaris*. The above steps were not significantly affected by DBM infestation. In *Arabidopsis*, there is good evidence that the sulfur donor is glutathione, γ -Glut-Cys-Gly, and its biosynthesis involves five committed enzymes, including ATPs, APR, OASTL, GSH1, and GSH2 (Geu-Flores et al., 2009, 2011). One unigene was discovered encoding GSH in G- and P-type, respectively. The GSH conjugate is then hydrolyzed by GGP (Geu-Flores et al., 2009) to form an S-alkyl-thiohydroximate, which is subsequently degraded by C-S lyase SUPERROOT1 (SUR1) to form a thiohydroximate, which is in turn S-glucosylated by glucosyltransferase UGT74 to form a desulfoglucosinolate. One *GGP*, one *SUR1* and two *UGT74* homolog sequences were discovered in G- and P-type *B. vulgaris*, respectively. All of them were significantly induced by DBM infestation in both types of plants, except *UGT74B1*, in G-type plants. The final step is catalyzed by desulfoglucosinolate sulfotransferase (SOT) to generate the glucosinolate itself, with 3-phosphoadenosine-5'-phosphosulfate (PAPS) as the sulfate donor. Four and three unigenes were identified as SOTs in G- and P-type *B. vulgaris*. One of them, T_Unigene_BMK.12426 in the P-type had the highest expression level and was upregulated by DBM (Figure 4B and Table S3). PAPS is produced from adenosine-5'-phosphosulfate (APS) through a two-step catalysis by ATP sulfurylase (ATPS) and APS kinase (APK; Sønderby et al., 2010). In the present study, two DBM inducible unigenes were discovered encoding APKs in G- and P-type *B. vulgaris*, respectively. By the end of these steps, the parent phenethyl glucosinolate, 2-phenylethylglucosinolate (1) would be produced.

Both methionine derived glucosinolates and phenethyl glucosinolates are further subjected to hydroxylation modification, resulting in increased structural diversity. However, existing genetic and enzymological knowledge concerns methionine-derived glucosinolates. The flavin-monooxygenase glucosinolate S-oxygenase (FMO-GSOX), alkenyl hydroxalkyl producing (AOP), Fe (II)-dependent oxygenase superfamily protein (GS-OH) and CYP81F2 are reported to take part in these processes and other side chain modification (Hansen et al., 2007; Wentzell et al., 2007; Li et al., 2008; Bednarek et al., 2009; Clay et al., 2009; Pfalz et al., 2009). Assuming that related genes would be responsible for hydroxylation of phenethyl glucosinolates, we searched for homologs in our transcriptome. Two FMO-GSOX and one CYP81F2 homolog sequences were discovered in G- and

P-type *B. vulgaris*, respectively. These CYPs were classified and named *CYP81F1v1* (G-type) and *CYP81F1v2* (P-type). However, unigenes responsible for AOP were not detected. In this study, CL12207.Contig1_All in G- and T_Unigene_BMK.14596 in P-type *B. vulgaris* were homologous to the *Arabidopsis GS-OH*, which is responsible for modification of 3-but enyl glucosinolate to produce 2-hydroxy-3-but enyl glucosinolate. These two genes were expressed at high levels and were upregulated by 2.5- and 4.4-fold in G- and P-types, respectively, under DBM infestation (Figure 4B, Tables S2, S3).

Five unigenes in G-type plants were identified as homologs of genes encoding myrosinase, while only one unigene was found in P-type. All of these genes were slightly upregulated by DBM but did not fulfill the two-fold cutoff in both types (Figure 4B and Table S3).

Candidate Regulatory Transcription Factor Genes and their Response to DBM Infestation

It was reported that three transcription factors (IQD1-1, Dof1.1 and MYB) could regulate the expression of genes involved in glucosinolate metabolism in *A. thaliana* (Wang et al., 2011). Two and one homolog sequences were discovered encoding Dof1.1 in G- and P-type *B. vulgaris*, respectively. One unigene was predicted to encode IQD1-1 in both plant types. These two types of transcription factors were not significantly affected by DBM in both plant types (Figure 4C and Table S3). Six members of the MYB family (MYB28, 29, 34, 51, 76, and 122) are reported to regulate the biosynthesis of glucosinolates (Gigolashvili et al., 2008; Sonderby et al., 2010). From our *B. vulgaris* transcriptome, one member of each of *MYB28*, 29, 34, and 51 homolog was found in the G-type and one homolog of each of *MYB28*, 34, 51, and 76 and two homologs of *MYB29* were identified in P-type plants. *MYB28* was downregulated in the P-type but not in the G-type *B. vulgaris*. *MYB29* was significantly upregulated in both types, but accumulated more than 27-fold higher in the P-type compared with the G-type plants after 4 h of infestation by DBM. While *MYB34* accumulated more than nine-fold more in the G-type than in the P-type. *MYB51* was downregulated in both plant types. *MYB76* was only identified in P-type plants and was induced by DBM (Figure 4C and Table S3). Thus, the induction of the glucosinolate pathway is possibly regulated by *MYB34* in the G-type and by *MYB29* and *MYB76* in the P-type.

qPCR Confirmation of Glucosinolate Genes Expression Patterns

We confirmed the expression patterns of six genes responsible for glucosinolates biosynthesis in leaves of *B. vulgaris* before and after (4 h) DBM infestation using qPCR. The expression patterns of these genes are shown in Figure 5. Most genes showed good correlation with the profiles from transcriptome sequencing. The unigenes encoding MAM1, GGP1, and UGT74B1 showed the same expression patterns between the two genotypes, both were upregulated at 4 h after DBM infestation. While the transcription of myrosinases was suppressed in both plant types. The BCAT4 genes were significantly downregulated in G-type plants, but

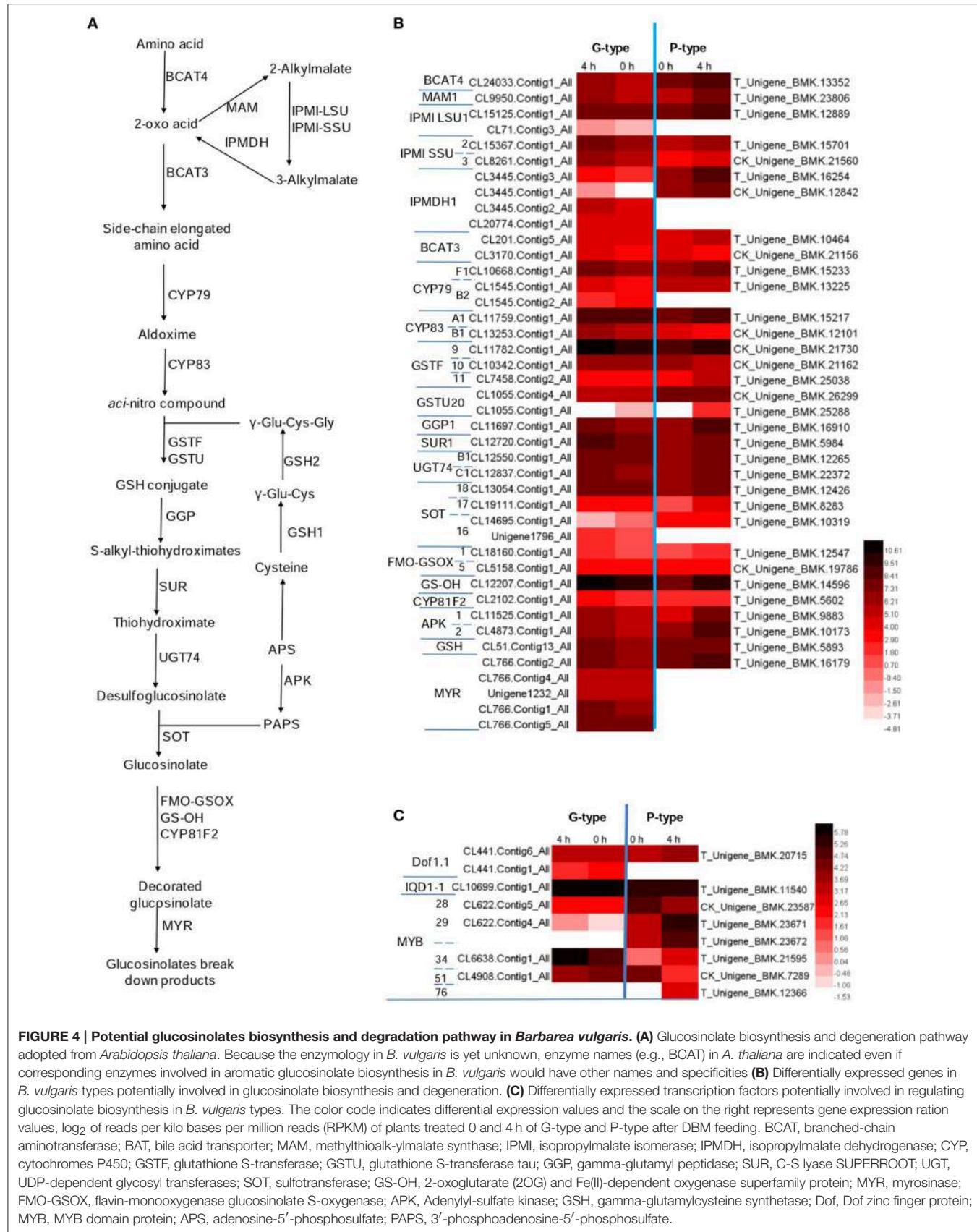


FIGURE 4 | Potential glucosinolates biosynthesis and degradation pathway in *Barbarea vulgaris*. (A) Glucosinolate biosynthesis and degeneration pathway adopted from *Arabidopsis thaliana*. Because the enzymology in *B. vulgaris* is yet unknown, enzyme names (e.g., BCAT) in *A. thaliana* are indicated even if corresponding enzymes involved in aromatic glucosinolate biosynthesis in *B. vulgaris* would have other names and specificities. **(B)** Differentially expressed genes in *B. vulgaris* types potentially involved in glucosinolate biosynthesis and degeneration. **(C)** Differentially expressed transcription factors potentially involved in regulating glucosinolate biosynthesis in *B. vulgaris* types. The color code indicates differential expression values and the scale on the right represents gene expression ration values, log₂ of reads per kilo bases per million reads (RPKM) of plants treated 0 and 4 h of G-type and P-type after DBM feeding. BCAT, branched-chain aminotransferase; BAT, bile acid transporter; MAM, methylthioalkylmalate synthase; IPMI, isopropylmalate isomerase; IPMDH, isopropylmalate dehydrogenase; CYP, cytochromes P450; GSTF, glutathione S-transferase; GSTU, glutathione S-transferase tau; GGP, gamma-glutamyl peptidase; SUR, C-S lyase SUPERROOT; UGT, UDP-dependent glycosyl transferases; SOT, sulfotransferase; GS-OH, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily protein; MYR, myrosinase; FMO-GSOX, flavin-monooxygenase glucosinolate S-oxygenase; APK, Adenyllyl-sulfate kinase; GSH, gamma-glutamylcysteine synthetase; Dof, Dof zinc finger protein; MYB, MYB domain protein; APS, adenosine-5'-phosphosulfate; PAPS, 3'-phosphoadenosine-5'-phosphosulfate.

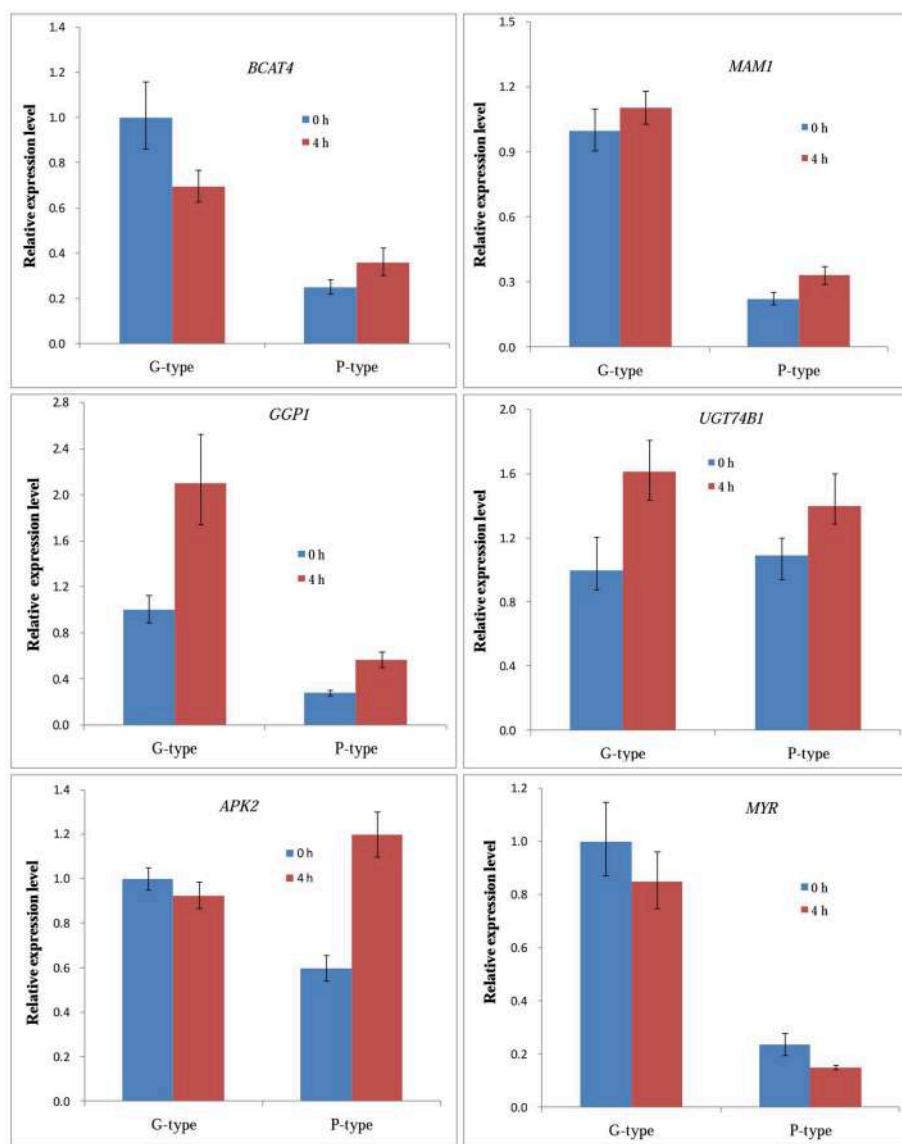


FIGURE 5 | qRT-PCR expression analysis of expression levels of six selected genes in leaves of *Barbarea vulgaris* at 0 h (control) and 4 h after being infested by diamondback moth larvae.

upregulated in P-type. In contrast to *BCAT4*, the *APK2* gene was stable in the G-type, while they were significantly upregulated in the P-type by DBM infestation.

Molecular Cloning and Comparison of Genes Involved in Glucosinolates Metabolism

Several RT-PCR cloning experiments were performed to check the quality of the unigene assembly. cDNA sequences of six selected genes [*BCAT4*, *CYP83A1*, *GGP1*, *SUR1*, *GS-OH* (*SHO* in G-type and *RHO* in P-type)] from the glucosinolate biosynthetic pathway were isolated from both G- and P-type *B. vulgaris* by blunt end cloning and were subsequently sequenced using the

Sanger method. The sequence similarities between the clones and corresponding unigenes were more than 99.2% pairwise identity, which validated the reliability of RNA-seq assembly (Table 2). Additionally, sequences similarities between G- and P-type were also compared, showing that the genes in both types were highly conserved (>99.0% pairwise identity) except for *GS-OH*, which showed significantly sequence variation (77.50% pairwise identity in coding DNA sequences and 65.48% identity in deduced amino acid sequences) between the two types (Table 2, Figure 6). The sequence diversity of *GS-OH* could be responsible for the different glucobarbarin epimers of the G-type and P-type glucosinolates (glucobarbarin, **2S**, vs. epiglucobarbarin, **2R**). Furthermore, the known biochemical function of *GS-OH* is equivalent to the functions envisioned for *SHO* and *RHO*:

TABLE 2 | Sequence analyses of the six genes putatively involved in glucosinolate biosynthesis in two types of *B. vulgaris*.

Gene	<i>B. vulgaris</i> type	Sequence length	Similarity with transcriptome (%)	Similarity between G- and P-type (%)
BCAT4	G-type	1064	99.4	99.0
	P-type	1064	99.2	
CYP83A1	G-type	1506	99.7	98.9
	P-type	1506	99.7	
GGP1	G-type	750	99.6	99.7
	P-type	750	100.0	
SUR1	G-type	1344	100.0	99.0
	P-type	1344	100.0	
SHO	G-type	1095	100.0	78.0 (compared with RHO)
	P-type	—	—	
RHO	G-type	—	—	—
	P-type	1071	99.5	

β -hydroxylation (Figure 1) of a glucosinolate side chain (Hansen et al., 2008). Thus, we named the GS-OH in G- and P-type separately as SHO and RHO, respectively, in accordance with a previous hypothesis put forward by Agerbirk et al. (2015).

A Genetic Model in Which SHO and RHO Produce the S and R Epimers of Glucobarbarins in *B. vulgaris*

To test the hypothesis that CL12207.Contig1_All and T_Unigene_BMK.14596 are the GS-OH genes responsible for the glucosinolate epimers in the G-type (SHO) and P-type (RHO) *B. vulgaris*, we generated F₁ plants by hybridization of G- and P-type plants and analyzed the correlation between gene expression and glucosinolate production. The SHO and 2S, RHO and 2R co-occurred in G-, P-type, and F₁ plants (Figure 7). For testing co-segregation, cooccurrence of gene and suggested glucosinolate product in F₂ plants was needed. Unfortunately, F₂ plants were not available in this project. Indeed, an F₂ generation from a G-type \times P-type cross appears to have been obtained only once in the literature (Kuzina et al., 2011), while further F₂ progenies have not been published, possibly due to a highly frequent sterility barrier between the accessions of the types that have so far been tested. Based on the available results, we established a double-codominance gene model to explain the 2S and 2R inheritance in the two types of *B. vulgaris*. In this model, the double heterozygote SsRr plants (F₁) generated by hybridization of SSrr (G-type, mainly containing 2S) and ssRR (P-type, mainly containing 2R) will contain both epimers (2S and 2R; Figures 3, 7). Therefore, F₂ populations should contain recessive homozygous plants (ssrr), which only accumulated precursor glucosinolate, 1 (or traces of the glucobarbarin epimers if additional minor genes were present). A previous study showed that the profile of five plants in 129 F₂ *B. vulgaris* population were dominated by 1 (Kuzina et al., 2011), which supports our codominance-gene model. The model is further supported by observation of low levels of epiglucobarbarin in

so-called “NAS-forms” of the G-type devoid of glucobarbarin (van Leur et al., 2006; Agerbirk et al., 2015).

DISCUSSION

Glucosinolates can be divided into groups according to their amino acid precursor, of which we consider three: methionine (Met), phenylalanine (Phe), and tryptophan (Trp) including chain-elongated homologs of the former two. Glucosinolates derived from Met (including chain elongated homologs) and Trp are well-studied and their biosynthetic pathways have been identified in the model plant *A. thaliana* (Sønderby et al., 2010). These served as a model for our search for genes involved in the biosynthetic pathway of aromatic Phe-derived glucosinolates. This is currently unknown, although a recent review lists a couple of genes that may be involved in their biosynthesis in natural or engineered systems (CYP79A2, CYP83A1, CYP83B1, SUR1, UGT74B1, and SOT16; Baskar et al., 2012). In an evolutionary perspective, Phe-derived glucosinolates should be divided in non-chain elongated glucosinolates directly derived from Phe and named “benzyl glucosinolates,” and chain elongated derived from homoPhe and named “phenethyl glucosinolates.” The former, without chain elongation, are believed to be an ancient character, while the latter seem more recent and occur as the core structure gluconasturtiin (1) in roots of most cruciferous crops, and as oxidized derivatives in *Barbarea* and occasionally elsewhere. *B. vulgaris* is dominated by oxidized phenethyl glucosinolates (Agerbirk et al., 2015), making it an ideal plant for the study of the phenethyl glucosinolate biosynthetic pathway including side chain decoration. In this study, a potential glucosinolate biosynthesis pathway was manually predicted by homology with biosynthesis of Met-derived glucosinolates in *Arabidopsis* and a number of candidate genes in this pathway were discovered in G- and P-type *B. vulgaris*. As Met-derived glucosinolates are not known from *Barbarea*, the genes would seem to be involved in biosynthesis of phenethyl glucosinolates. Our model will serve as a starting point for exploring the biosynthesis of phenethyl glucosinolates at the molecular level. Furthermore, we found the GS-OH candidate genes SHO and RHO in G- and P-type plants, which are most likely responsible for the stereospecific biosynthesis of glucobarbarin and its epimer, epiglucobarbarin. The functions of the genes identified in this study require confirmation by further studies, such as expression in *A. thaliana*. The transcription factors involved in regulating the glucosinolate biosynthetic pathway, including Dof1.1, IQD1-1, and MYB, were also identified successfully in G- and P-type *B. vulgaris*. Based on their expression patterns, the MYB34 in the G-type and MYB29 and MYB76 in the P-type were deduced as regulators of the glucosinolate induction in the DBM response.

Recently, it has been speculated that resistant and susceptible plant types could have diverged during the ice age because of geographical isolation and adaption to their new environment, leading to the formation of different evolutionary lineages and taxa, which differ in their resistance, hairiness, saponins, flavonoids, and glucosinolates (Hauser et al., 2012; Christensen et al., 2014). The results of the present study showed that

A	G-type P-type	ATGGACTCTGAGGATAACAATGGCTGAATAATACGACCGTGTAGTGAGTTAACGGCTTCGACGAGACAAAGCGGGGTGAAGGGTCTTGTAGAAGCTGATGGACTCTGAGTCTACGATCGTGCAGTGAGTTAACGGCTTCGACGAGACAAAGCTGGGTGAAAGGTCTTGTCAAGCTGAGCTG	100 79	
	G-type P-type	GAATACAAAAATCCCAGCATTTCATCACCCGCTTGTCACAGAAACAAACAGTAACCTGGCTCAAGG...GTGACGTGCCAGAAATCGATCTAGG GAATACAAAAATCCCAGCATTTCATCACCCGCTTGTCACAGAAACAACTAAACCATCTCAATGATGAGTCAAGCTGAGATATGA.	197 178	
	G-type P-type	AGGTGGCGTGTGGAAATCCCAGCATTGCAGAGAGGGTGGTTGATGAGAATAAACGAGTGGAGAAAGTATGGGTTTTCTACGGGTTAACCATGGG ..GTGGCGTGTGGAAATCCCAGCATTGCAGAGAGGGTGGTTGCAAGGCTAAAGGCAACGGAGAAGTTGGGTTTTCTACGGGTTAACCATGGG	297 276	
	G-type P-type	ATTCACACTACATGTATGGAGAAAGATGAAGATGCGTTCTGAGGTTTCACAGAGCAAGATCTGAAGTGAGGAAAATCTCTACACCCGAGACAAACCA ATTCCTATGGAACTTATGGAGAAAATGATAGATGTCAGTGTGAGGTTTCACAGAGCAAGATCCAGAAGTGAGGAAAACGGTCTATAACCCGAGACAAACCA	397 376	
	G-type P-type	AAABAGTTAGTATAACTCAATGCTGATCTCCATGAAATCTCCTGCTGCGAGTTGAAAGATACTTTGACTACGATAATGGCTCCCTGATGCTCCAAAGGC AAACAGTTAGTATAACTCAATGATGTCAGTGTGAGGTTTCACCTGCTGCGAGTTGAAAGATACTTTGACTACGATAATGGCTCCCTGATGCTTCCAAAGCT	497 476	
	G-type P-type	AGAGGAGTTGCCAAAGGTTGGGGAGATCATGTTGGAGTAECTAAAGGAAGCGATGAAGTTACAGAGTTAACCTTCAACTTAAATCAGAAGCTTA AGAGGAGTTGCCAAAGGTTGGGGAGATCATGTTGGAGTAECTAAAGGAAGCGATGAAGTTACAGAGTTAACCTTCAACTTAAATCAGAAGCTTA	597 576	
	G-type P-type	GGGTTGAGTTCTAACCACTCAAAGAAATGGATTGCAACAAAGGTTAGTCATGCTCAATGTTTACTACCCGCCCTGCTCTGAGCAAAATCTAACATTAC GGGTTGAGTTCTAACCACTCAAAGATATGGATTGCGAGAAGGTTAAATGCTGCTATGTCATGTTTACTACCCGCCCTGCTCTGAGCAACACCTG	697 676	
	G-type P-type	GGGGCGCTCCTCACACGGACAGATCTTCATCACTATCTTCTTCAGACCACATTGAAACTATTCAGTTTCCGTATGGATCCTGGATCGATGTT GGGGCACTCCTCACACGGACAGATCTTCATCACTATTCTTCTTCAGACCACATTGGAGGACTTCAGTTCTCAGGATGGATACTGGATCGATGTT	797 776	
	G-type P-type	TCTTAATCCAAAGCTCTCTCATTAACGTTGGAGATCTCTACAGCTTATATCGAAATGACAAGTTATAAGTGTGGAGCATAGGTTTGGCAAATAGA TCTTACTCGGGAGCTTTATCTCTAACGTTGGAGATCTTATACAGCTTTAACAAATGACAAGTTGAAGTGTGGAGCATAAAATCTGGCAAACGAA	897 876	
	G-type P-type	CATCAAGAGCCGGCAATTCCATCGGTGTTCTTCATCCATCCTTCCAGGTTOAGAAAATATGGACCCATTAAAGAGTTTGTCTGAACAAACACC GATCAAGAGCCCAAGCTCATCGGTCTTCTTGTATCCATCCTTCCAGGTTOAGAAAATATGGACCCATTAAAGAGTTTGTCTGAACAAACACC	997 976	
	G-type P-type	CTCCAAGTACAGAGAGACCACCGCGGAAACCTCTAGCCACTATGTGGCTAGACAACTTCACTGGAAATGCTCGTTGCTTCACTTAAGGATCTGA CTCCAAGTACAGAGACTCACTGGAAAGCTCTAACCACTCTCGTGGATAGAAAACCTPATGTSAAATTAATCTGTTGAGCATTAAAGGATCTGA	1095 1071	
B	G-type P-type	MDSEDTMAEIYDRASELKAFDETIGVKGLVETGITKIPRIFHNPFLVETTSKPGSR.VTFPEIDLGGGVIESPAMRERVVDEIKYAMEKYMESYDRASELKAFDETIGVKGLVETGITKIPRIFHDERATSRNTKESSMMVITPTIDMSG.VFESMDTRKSVVAKVKEATEKEFG	91 84	
	G-type P-type	FFYAVNHGIPPLVMEKMKDGVRRFHEQDPEVRKMFYTRDKTKVRYNSNADLHESPAASWKTDLITIMAPDAPKAEELPKVCGEIMLEYSKE FFQAIINHGIPPMELMEKMDVTRRFHEQDPEVRKTFYTSRDKTKQFKYNSNNLDLGSPAAEWRDFTCEMAPNVPKLEDLPKICGEVIMLEYSKE	183 176	
	G-type P-type	AMKLAELIFQLISEALGISSNHIKEMDCTKGIVMLNLYYPPCPFPNLTLGGAPHTDRSFITILLQDHIEVFQVFRDGWSIDVAENPKALLIN VMKFGELIFQLISEALGINPNHLKDMDCAEGLMLLCHFYYPCCFPDRTLGGTPHTDRSFITILLQDHIGGLQLQDGWVIDVPTPGALLIN	275 268	
	G-type P-type	VGDLIQLISNDKEISVEHRLVLANRQEPRISIACFVPHFPGSKRYGPIKEILSELQNPPKYRETTAETSSHVARQLDGKNASLHLRI VGDLIQLUTNDKEISVEHRLVLANRQEPRISIAFVPHPTSSKVYGPIKEILSELQNPLKYRDSTAKVSNNPVDRKPN.VNNSLSHLRI	364 356	

FIGURE 6 | Sequence alignment of the full-length coding DNA sequences (A) and deduced amino acid sequences (B) of GS-OH homologs of G-type (*SHO*) and P-type (*RHO*) *Barbarea vulgaris*.

upstream genes of GS-OH were highly conserved during evolution: the sequence similarity between the two genotypes exceeded 98.9%. Unexpectedly, the *SHO* and *RHO* have significantly sequence variation between G- and P-type *B. vulgaris*, as low as 77.5% in coding DNA sequences and 65.48% identity in deduced amino acid sequences (Figure 6). Thus, we consider them as two independent genes that may have diverged during the separation of the two types.

There is ample evidence that glucosinolates not only function in defense against generalist herbivores, but also play a key role in host recognition for crucifer specialist insects (Mewis et al., 2005; Badenes-Pérez et al., 2011). Previous reports on *A. thaliana* indicated that both generalist and specialist insects can induce glucosinolate synthesis pathways, while the transcription of myrosinases was suppressed (Kuśnierszyk et al., 2007). In our previous study using the same data set, the P-type

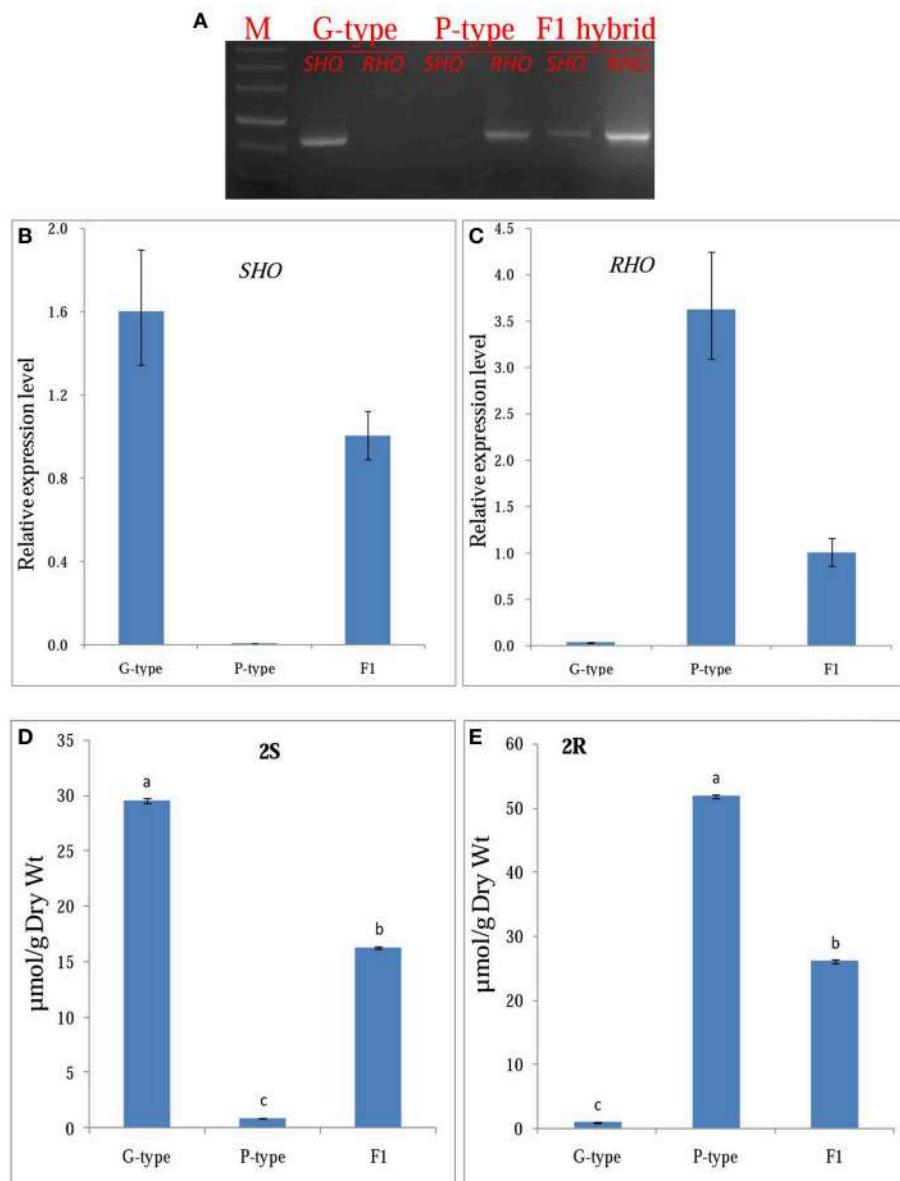


FIGURE 7 | Functional confirmation of candidate GS-OH gene. (A) gene expression of the SHO, RHO in *Barbarea vulgaris* leaves of G-type, P-type, and F₁ (generated by hybridization of G- and P-type plants). **(B,C)**, qRT-PCR expression analysis of SHO, RHO in *Barbarea vulgaris* leaves of G-type, P-type and F₁, respectively. **(D,E)**, Mean (\pm SE) concentration in rosette leaves of *Barbarea vulgaris* of G- and P-type. **2S**, (2S)-2-hydroxy-2-phenylethyl GSL (glucobarbarin); **2R**, (2R)-2-hydroxy-2-phenylethyl GSL (epiglucobarbarin).

glucosinolate biosynthesis pathway was not over-represented among the upregulated pathways by a hypergeometric test, mainly because many differentially expressed genes have not been annotated to the glucosinolate biosynthesis pathway by the automatic KEGG annotation pipeline. In our present study, the glucosinolate biosynthesis pathways were constructed manually and refined, and the expression level of the most glucosinolate synthesis genes were increased in G- and P-type *B. vulgaris* after DBM infestation, as revealed by transcriptome and qPCR experiments, which is consistent with previous

reports in *Arabidopsis* and *Brassica* plants (Kuśnierszyk et al., 2007).

Herbivory is probably a multifaceted challenge of plants given that the wounds from the herbivore provide a direct access for pathogenic microbes as well as increased evaporation, stress from released phytochemicals, etc. Hence, it is likely that plant responses to insect herbivory should include not only defenses against the herbivore, but also defenses against a variety of microbes and other stresses. Furthermore, induction responses may not be specific for each herbivore, but produce

a response that on average has defensive properties against a range of frequent herbivores. For these reasons, it is not surprising that some glucosinolates were induced by DBM larvae despite the resistance of the larvae to this defense. Indeed, a similar induction of **2S** and **2R** by a flea beetle was recently reported (van Mölken et al., 2014). Quantitatively, the reported induction was similar to the induction reported here. However, we find it striking that the massive tissue damage in the P-type (**Figure 1**) did not result in induction of the indole glucosinolate **4**, which is in many plant species a highly inducible glucosinolate (Bodnaryk, 1992; Hopkins et al., 1998; Bartlet et al., 1999). In contrast, the modest tissue damage in the G-type (**Figure 2**) never-the-less induced **4**. Furthermore, it is interesting that the phenolic **3R**, believed to be biosynthesized from **2R**, was not induced in the P-type, although the apparent precursor was induced. In contrast, the phenolic **3R** was reported to be many fold induced in the transition from summer to fall (Agerbirk and Olsen, 2015) suggesting that environmental regulation of glucosinolate hydroxylation in *B. vulgaris* is complex. A gene sequence (*PHO*) for this hydroxylation of **2R** to **3R** is not suggested here, but the availability of the P-type transcriptome (Zhang et al., 2015), and the present refinement, now provide candidates for future investigations of glucosinolate regulation in the species. From this investigation, the relevant gene would be expected to be unique for the P-type and not be induced by DBM herbivory. This hydroxylation is known to have functional significance, as the hydrolysis product of **3R** is a thiazolidine-2-one, in contrast to the oxazolidine-2-thione produced from **2R** and **2S**.

The DBM is one of the most destructive pests of crucifer crops causing about \$ 4-5 billion loss annually in the world (Furlong et al., 2013). It is reported to have developed resistance to all major classes of insecticides including *Bacillus thuringiensis* (*Bt*) insecticidal proteins (Shelton, 2004; Furlong et al., 2013). In China, high dose of insecticide with short interval time are commonly used to control DBM, which causes severe environmental damage and food contamination. Therefore, an integrated pest management is urgently needed. Previous studies on agricultural uses of *B. vulgaris* mostly focus on the use as a “dead-end” trap crop (Lu et al., 2004; Badenes-Pérez et al., 2005) and mining resistance genes for breeding insect resistant cultivars (Wei et al., 2013; Khakimov et al., 2015; Zhang et al., 2015). In recent reports, we have confirmed the resistance-properties of G-type *B. vulgaris* to a contemporary Chinese field-isolate

of DBM (Wei et al., 2013; Liu et al., 2015b; Zhang et al., 2015), further implying the application potential of this wild crucifer in the DBM controlling. Our present research shows that DBM infection induced the content of glucosinolate, an oviposition attracting signal for DBM, in agreement with usage of *B. vulgaris* as a “dead-end” trap for DBM control. On the other hand, DBM chewing can be envisioned to expose the plant to pathogenic microbes which may lead to additional loss for Brassicaceae vegetable production. The DBM induction of glucosinolates possibly could reduce pathogenic microbes’ access via the wounds rather than waste of resources, thus improving insect resistance or tolerance, respectively, of G-type and P-type *B. vulgaris*.

In conclusion, the present study identified genes involved in glucosinolate biosynthesis of G- and P-type *B. vulgaris*, and characterized the relationship between gene expression patterns and glucosinolate contents in response DBM. These findings will deepen our understanding of the biosynthesis of the phenethyl group of aromatic glucosinolates at the molecular level and provide the basis for further investigation of the molecular ecology of insect resistance in *B. vulgaris* plants.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: XL, TL, XZ. Performed the experiments: TL, XZ, HY, JS. Analyzed the data: TL, XZ, XL, NA, YQ, HW, DS. Wrote the paper: TL, XZ, XL, NA. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00083>

REFERENCES

- Agerbirk, N., and Olsen, C. E. (2012). Glucosinolate structures in evolution. *Phytochemistry* 77, 16–45. doi: 10.1016/j.phytochem.2012.02.005
- Agerbirk, N., and Olsen, C. E. (2015). Glucosinolate hydrolysis products in the crucifer *Barbarea vulgaris* include a thiazolidine-2-one from a specific phenolic isomer as well as oxazolidine-2-thiones. *Phytochemistry* 115, 143–151. doi: 10.1016/j.phytochem.2014.11.002
- Agerbirk, N., Olsen, C. E., Cipollini, D., Ørgaard, M., Linde-Laursen, I., and Chew, F. S. (2014). Specific glucosinolate analysis reveals variable levels of epimeric glucobarbarins, dietary precursors of 5-phenyloxazolidine-2-thiones, in watercress types with contrasting chromosome number. *J. Agric. Food Chem.* 62, 9586–9596. doi: 10.1021/jf5032795
- Agerbirk, N., Olsen, C. E., Heimes, C., Christensen, S., Bak, S., and Hauser, T. P. (2015). Multiple hydroxyphenethyl glucosinolate isomers and their tandem mass spectrometric distinction in a geographically structured polymorphism in the crucifer *Barbarea vulgaris*. *Phytochemistry* 115, 130–142. doi: 10.1016/j.phytochem.2014.09.003
- Badenes-Pérez, F. R., Reichelt, M., Gershenson, J., and Heckel, D. G. (2011). Phyloplane location of glucosinolates in *Barbarea* spp. (Brassicaceae) and misleading assessment of host suitability by a specialist herbivore. *New Phytol.* 189, 549–556. doi: 10.1111/j.1469-8137.2010.03486.x

- Badenes-Pérez, F. R., Reichelt, M., and Heckel, D. G. (2010). Can sulfur fertilisation improve the effectiveness of trap crops for diamondback moth, *Plutella xylostella* (L.) (Lepidoptera: Plutellidae)? *Pest Manage. Sci.* 66, 832–838. doi: 10.1002/ps.1949
- Badenes-Pérez, F. R., Shelton, A. M., and Nault, B. A. (2005). Using yellow rocket as a trap crop for diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 98, 884–890. doi: 10.1603/0022-0493-98.3.884
- Bartlett, E., Kiddle, G., Williams, I., and Wallsgrove, R. (1999). “Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist,” in *Proceedings of the 10th International Symposium on Insect-Plant Relationships* (Oxford: Springer), 163–167.
- Baskar, V., Gururani, M. A., Yu, J. W., and Park, S. W. (2012). Engineering glucosinolates in plants: current knowledge and potential uses. *Appl. Biochem. Biotechnol.* 168, 1694–1717. doi: 10.1007/s12010-012-9890-6
- Bednarek, P., Piślewska-Bednarek, M., Svatoš, A., Schneider, B., Doubský, J., Mansurova, M., et al. (2009). A glucosinolate metabolism pathway in living plant cells mediates broad-spectrum antifungal defense. *Science* 323, 101–106. doi: 10.1126/science.1163732
- Bodnaryk, R. P. (1992). Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31, 2671–2677. doi: 10.1016/0031-9422(92)83609-3
- Christensen, S., Heimes, C., Agerbirk, N., Kuzina, V., Olsen, C. E., and Hauser, T. P. (2014). Different geographical distributions of two chemotypes of *Barbarea vulgaris* that differ in resistance to insects and a pathogen. *J. Chem. Ecol.* 40, 491–501. doi: 10.1007/s10886-014-0430-4
- Clay, N. K., Adio, A. M., Denoux, C., Jander, G., and Ausubel, F. M. (2009). Glucosinolate metabolites required for an *Arabidopsis* innate immune response. *Science* 323, 95–101. doi: 10.1126/science.1164627
- Dalby-Brown, L., Olsen, C. E., Nielsen, J. K., and Agerbirk, N. (2011). Polymorphism for novel tetraglycosylated flavonols in an eco-model crucifer, *Barbarea vulgaris*. *J. Agric. Food Chem.* 59, 6947–6956. doi: 10.1021/jf200412c
- Furlong, M. J., Wright, D. J., and Dossall, L. M. (2013). Diamondback moth ecology and management: problems, progress, and prospects. *Annu. Rev. Entomol.* 58, 517–541. doi: 10.1146/annurev-ento-120811-153605
- Geu-Flores, F., Moldrup, M. E., Böttcher, C., Olsen, C. E., Scheel, D., and Halkier, B. A. (2011). Cytosolic γ-glutamyl peptidases process glutathione conjugates in the biosynthesis of glucosinolates and camalexin in *Arabidopsis*. *Plant Cell* 23, 2456–2469. doi: 10.1105/tpc.111.083998
- Geu-Flores, F., Nielsen, M. T., Nafisi, M., Møldrup, M. E., Olsen, C. E., Motawia, M. S., et al. (2009). Glucosinolate engineering identifies a γ-glutamyl peptidase. *Nat. Chem. Biol.* 5, 575–577. doi: 10.1038/nchembio.185
- Gigolashvili, T., Engqvist, M., Yatusevich, R., Mueller, C., and Fluegg, U. I. (2008). Hag2/myb76 and hag3/myb29 exert a specific and coordinated control on the regulation of aliphatic glucosinolate biosynthesis in *Arabidopsis thaliana*. *New Phytol.* 177, 627–642. doi: 10.1111/j.1469-8137.2007.02295.x
- Grubb, C. D., and Abel, S. (2006). Glucosinolate metabolism and its control. *Trends Plant Sci.* 11, 89–100. doi: 10.1016/j.tplants.2005.12.006
- Hansen, B. G., Kerwin, R. E., Ober, J. A., Lambrix, V. M., Mitchell-Olds, T., Gershenson, J., et al. (2008). A novel 2-oxoacid-dependent dioxygenase involved in the formation of the goiterogenic 2-hydroxybut-3-enyl glucosinolate and generalist insect resistance in *Arabidopsis*. *Plant Physiol.* 148, 2096–2108. doi: 10.1104/pp.108.129981
- Hansen, B. G., Kliebenstein, D. J., and Halkier, B. A. (2007). Identification of a flavin-monooxygenase as the S-oxygenating enzyme in aliphatic glucosinolate biosynthesis in *Arabidopsis*. *Plant J.* 50, 902–910. doi: 10.1111/j.1365-313X.2007.03101.x
- Hauser, T. P., Toneatto, F., and Nielsen, J. K. (2012). Genetic and geographic structure of an insect resistant and a susceptible type of *Barbarea vulgaris* in western europe. *Evol. Ecol.* 26, 611–624. doi: 10.1007/s10682-011-9515-5
- Hopkins, R., Griffiths, D., Birch, A., and McKinlay, R. (1998). Influence of increasing herbivore pressure on modification of glucosinolate content of swedes (*Brassica napus* spp. *rapifera*). *J. Chem. Ecol.* 24, 2003–2019. doi: 10.1023/A:1020729524818
- Khakimov, B., Poulsen, V. K., Erthmann, P. Ø., Fukushima, E. O., Augustin, J. M., Olsen, C. E., et al. (2015). Identification and genome organization of saponin pathway genes from a wild crucifer, and their use for transient production of saponins in *Nicotiana benthamiana*. *Plant J.* 84, 478–490. doi: 10.1111/tpj.13012
- Kuchernig, J. C., Burow, M., and Wittstock, U. (2012). Evolution of specifier proteins in glucosinolate-containing plants. *BMC Evol. Biol.* 12:127. doi: 10.1186/1471-2148-12-127
- Kuśnierczyk, A., Winge, P., Midelfart, H., Armbruster, W. S., Rossiter, J. T., and Bones, A. M. (2007). Transcriptional responses of *Arabidopsis thaliana* ecotypes with different glucosinolate profiles after attack by polyphagous myzus persicae and oligophagous *Brevicoryne brassicae*. *J. Exp. Bot.* 58, 2537–2552. doi: 10.1093/jxb/erm043
- Kuzina, V., Ekstrom, C. T., Andersen, S. B., Nielsen, J. K., Olsen, C. E., and Bak, S. (2009). Identification of defense compounds in *Barbarea vulgaris* against the herbivore *Phyllotreta nemorum* by an ecometabolomic approach. *Plant Physiol.* 151, 1977–1990. doi: 10.1104/pp.109.136952
- Kuzina, V., Nielsen, J. K., Augustin, J. M., Torp, A. M., Bak, S., and Andersen, S. B. (2011). *Barbarea vulgaris* linkage map and quantitative trait loci for saponins, glucosinolates, hairiness and resistance to the herbivore *Phyllotreta nemorum*. *Phytochemistry* 72, 188–198. doi: 10.1016/j.phytochem.2010.11.007
- La, G. X., Fang, P., Teng, Y. B., Li, Y. J., and Lin, X. Y. (2009). Effect of CO₂ enrichment on the glucosinolate contents under different nitrogen levels in bolting stem of Chinese kale (*Brassica alboglabra* L.). *J. Zhejiang Univ. Sci. B* 10, 454–464. doi: 10.1631/jzus.B0820354
- Li, J., Hansen, B. G., Ober, J. A., Kliebenstein, D. J., and Halkier, B. A. (2008). Subclade of flavin-monooxygenases involved in aliphatic glucosinolate biosynthesis. *Plant Physiol.* 148, 1721–1733. doi: 10.1104/pp.108.125757
- Liu, T. J., Zhang, X. H., Li, X. X., Shen, D., Wang, H. P., Qiu, Y., et al. (2015a). Advances on research and utilization of elite resistant resource - *Barbarea vulgaris*. *Acta Hortic. Sin.* 42, 1719–1731. doi: 10.16420/j.issn.0513-353x.2015-0178
- Liu, T. J., Zhang, X. H., Shen, D., Wang, H. P., Qiu, Y., Song, J. P., et al. (2015b). Analysis on genetic diversity of *Barbarea vulgaris* germplasm resources based on phenotypic traits. *J. Plant Genet. Resour.* 16, 528–534. doi: 10.13430/j.cnki.jngr.2015.03.014
- Livak, K. J., and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 25, 402–408. doi: 10.1006/meth.2001.1262
- Lu, J. H., Liu, S. S., and Shelton, A. M. (2004). Laboratory evaluations of a wild crucifer *Barbarea vulgaris* as a management tool for the diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae). *Bull. Entomol. Res.* 94, 509–516. doi: 10.1079/BER2004328
- Mewis, I., Appel, H. M., Hom, A., Raina, R., and Schultz, J. C. (2005). Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiol.* 138, 1149–1162. doi: 10.1104/pp.104.053389
- Nielsen, J. K. (1997). Variation in defences of the plant *Barbarea vulgaris* and in counter adaptations by the flea beetle *Phyllotreta nemorum*. *Entomol. Exp. Appl.* 82, 25–35. doi: 10.1046/j.1570-7458.1997.00110.x
- Pedras, M. S. C., Alavi, M., and To, Q. H. (2015). Expanding the nasturlexin family: nasturlexins C and D and their sulfoxides are phytoalexins of the crucifers *Barbarea vulgaris* and *B. verna*. *Phytochemistry* 118, 131–138. doi: 10.1016/j.phytochem.2015.08.009
- Pfalz, M., Vogel, H., and Kroymann, J. (2009). The gene controlling the indole glucosinolate modifier1 quantitative trait locus alters indole glucosinolate structures and aphid resistance in *Arabidopsis*. *Plant Cell* 21, 985–999. doi: 10.1105/tpc.108.063115
- Rasmann, S., Chassin, E., Bilat, J., Glauser, G., and Reymond, P. (2015). Trade-off between constitutive and inducible resistance against herbivores is only partially explained by gene expression and glucosinolate production. *J. Exp. Bot.* 66, 2527–2534. doi: 10.1093/jxb/erv033
- Ratzka, A., Vogel, H., Kliebenstein, D. J., Mitchell-Olds, T., and Kroymann, J. (2002). Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. U.S.A.* 99, 11223–11228. doi: 10.1073/pnas.172112899
- Shelton, A. M. (2004). “Management of the diamondback moth: déjà vu all over again? in the management of diamondback moth and other crucifer pests,” in *Diamondback Moth and Other Crucifer Pests: Proceedings of the Fourth International Workshop Management*, eds N. M. Endersby and P. M. Ridland (Melbourne, VIC: Regional Institute), 3–8.

- Shinoda, T., Nagao, T., Nakayama, M., Serizawa, H., Koshioka, M., Okabe, H., et al. (2002). Identification of a triterpenoid saponin from a crucifer, *Barbarea vulgaris*, as a feeding deterrent to the diamondback moth, *Plutella xylostella*. *J. Chem. Ecol.* 28, 587–599. doi: 10.1023/A:1014500330510
- Sønderby, I. E., Burow, M., Rowe, H. C., Kliebenstein, D. J., and Halkier, B. A. (2010). A complex interplay of three R2R3 MYB transcription factors determines the profile of aliphatic glucosinolates in *Arabidopsis*. *Plant Physiol.* 153, 348–363. doi: 10.1104/pp.109.149286
- Sønderby, I. E., Geu-Flores, F., and Halkier, B. A. (2010). Biosynthesis of glucosinolates—gene discovery and beyond. *Trends Plant Sci.* 15, 283–290. doi: 10.1016/j.tplants.2010.02.005
- Toneatto, F., Nielsen, J. K., Orgaard, M., and Hauser, T. P. (2010). Genetic and sexual separation between insect resistant and susceptible *Barbarea vulgaris* plants in Denmark. *Mol. Ecol.* 19, 3456–3465. doi: 10.1111/j.1365-294X.2010.04760.x
- Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B. C., Remm, M., et al. (2012). Primer3-new capabilities and interfaces. *Nucleic Acids Res.* 40, e115–e115. doi: 10.1093/nar/gks596
- van Leur, H., Raaijmakers, C. E., and van Dam, N. M. (2006). A heritable glucosinolate polymorphism within natural populations of *Barbarea vulgaris*. *Phytochemistry* 67, 1214–1223. doi: 10.1016/j.phytochem.2006.04.021
- van Leur, H., Vet, L. E., Van der Putten, W. H., and van Dam, N. M. (2008). *Barbarea vulgaris* glucosinolate phenotypes differentially affect performance and preference of two different species of lepidopteran herbivores. *J. Chem. Ecol.* 34, 121–131. doi: 10.1007/s10886-007-9424-9
- van Mölken, T., Kuzina, V., Munk, K. R., Olsen, C. E., Sundelin, T., van Dam, N. M., et al. (2014). Consequences of combined herbivore feeding and pathogen infection for fitness of *Barbarea vulgaris* plants. *Oecologia* 175, 589–600. doi: 10.1007/s00442-014-2928-4
- Wang, H., Wu, J., Sun, S., Liu, B., Cheng, F., Sun, R., et al. (2011). Glucosinolate biosynthetic genes in *Brassica rapa*. *Gene* 487, 135–142. doi: 10.1016/j.gene.2011.07.021
- Wang, Y., Pan, Y., Liu, Z., Zhu, X., Zhai, L., Xu, L., et al. (2013). *De novo* transcriptome sequencing of radish (*Raphanus sativus* L.) and analysis of major genes involved in glucosinolate metabolism. *BMC Genomics* 14:836. doi: 10.1186/1471-2164-14-836
- Wei, X. C., Zhang, X. H., Shen, D., Wang, H. P., Wu, Q. J., Lu, P., et al. (2013). Transcriptome analysis of *Barbarea vulgaris* infested with diamondback moth (*Plutella xylostella*) larvae. *PLoS ONE* 8:e64481. doi: 10.1371/journal.pone.0064481
- Wentzell, A. M., Rowe, H. C., Hansen, B. G., Ticconi, C., Halkier, B. A., and Kliebenstein, D. J. (2007). Linking metabolic QTLs with network and cis-eQTLs controlling biosynthetic pathways. *PLoS Genet.* 3:e162. doi: 10.1371/journal.pgen.0030162
- Wittstock, U., and Halkier, B. A. (2002). Glucosinolate research in the *Arabidopsis* era. *Trends Plant Sci.* 7, 263–270. doi: 10.1016/S1360-1385(02)02273-2
- Zhang, X. H., Liu, T. J., Wei, X. C., Qiu, Y., Song, J. P., Wang, H. P., et al. (2015). Expression patterns, molecular markers and genetic diversity of insect-susceptible and resistant *Barbarea* genotypes by comparative transcriptome analysis. *BMC Genomics* 16:486. doi: 10.1186/s12864-015-1609-y

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Development of a Susceptibility Index of Apple Cultivars for Codling Moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) Oviposition

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Codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae) is a major fruit feeding pest of apples. Understanding susceptibility differences of various apple cultivars to CM oviposition is an important step in developing resistant varieties as well as monitoring and management strategies for this pest in apple orchards planted with mixed-cultivars. In this context, oviposition preferences of CM for the fruits of different apple cultivars were studied in laboratory bioassays using a series of no-choice and multiple-choice tests in 2006, 2007, and 2008. In 2006 and 2007, 10 apple cultivars, viz., Arlet, Fuji, Gala, Golden Delicious, Honeycrisp, Pristine, Delicious, Stayman, Sunrise, and York Imperial were evaluated, while in the 2008 tests, Golden Delicious, Honeycrisp, and York Imperial were evaluated. During the 2006 tests, preferred apple cultivars for CM oviposition were Golden Delicious and Fuji, while the least preferred were Arlet, Pristine, Sunrise, and Honeycrisp. Similarly, during the 2007 tests, Golden Delicious, Fuji and Stayman remained the preferred cultivars, while Arlet, Honeycrisp, Pristine, and Sunrise remained the least preferred cultivars. In the 2008 tests, Golden Delicious and Honeycrisp were the most and least preferred cultivars, respectively. Based on the oviposition preferences from these bioassays, a susceptibility index for each cultivar was developed. This index may be used as a standard measure in cultivar evaluations in breeding programs, and may assist fruit growers and crop consultants to select the most appropriate cultivar(s) for monitoring and detecting the initial signs of fruit injury from CM in an apple orchard planted with mixed-cultivars.

Keywords: apple cultivars, codling moth, oviposition, susceptibility, host preference, Honeycrisp, Gala, Golden Delicious

INTRODUCTION

The codling moth (CM), *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), probably originating in Europe (Pashely and Bush, 1979), is a serious pest of apples worldwide (Dean, 1989; Barnes, 1991; Witzgall et al., 2008), and causes significant economic damage to pome fruits. CM is closely associated with apple, *Malus pumila* Miller (Rosaceae), however, other species belonging to various plant families, such as pears (*Pyrus* sp.), quinces (*Cydonia oblonga* Mill.), peaches (*Prunus persica*)

(L.), wild haws (*Crataegus* sp.), English walnuts (*Juglans regia* L.) (Shelford, 1927), plum (*Prunus* sp.), nectarines (*Prunus* sp.), and sweet cherry (*Prunus avium* L.) are also reported as host plants (Madsen and Borden, 1954; Barnes, 1991).

CM completes its life cycle in four different stages, viz., egg, larva, pupa, and adult. The eggs of CM are disk-shaped, flattened, or ovate, and measure about 0.98 by 1.25 mm in diameter (Putman, 1963; Dean, 1989). Development time for eggs largely depends on temperature, and upon hatching on or near fruits, the CM larva penetrates the epidermis of the fruit, feeds on the fruit pulp and eventually making its way to the core, where the larva feeds on the seeds. After feeding on the seeds, mature larvae (fifth instar) make their way to the periphery of the fruit and make a hole to exit from the fruit. Larvae then search for a suitable place for spinning a cocoon to pupate or enter into diapause in order to avoid unfavorable environmental conditions (for instance, winter). Upon emergence, adult moths feed on the exudates from fruits and other parts of their host plants (Geier, 1963), and copulate during the dusk period (Van Leeuwen, 1929). Multiple mating occurs in both sexes (Gehring and Madsen, 1963; Howell et al., 1978), and fecundity of the female varies from host to host (Phillips and Barnes, 1975).

The fruits and leaves of apple tree release different volatiles that attract female moths to the host tree and regulate host-finding mechanisms (Wearing et al., 1973; Sutherland et al., 1974; Hern and Dorn, 1999). The main source of attraction of CM to apple trees and other host plants are kairomones (i.e., *E, E* α -farnesene and *Z, E* α -farnesene), which are naturally occurring sesquiterpene compounds (Wearing and Hutchins, 1973). Kairomones likely induce female moths to lay their eggs directly on fruit or in close vicinity of fruits and fruit clusters (Wildbolz, 1958). Oviposition in CM is mainly stimulated by a sesquiterpene compound known as α -farnesene (Wearing and Hutchins, 1973). Most eggs (up to 90%) are laid within 10 cm of a fruit (Blomfield et al., 1997). The size of fruit clusters also has significant impacts on the distribution of eggs. The number of eggs laid on fruit and nearby leaves increases with an increase in the size of the fruit cluster (Jackson, 1979; Blomfield et al., 1997). In a field environment with different apple cultivars, CM females deposit eggs on fruits, as well as both sides of spur and shoot leaves (Joshi et al., 2009; Joshi, 2011).

Female CM may discriminate among apple cultivars for oviposition as they do for other hosts such as walnut (Shelton and Anderson, 1990). The fruit size of walnut and its chemical composition varies across different commercial cultivars (Tulecke and McGranahan, 1994) and are known to affect oviposition preferences (Bezemer and Mills, 2001). In addition, the maturity level of fruits of different walnut cultivars is also known to affect the oviposition preferences of CM, as the female moths prefer to oviposit on mature rather than immature fruits (Olson, 1977; Shelton and Anderson, 1990). However, in the case of apple, such studies related to oviposition/host preference are restricted to several cultivars with very few published reports (Phillips and Barnes, 1975; Blomfield et al., 1997). Considering the importance of oviposition preferences in understanding host plant resistance, in this study we investigated susceptibility of 10 apple cultivars for CM oviposition in the

laboratory. In particular, we determined if oviposition and oviposition-site preference of CM varies among apple cultivars, and if there are any differences in the susceptibility of apple cultivars for CM oviposition during the early and late crop season. Based on the results from these studies, a susceptibility index of apple cultivars for CM oviposition was developed. This index may be used as a standard measure in cultivar evaluations and breeding programs to develop future resistant varieties as well as assisting fruit growers and pest management consultants select the most appropriate cultivar(s) for monitoring and detecting the initial signs of a CM infestation.

MATERIALS AND METHODS

Over three years, a series of laboratory experiments were conducted to study the susceptibility of 10 commercial apple cultivars, viz., 'Arlet,' 'Gala,' 'Golden Delicious,' 'Fuji,' 'Honeycrisp,' 'Pristine,' 'Delicious,' 'Stayman,' 'Sunrise,' and 'York Imperial' for CM oviposition. Two sets of experiments, based on fruit maturity, were conducted each year with the fruits collected from trees during the second week of July and either the first or second week of August in 2006, 2007, and 2008. General descriptions of bloom time, harvest time and an estimated range of fruit maturity in days after full bloom of the apple cultivars used in this study are given in Table 1.

Experimental Fruits

Fruits of all cultivars were collected from unsprayed (without insecticide application) trees (10–33 years old) in apple orchards established in south facing slopes with typical well-drained soils of the Appalachian region. Fruits were stored in small cardboard boxes in a cold room (0°C). Fruits were removed from the cold room approximately 4–5 h before the start of each experiment. All fruits were washed three times with clean cold water and were carefully inspected via a 10X Opti-Visor® lens (Donegan

TABLE 1 | Description of bloom time, harvest time and an estimated range of fruit maturity in terms of days after full bloom of apple cultivars used in multiple-choice and no-choice experiments.

Apple cultivars	Bloom time	Harvest time	DAFB*
			(estimate range)
Arlet	Early – midseason	Mid September	125–130
Fuji	Mid – late season	Late October – Mid November	170–185
Gala	Midseason	Late August	110–120
Golden Delicious	Midseason	Mid September – Early October	135–150
Honeycrisp	Early season	Mid September	125–140
Pristine	Early season	Early August	90–100
Delicious	Mid season	Late September	135–155
Stayman	Early season	Late October	165–175
Sunrise	Midseason	Mid August	95–105
York Imperial	Midseason	Late October	170–180

*DAFB, Days after full bloom.

Source: Pennsylvania Tree Fruit Production Guide (2006–2007).

Optical Co., Lenexa, KS, USA) for field oviposition/infestation by CM and other insects. Fruits of approximately similar size were vertically suspended in oviposition chambers by tying the stem to the top of the oviposition chamber using aluminum wire. Fruits damaged while being placed in oviposition chambers were discarded and replaced by new fruits from the same lot.

Experimental Insects

Codling moth adults used in this study were obtained from a laboratory colony established from adults or larvae collected from a block of apples located at The Pennsylvania State University, Fruit Research and Extension Center (FREC), Biglerville, PA, USA. Green thinning apples of various cultivars were used to maintain the laboratory colony/insect culture throughout the year during this study year. Pupae were collected from rearing containers in cardboard strips, and kept in environmentally controlled chambers (18–20°C) till their use. CM pupae of similar age were selected and sexed, and placed into the oviposition chambers. The adult moths were allowed to emerge, mate, and freely oviposit on fruits. Pupae were regularly monitored for adult emergence. If there was no adult emergence from a pupa within 3 days of release, then it was replaced by an adult (2–3 days old) of the same sex from the same pupal lot.

Experimental Design (Multiple-choice and No-choice Tests)

Multiple-choice oviposition preference tests and no-choice preference tests were conducted for both fruit maturity sets. In the no-choice tests, the oviposition chamber consisted of transparent plastic cups (1.0 L) internally lined with charcoal-colored fiberglass screen. In the multiple-choice tests, a cylindrical chamber (length = 0.81 m, diameter = 0.17 m) made of transparent fiberglass internally lined with fine aluminum mesh screening served as the oviposition chamber. In the multiple-choice tests, fruits of each cultivar were allocated to one of several locations at random in the oviposition chamber. During the study period, insects were maintained under laboratory conditions (temperature ~21–23°C, relative humidity ~70%, and photoperiod 11:10 h light:dark with an ~3 h period of dim light for oviposition induction). The year-wise description of these bioassays is as follows:

2006 Bioassays

Nine cultivars ('Arlet,' 'Golden Delicious,' 'Fuji,' 'Honeycrisp,' 'Pristine,' 'Delicious,' 'Stayman,' 'Sunrise,' and 'York Imperial') were evaluated in multiple-choice and no-choice tests during the first set (July) of experiments. In the second set of experiments (August), all cultivars (except 'Pristine' which was replaced by 'Gala') were again evaluated. Each treatment (cultivar) was replicated at least eight times in the multiple-choice tests and 10 times in the no-choice tests. All fruits were collected during 14–17 July and 12–15 August for the first set (19 July) and second set (25 August) of experiments, respectively. Fruits of all cultivars (except 'Arlet,' 'Pristine,' and 'Sunrise') were collected from non-insecticide sprayed trees at FREC, Biglerville. Fruits of 'Arlet,' 'Pristine,' and 'Sunrise' cultivars (collected from an orchard partially sprayed with common orchard pesticides for the

purpose of general maintenance) were received from the USDA Appalachian Fruit Research Station, Kearneysville, WV, USA. In both no-choice experiments (early and late), one pair of unmated male and female adults was placed per cup, and the number of deposited eggs was counted after 8 days. In multiple-choice tests, seven and six pairs of unmated adults were utilized in the early and late experiments, respectively. Total numbers of eggs were counted after 15 days (early), and 10 days (late). The position of each egg on fruit (stem, calyx, or lateral) was recorded.

2007 Bioassays

All 10 cultivars were evaluated in multiple-choice and no-choice tests conducted during the months of July and August. Each treatment (cultivar) had 8 and 10 replicates in the no-choice and multiple-choice tests, respectively. Fruits were collected during 12–15 July (early) 11–14 August (late). Fruits of all cultivars (except 'Arlet,' 'Pristine,' and 'Sunrise') were collected from non-insecticide sprayed trees at FREC, Biglerville. Fruits of 'Arlet,' 'Pristine,' and 'Sunrise' cultivars (collected from partially sprayed orchards) were received from the USDA Appalachian Fruit Research Station, Kearneysville, WV, USA for the early set of experiments, and for the late set of experiments from The Russell E. Larson Agricultural Research farm, Rock Springs, PA, USA. The early set of multiple-choice and no-choice tests were conducted on 17 July, while the late set of experiments were conducted on 16 August. In the no-choice tests, two pairs of unmated male and female adults were used in both sets of no-choice tests. In multiple-choice tests, three pairs of unmated male and female adults were used in both sets of multiple-choice tests. In all tests, the total numbers of deposited eggs on fruits were counted after 10 days. The position of eggs on the fruits was recorded as per the procedure used in the 2006 bioassays.

2008 Bioassays

Based on the results of bioassays conducted during the first two years, only three cultivars ('Golden Delicious,' 'Honeycrisp,' and 'York Imperial') were further evaluated in the third year. Fruits of similar size were collected from non-insecticide sprayed trees at FREC, Biglerville, and utilized the same day for both the no-choice and multiple-choice experiments. The study was replicated 15 and 8 times in the no-choice and multiple-choice tests, respectively. In the multiple-choice tests, two fruits of each treatment/cultivar were used in each replication. In the early experiment, fruits were collected on 22 July, and used in both types of tests on the same day, and observations on eggs were taken after 10 days. In the late set, fruits were collected on 28 August, and observations were recorded after 11 days in both no-choice and multiple-choice tests. Similar to previous years, the position of eggs on the fruits was recorded in 2008.

Statistical Analysis and Development of Oviposition-based Susceptibility Index

A general linear mixed-model analysis of variance (ANOVA) was used to analyze the data. In the analysis, two similar statistical models were used to address the study objectives. The first model (**Table 2**) was used to determine: (a) the oviposition preference of CM among apple cultivars; (b) differences in oviposition

TABLE 2 | Mix model ANOVA results of the sum of number of eggs per pair of codling moth and covariates (year, apple cultivar, and season [early and late]).

Covariates	df	F-value	P-value
Year	2	155.7609	0.000
Season	1	63.7488	0.000
Cultivar	9	49.2121	0.000
Year:Season	2	15.4542	0.000
Year:Cultivar	11	5.5816	0.000
Season:Cultivar	9	4.0555	0.000
Year:Season:Cultivar	9	2.9684	0.002
Residuals	778		

Response variable in this analysis is eggs per pair of codling moth per fruit.

All data and all years pooled together.

preferences during the early and late season (i.e., based on time of fruit collection: early [July] versus late season [August]); and (c) differences in oviposition preferences in the multiple-choice and no-choice tests. The second model, which includes the egg counts by position on the fruit, was used to determine CM oviposition-site preferences across different cultivars (**Table 3**). The mixed-model ANOVA analysis was performed using R software (ISBN 3-900051-07-0; R Development Core Team, 2005).

Oviposition preference based on the mean number of eggs (per pair of CM per fruit) was determined for each cultivar. The data sets were transformed (to achieve the assumptions of parametric analysis) by taking the natural log of the “eggs per pair” variable. Pairwise comparisons were done among all cultivars, and means were separated using Tukey’s honest significant differences *post hoc* test ($P < 0.05$) when ANOVA was significant (Zar, 1999).

TABLE 3 | Mix model ANOVA results of the sum of mean number of eggs per pair of codling moth and covariates (year, apple cultivar, position of eggs on apple [calyx, stem, and lateral sites], and season [early and late]).

Covariates	df	F-value	P-value
Site	2	523.0084	0.000
Cultivar	9	126.9134	0.000
Year	2	217.1037	0.000
Season	1	95.9429	0.000
Site:Cultivar	18	2.8307	0.000
Site:Year	4	4.1102	0.003
Cultivar:Year	11	10.6993	0.000
Site:Season	2	0.7818	0.458
Cultivar:Season	9	8.6888	0.000
Year:Season	2	17.0352	0.000
Site:Cultivar:Year	22	1.9029	0.007
Site:Cultivar:Season	18	1.4118	0.115
Site:Year:Season	4	8.6915	0.000
Cultivar:Year:Season	9	4.7172	0.000
Site:Cultivar:Year:Season	18	0.8713	0.615
Residuals	2334		

Response variable in this analysis is eggs per pair of codling moth per fruit.

All data and all years pooled together.

The CM oviposition susceptibility index (based on oviposition preferences of CM) for each cultivar was characterized as:

$$SI = \frac{1}{n} \sum_{i,j,k,t} SEPP(i, j, k, t) \quad (1)$$

Where, SI = Susceptibility index; $SEPP$ = Standardized mean eggs per pair of moths for an apple cultivar [i]; j = Time of fruit collection (early or late); k = year of observation; and t = type of tests (i.e., no-choice and multiple-choice tests).

Standardized mean eggs per pair of moths for an apple cultivar were determined as following:

$$SEPP[i] = \frac{EPP[i]}{EPP_{\max}[i]} \quad (2)$$

Where, $SEPP$ = Standardized mean eggs per pair of moths for an apple cultivar [i]; EPP [i] = Mean number of eggs per pair of moths on an apple cultivar [i]; and EPP_{\max} [i] = Maximum number of eggs per pair of moths on an apple cultivar [i].

Mean total number of eggs per pair of moths (EPP) on a cultivar was calculated by the following equation:

$$EPP[i] = EPP_C[i] + EPP_S[i] + EPP_L[i] \quad (3)$$

Where EPP [i] = Mean number of eggs per pair of moths on an apple cultivar [i]; $EPP_C[i]$ = Mean number of eggs per pair of moths on calyx side of an apple cultivar [i]; $EPP_S[i]$ = Mean number of eggs per pair of moths on stem side of an apple cultivar [i]; and $EPP_L[i]$ = Mean number of eggs per pair of moths on lateral side of an apple cultivar [i].

Oviposition susceptibility index of all cultivars was compared and means were separated using Fisher’s protected least significant differences *post hoc* test ($P < 0.05$) when ANOVA was significant (Zar, 1999). The analysis was performed using SPSS-13 statistical software (SPSS Inc., Chicago, IL, USA).

RESULTS

2006 Early Season (July)

In the multiple-choice test, on the calyx site of fruits (**Table 4**), CM females laid significantly higher numbers of eggs on ‘York Imperial,’ ‘Golden Delicious,’ and ‘Delicious’ than other cultivars ($P < 0.05$; **Figure 1A**). In contrast, the lowest numbers of eggs were laid on the ‘Honeycrisp’ cultivar ($P < 0.05$; **Figure 1A**). On the stem site of fruits, CM females laid significantly more eggs on ‘Stayman,’ ‘York Imperial,’ ‘Golden Delicious,’ and ‘Delicious’ than all other cultivars ($P < 0.05$; **Figure 1B**). On the lateral site of fruits, CM females preferred ‘York Imperial,’ ‘Golden Delicious,’ and ‘Delicious’ than the cultivars ‘Pristine,’ ‘Honeycrisp,’ ‘Arlet,’ and ‘Sunrise’ ($P < 0.05$; **Figure 1C**).

In the no-choice test, on the calyx site (**Table 4**), the female moths significantly preferred to oviposit on ‘Golden Delicious’ ($P = 0.009$), ‘Fuji’ ($P = 0.005$), and ‘Delicious’ ($P = 0.008$), compared to ‘Pristine’ (**Figure 1D**). On the stem site, ‘Golden Delicious’ was significantly more preferred than

TABLE 4 | Statistical details of oviposition site preferences of codling moth across different cultivars.

Year	Season/Time	Test type	df*	Oviposition Sites on fruits					
				Calyx		Stem		Lateral	
				F-value	P-value	F-value	P-value	F-value	P-value
2006	Early	Multiple-choice	8	9.18	< 0.001	5.92	< 0.001	8.56	< 0.001
2006	Early	No-choice	8	3.51	0.002	4.86	< 0.001	2.88	0.007
2006	Late	Multiple-choice	8	3.67	0.001	8.26	< 0.001	9.18	< 0.001
2006	Late	No-choice	8	2.46	0.019	6.03	< 0.001	6.57	< 0.001
2007	Early	Multiple-choice	9	3.43	0.002	15.64	< 0.001	17.12	< 0.001
2007	Early	No-choice	9	5.38	< 0.001	11.04	< 0.001	16.57	< 0.001
2007	Late	Multiple-choice	9	2.94	0.005	10.55	< 0.001	12.14	< 0.001
2007	Late	No-choice	9	2.16	0.032	12.37	< 0.001	10.76	< 0.001
2008	Early	Multiple-choice	2	8.26	0.002	15.97	< 0.001	9.23	0.001
2008	Early	No-choice	2	0.76	0.474	36.87	< 0.001	16.43	< 0.001
2008	Late	Multiple-choice	2	7.12	0.004	14.44	< 0.001	19.66	< 0.001
2008	Late	No-choice	2	16.91	< 0.001	29.67	< 0.001	34.68	< 0.001

*df values are same across different oviposition sites.

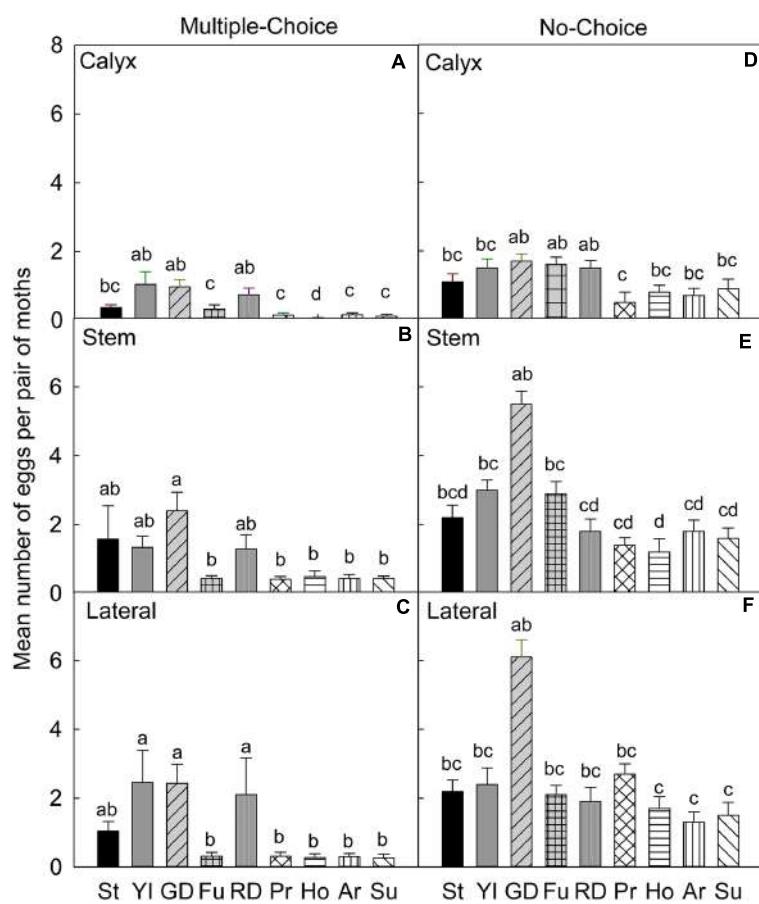


FIGURE 1 | Relative susceptibility of different apple cultivars for oviposition of codling moth during July 2006 (early season/Set 1). Mean number of eggs per pair of moths per fruit on calyx, stem, and lateral sides of fruits of different cultivars are shown in multiple-choice tests (**A–C**) and no-choice tests (**D–F**). St, Stayman; YI, York Imperial; GD, Golden Delicious; Fu, Fuji; RD, Delicious; Pr, Pristine; Ho, Honeycrisp; Ar, Arlet; Su, Sunrise. N = 8 for all the multiple-choice tests, and N = 10 for all the no-choice tests. Each bar represents standard error of mean. Different letters over bars indicate significant difference (P < 0.05).

'Delicious' ($P = 0.014$), 'Pristine' ($P = 0.013$), 'Honeycrisp' ($P < 0.001$), 'Arlet' ($P = 0.047$), and 'Sunrise' ($P = 0.023$; **Figure 1E**). On the lateral site, 'Golden Delicious' was significantly more preferred than 'Honeycrisp' ($P = 0.049$), 'Arlet' ($P = 0.004$), and 'Sunrise' ($P = 0.008$; **Figure 1F**); however, the total number of eggs on 'Golden Delicious' was not significantly different from that of all other cultivars ($P > 0.05$; **Figure 1F**).

2006 Late Season (August)

In the multiple-choice test, on the calyx site (**Table 4**), the oviposition preference of CM was not significantly different for all cultivars ($P > 0.05$), except for 'Fuji', when compared to 'Delicious' ($P = 0.042$), and 'Sunrise' ($P = 0.015$; **Figure 2A**). On the stem (**Figure 2B**) and lateral (**Figure 2C**) sites of fruits, 'Stayman,' 'Golden Delicious,' 'Fuji,' 'Honeycrisp,' and 'Arlet' rather than 'Delicious,' 'Gala,' and 'Sunrise' were the significantly preferred cultivars ($P < 0.05$).

In the no-choice test, on the calyx site (**Table 4**), CM deposited more eggs on 'Golden Delicious' than on 'Gala' ($P = 0.011$), otherwise, there was no significant differences between 'Golden Delicious' and all other cultivars ($P > 0.05$; **Figure 2D**). On the stem (**Figure 2E**) and lateral (**Figure 2F**) sites of fruits, CM deposited more eggs on 'Golden Delicious' and 'Fuji' than on 'Gala,' 'Honeycrisp,' and 'Sunrise' ($P < 0.05$).

2007 Early Season (July)

In the multiple-choice test of early season 2007, on the calyx site (**Table 4**), CM significantly preferred 'Golden Delicious' for oviposition over 'Pristine' ($P = 0.006$), 'Arlet' ($P = 0.032$), 'Sunrise' ($P = 0.006$), and 'Gala' ($P = 0.032$; **Figure 3A**). On the stem site of fruits, 'Golden Delicious' was again the significantly preferred cultivar for oviposition over other cultivars, *viz.*, 'Pristine' ($P < 0.001$), 'Honeycrisp' ($P < 0.001$), 'Arlet' ($P < 0.001$), 'Sunrise' ($P < 0.001$), and 'Gala' ($P < 0.001$; **Figure 3B**). However, the preference for 'Golden Delicious' was similar to 'Stayman' ($P = 0.092$), 'York Imperial' ($P = 0.457$),

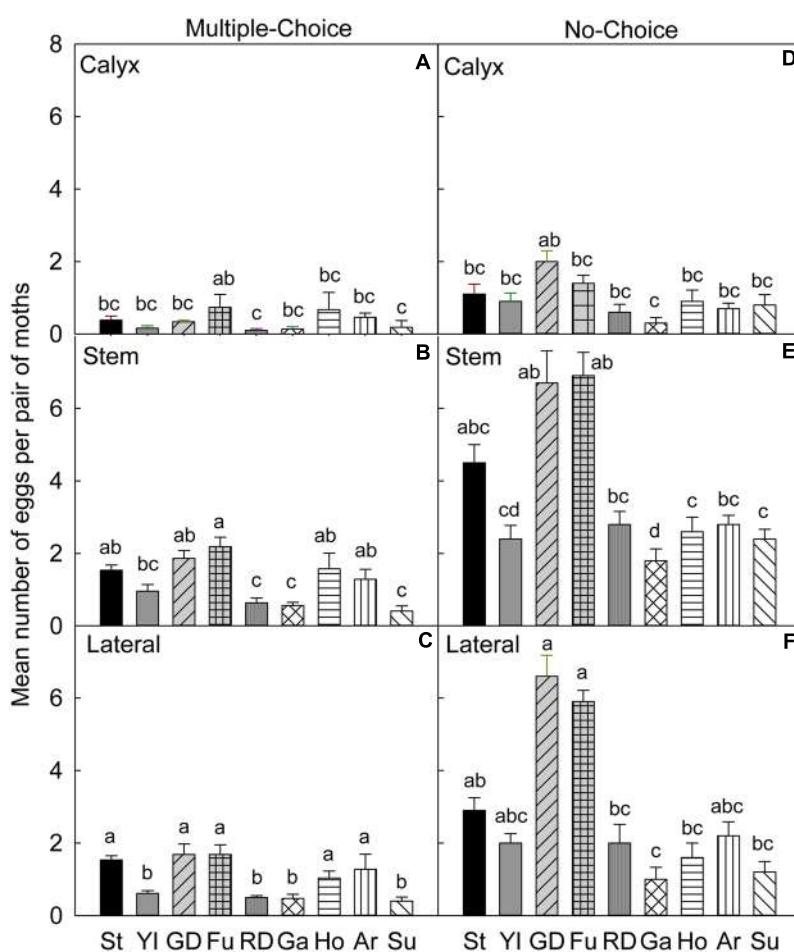
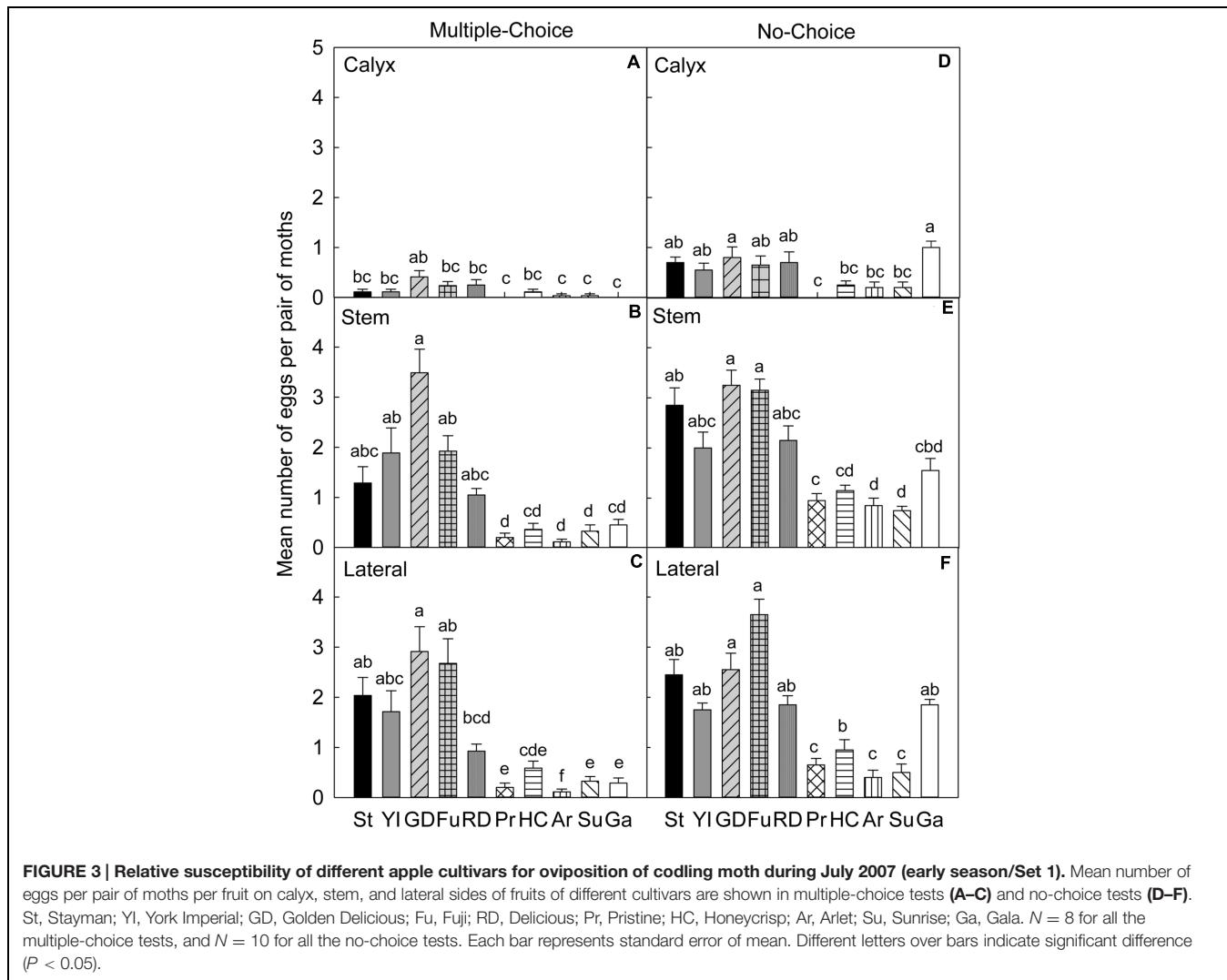


FIGURE 2 | Relative susceptibility of different apple cultivars for oviposition of codling moth during August 2006 (late season/Set 2). Mean number of eggs per pair of moths per fruit on calyx, stem, and lateral sides of fruits of different cultivars are shown in multiple-choice tests (A–C) and no-choice tests (D–F). St, Stayman; YI, York Imperial; GD, Golden Delicious; Fu, Fuji; RD, Delicious; Ho, Honeycrisp; Ar, Arlet; Su, Sunrise; Ga, Gala. $N = 8$ for all the multiple-choice tests, and $N = 10$ for all the no-choice tests. Each bar represents standard error of mean. Different letters over bars indicate significant difference ($P < 0.05$).



'Fuji' ($P = 0.777$), and 'Delicious' ($P = 0.064$; **Figure 3B**). On the lateral site, 'Golden Delicious' was significantly more preferred than all other cultivars ($P < 0.05$), except 'Stayman' ($P = 0.996$), 'York Imperial' ($P = 0.777$), and 'Delicious' ($P = 0.109$; **Figure 3C**). In contrast, 'Arlet' was the least preferred cultivar for oviposition on the lateral site of fruits ($P < 0.05$; **Figure 3C**).

In the no-choice test (July 2007), on the calyx site of fruits (**Table 4**), CM deposited higher numbers of eggs on 'Golden Delicious' and 'Gala' than on 'Pristine,' 'Honeycrisp,' 'Arlet,' and 'Sunrise' ($P < 0.05$; **Figure 3D**). On the stem (**Figure 3E**) and lateral (**Figure 3F**) sites of fruits, 'Golden Delicious' and 'Fuji' received the highest number of eggs over 'Pristine,' 'Honeycrisp,' 'Arlet,' and 'Sunrise' ($P < 0.05$).

2007 Late Season (August)

In the multiple-choice test conducted during the late season study of 2007, on the calyx site (**Table 4**), 'Golden Delicious' was more preferred for oviposition than 'York Imperial' ($P = 0.036$) and 'Sunrise' ($P = 0.022$; **Figure 4A**). On the stem site of fruits, the

moths again preferred 'Golden Delicious' for oviposition over all other cultivars ($P < 0.05$), except 'Stayman' ($P = 0.978$) and 'Fuji' ($P = 0.563$; **Figure 4B**). In contrast, 'Pristine' was the least preferred cultivar ($P < 0.05$; **Figure 4B**). On the lateral site, 'Stayman,' 'Golden Delicious,' and 'Fuji' were the most preferred cultivars for oviposition ($P < 0.05$), except for 'York Imperial' and 'Delicious' ($P > 0.05$; **Figure 4C**).

In the no-choice test, on the calyx site (**Table 4**), CM deposited less eggs on 'Pristine' than 'Gala' ($P = 0.047$), however, such lower preference for 'Pristine' was not significantly different from all other cultivars ($P > 0.05$; **Figure 4D**). On the stem (**Figure 4E**) and lateral (**Figure 4F**) sites of fruits, CM showed less preference for 'Pristine' ($P < 0.05$) than all other cultivars, except for 'Honeycrisp,' 'Arlet,' and 'Sunrise' ($P > 0.05$).

2008 Early Season (July)

In the multiple-choice test (**Table 4**), on the calyx (**Figure 5A**) and stem (**Figure 5B**) sites of fruits, 'Golden Delicious' was the most preferred cultivar for oviposition over the other two cultivars

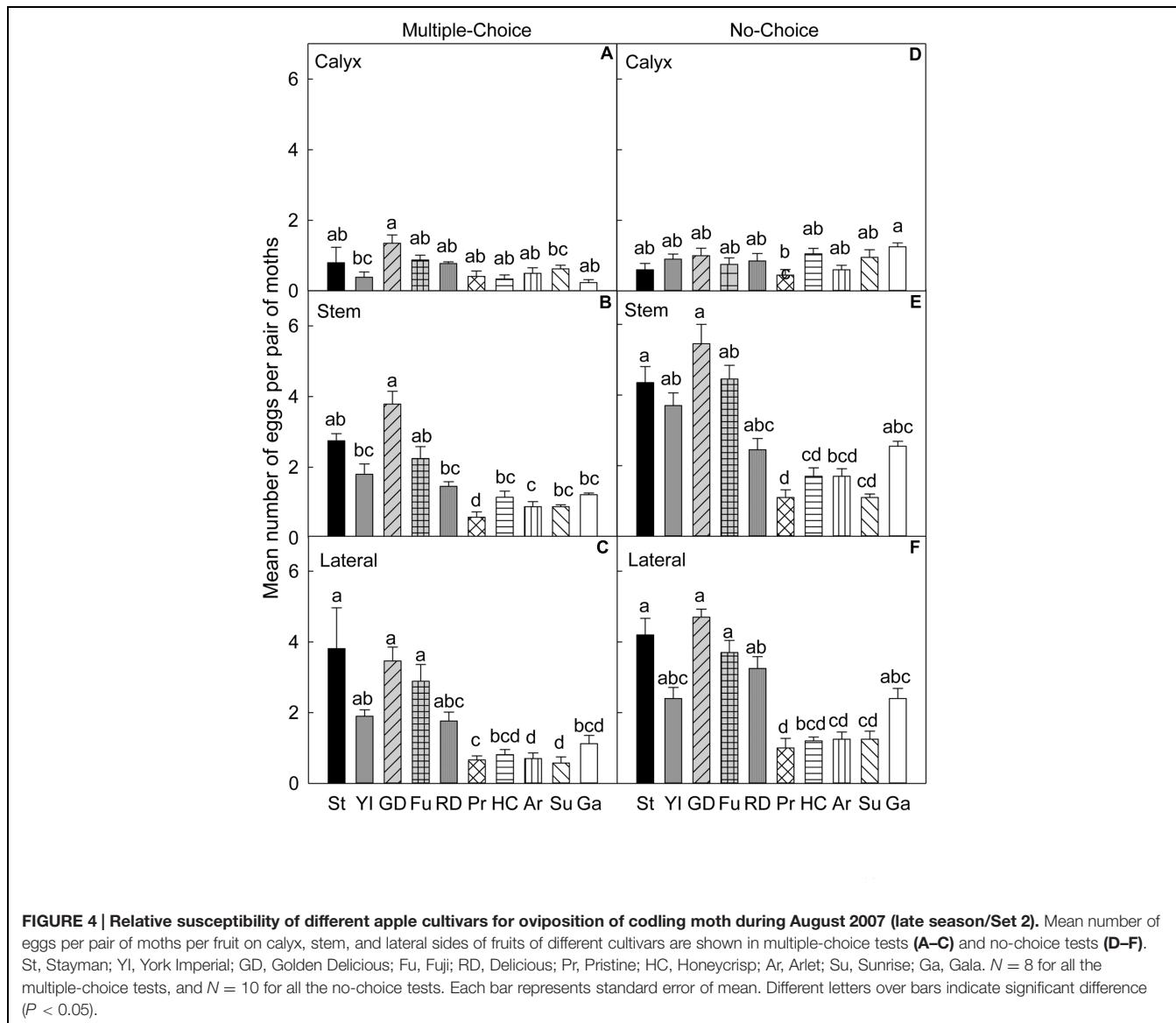


FIGURE 4 | Relative susceptibility of different apple cultivars for oviposition of codling moth during August 2007 (late season/Set 2). Mean number of eggs per pair of moths per fruit on calyx, stem, and lateral sides of fruits of different cultivars are shown in multiple-choice tests (**A–C**) and no-choice tests (**D–F**). St, Stayman; YI, York Imperial; GD, Golden Delicious; Fu, Fuji; RD, Delicious; Pr, Pristine; HC, Honeycrisp; Ar, Arlet; Su, Sunrise; Ga, Gala. $N = 8$ for all the multiple-choice tests, and $N = 10$ for all the no-choice tests. Each bar represents standard error of mean. Different letters over bars indicate significant difference ($P < 0.05$).

($P < 0.05$). On the lateral site, ‘Honeycrisp’ was less preferred for oviposition than ‘Golden Delicious’ ($P = 0.001$; **Figure 5C**).

In the no-choice test (July 2008), on the calyx site (**Table 4**), the oviposition preference of CM did not differ significantly across all the cultivars ($P > 0.05$; **Figure 5D**). On stem (**Figure 5E**) and lateral sites of fruits (**Figure 5F**), CM deposited more eggs on ‘Golden Delicious’ over ‘Honeycrisp’ and ‘York Imperial’ ($P < 0.05$).

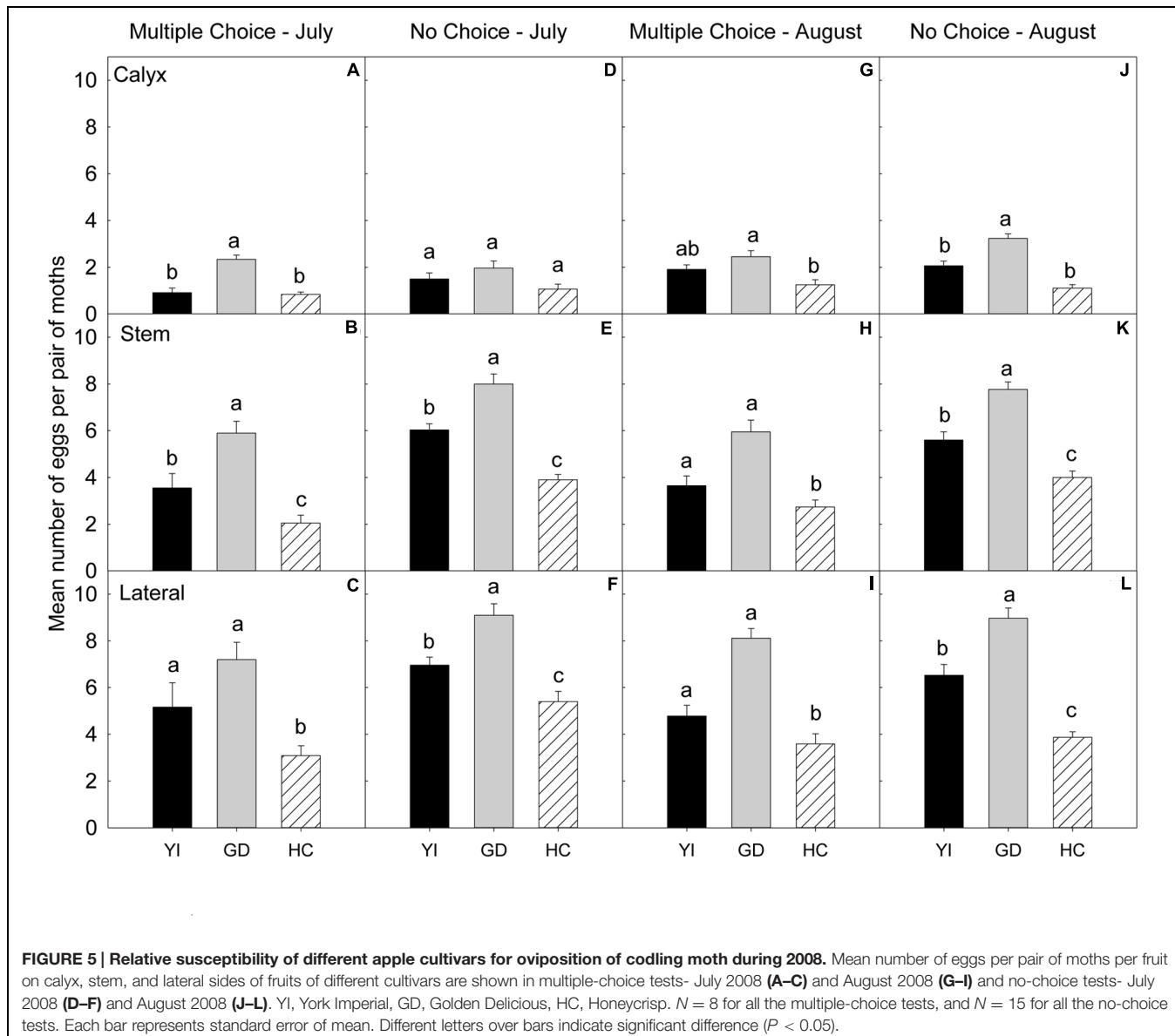
2008 Late Season (August)

In the multiple-choice test (**Table 4**), on the calyx site, ‘Golden Delicious’ was the most preferred cultivar over ‘Honeycrisp’ ($P = 0.004$), but it was not more preferred over ‘York Imperial’ ($P = 0.475$; **Figure 5G**). On stem (**Figure 5H**) and lateral (**Figure 5I**) sites of fruits, ‘Honeycrisp’ was the least preferred cultivar when compared to ‘Golden Delicious’ and ‘York Imperial’ ($P < 0.05$).

In the no-choice test (August 2008), on calyx (**Figure 5J**), stem (**Figure 5K**), and lateral (**Figure 5L**) sites of fruits, ‘Golden Delicious’ was the most preferred cultivar for oviposition over that of ‘Honeycrisp’ and ‘York Imperial’ ($P < 0.05$).

Interaction Effects of Apple Cultivar, Choice (Type of Test), Season (Early or Late), Study Year and Oviposition Sites on CM Oviposition

All covariates (cultivar, season [early or late], and study year) had a significant influence on the oviposition of CM on different apple cultivars ($P < 0.001$; **Table 2**). All types of interactions presented in **Table 2** had a significant impact on the oviposition preference of CM ($P < 0.05$). Oviposition sites (i.e., calyx, stem, and lateral) on fruits had a highly significant influence on CM oviposition ($P < 0.001$; **Table 3**). All the



interactions of oviposition sites with other covariates, except Site:Season ($P = 0.458$), Site:Cultivar:Season ($P = 0.115$) and Site:Cultivar:Year:Season ($P = 0.615$), displayed a significant interactive impact on the oviposition of CM ($P < 0.05$; Table 3).

Susceptibility Index for Different Apple Cultivars for CM Oviposition

In terms of the CM oviposition susceptibility index (on a scale of 0 – 1, where, '0' = the least susceptible and '1' = the most susceptible), 'Golden Delicious' had a significantly higher susceptibility index than 'Stayman' ($P = 0.002$), 'York Imperial' ($P = 0.002$), 'Fuji' ($P = 0.011$), 'Delicious' ($P < 0.001$), 'Pristine' ($P < 0.001$), 'Honeycrisp' ($P < 0.001$), 'Arlet' ($P < 0.001$), 'Sunrise' ($P < 0.001$), and 'Gala' ($P < 0.001$; Figure 6). In contrast, 'Pristine,' 'Honeycrisp,' 'Arlet,' and 'Sunrise' were noticeably less susceptible to oviposition by CM ($P < 0.05$; Figure 6).

DISCUSSION

In the majority of bioassays conducted across different years, CM females preferred to oviposit on 'Golden Delicious,' 'Fuji,' 'Delicious,' 'Stayman,' and 'York Imperial' over other cultivars, *viz.*, 'Pristine,' 'Honeycrisp,' 'Arlet,' 'Sunrise,' and 'Gala.' Different volatile fruit-coat constituents likely affect the ovipositional preferences by CM for apple fruits. For instance, the production of the sesquiterpene α -farnesene, an ovipositional stimulant for female CM, and an important constituent in the outer skin of apple fruits, varies greatly across different cultivars and changes as fruit mature (Wearing and Hutchins, 1973; Sutherland et al., 1977). Such variation could be an important factor in helping explain the differential cultivar ovipositional preferences of CM found in this study. However, such hypothesis needs further evaluation.

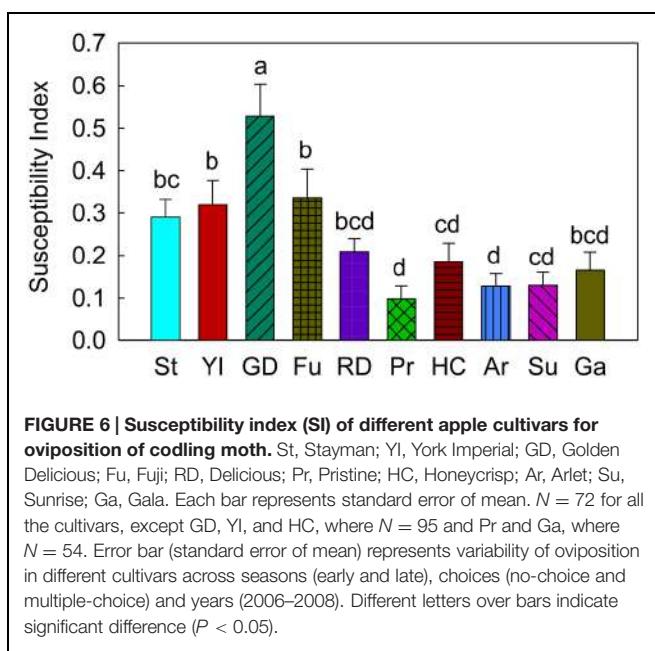


FIGURE 6 | Susceptibility index (SI) of different apple cultivars for oviposition of codling moth. St, Stayman; YI, York Imperial; GD, Golden Delicious; Fu, Fuji; RD, Delicious; Pr, Pristine; HC, Honeycrisp; Ar, Arlet; Su, Sunrise; Ga, Gala. Each bar represents standard error of mean. $N = 72$ for all the cultivars, except GD, YI, and HC, where $N = 95$ and Pr and Ga, where $N = 54$. Error bar (standard error of mean) represents variability of oviposition in different cultivars across seasons (early and late), choices (no-choice and multiple-choice) and years (2006–2008). Different letters over bars indicate significant difference ($P < 0.05$).

Results from the no-choice and multiple-choice tests across different years in this study showed that CM females deposited significantly more eggs on ‘Golden Delicious’ over other cultivars, *viz.*, ‘Pristine,’ ‘Honeycrisp,’ ‘Arlet,’ and ‘Sunrise.’ Similar trends in the ovipositional preferences of a closely related tortricid pest, oriental fruit moth [*Grapholita molesta* (Busck)] for these different apple cultivars are also reported (Joshi et al., 2007; Myers et al., 2007). In the one study by Joshi et al. (2007), the oriental fruit moth preferred ‘Golden Delicious’ for oviposition compared to the cultivars ‘Pristine,’ ‘Arlet,’ and ‘Sunrise.’ Based on the ovipositional preferences exhibited by CM, these preferred cultivars are highly likely more susceptible to CM infestations, especially if these laboratory results reflect field behaviors. The choice of a preferred suitable substrate or host for oviposition plays a key role in the survival and completion of different life stages of lepidopteran insects (Chew and Robbins, 1984; Renwick, 1989). Similarly, in the case of CM, judicious selection of an appropriate host for depositing eggs might play a key role in determining the initial fate of a neonate larva that feeds internally in fruits of the selected host(s). Upon hatching, the larva enters the fruit, and remains inside the fruit till the pre-pupal stage. The larva developing inside the fruit is usually incapable of moving from one fruit to other, so the oviposition preferences of female CM most likely determine larval survival by selecting the most suitable host/cultivar. In oviposition preference studies of a closely related fruit pest species (*i.e.*, oriental fruit moth), Myers et al. (2006a) found higher percentages of larval entry in fruits of preferred (in terms of oviposition) cultivars like ‘Golden Delicious’ and ‘Delicious’ during their early and late season experiments. Therefore, it is likely that the oviposition preference of CM for these different apple cultivars might be related to larval survival. The percent larval survival on the most preferred cultivar (*i.e.*, ‘Golden Delicious’) was higher than one of the less preferred cultivars (*i.e.*, ‘Arlet’) when neonate

larvae were individually exposed to these different cultivars (NKJ et al., unpublished data). Such preferences for ‘Golden Delicious’ were also revealed in the present oviposition bioassays, as ‘Golden Delicious’ was the preferred cultivar over ‘Arlet,’ ‘Sunrise,’ and ‘Pristine’ cultivars. In a related study on relative susceptibility of different apple cultivars to various arthropod pests that was conducted in an orchard, Hogmire and Miller (2005) reported ‘Golden Delicious’ as a highly susceptible cultivar to CM infestations versus other cultivars such as ‘Pristine,’ ‘Honeycrisp,’ ‘Arlet,’ and ‘Sunrise.’

Early maturing varieties have been considered less susceptible to CM infestations (Isely, 1943). In the present study, for the majority of oviposition bioassays, CM least preferred to oviposit on early maturing cultivars, *viz.*, ‘Pristine,’ ‘Honeycrisp,’ ‘Arlet,’ ‘Sunrise,’ and ‘Gala’ as compared to later maturing cultivars such as ‘Stayman,’ ‘York Imperial,’ ‘Golden Delicious,’ ‘Fuji,’ and ‘Delicious.’ Such preference could be related to the presence or emission of fruit volatiles from these cultivars, since fruit volatiles are known to play a crucial role in guiding female moths to oviposit on or near fruits (Wildbolz, 1958; Lombarkia and Derridj, 2002; Reed and Landolt, 2002). The oviposition preferences of CM across different cultivars may also vary in relation to the time during the season that an apple matures and to its fruit maturity at any specified time during the season, because the release of volatiles from fruits increases from early to late season (Sutherland et al., 1977; Mattheis et al., 1991). In general, more eggs per pair of CM adults per fruit were observed in bioassays conducted with fruits collected later in the season (August) than those collected earlier in the season (July). During the early stages of fruit development, fruits are reported to release only a few ester type compounds as compared to ripening and mature fruits (late season), which are reported to release many ester type compounds plus a few terpenoids (Bengtsson et al., 2001). Such changes in volatile emissions may be the reason for the variations in the ovipositional preferences of CM for fruits collected in July (early season) and August (late season). During the early stages of fruit development, CM females are reported to deposit more eggs on neighboring leaves (*i.e.*, shoot and spur) around fruits and fruit clusters (Wildbolz, 1958; Blomfield et al., 1997) than directly on fruits (NKJ et al. unpublished data), while during the fruit maturation and ripening period, more eggs are deposited directly on fruits compared to the early stages of fruit development (Summerland and Steiner, 1943). This type of oviposition pattern/preference may be helpful in increasing the likelihood of larval survival upon hatching. Sutherland et al. (1977) found that the production of α -farnesene (which is known to influence the oviposition behavior of CM) increases as fruit maturity increases. Consequently, CM females deposit more eggs on fruits as fruit maturity increases during the season. The variability among different apple cultivars in the production of the oviposition stimulant α -farnesene could be a major factor affecting the ovipositional preference of CM for different apple cultivars during the early and latter part of the growing season. Other strong possibilities causing such early and late season variation in the oviposition preferences of CM could be the differential developmental stages (maturity level) and other characteristics (such as chemical composition of fruit-coat, fruit

color, etc.) of fruits of these different cultivars during the two different time periods of a season.

Apart from the chemical constituents of the fruit coat, physical characteristics of the apple fruit surface may vary from one cultivar to other, as well as within the calyx, stem and lateral sides of fruit of different cultivars (Belding et al., 1998; Verardo et al., 2003). Such microtopographic properties can be categorized on the basis of roughness and smoothness of host surface, and play a crucial role in the attachment ability of CM (Al Bitar et al., 2010), and may influence its oviposition behavior, particularly the oviposition site selection (Al Bitar et al., 2014). Friction forces, which affect the attachment ability of CM eggs to these different types of surfaces, had been reported to be higher on oviposition substrates with smooth surfaces (Al Bitar et al., 2009, 2010), and could be main factors behind the CM oviposition preferences for the smooth substrates (e.g., fruits) over rough surfaces (e.g., leaves with trichomes). Variation in the CM oviposition on calyx, stem and lateral sides of fruits of apple cultivars in this study could be due to differences in fruit surface properties such as amorphous wax layer (comprised of microcracks and epicuticular wax crystals) favoring CM egg adhesion to oviposition substrates. Composition and abundance of microcracks (Al Bitar et al., 2014) and epicuticular wax (Belding et al., 1998) on fruit surfaces vary across different apple cultivars. CM egg adhesion to different oviposition substrates of the fruit of different cultivars (for instance, 'Golden Delicious,' 'Elstar,' 'Jonica,' 'Boskoop,' 'Topaz') had been reported to vary within upper (stem), middle (lateral), and lower (calyx) sections of fruits (Al Bitar et al., 2014). Regardless of test type, year and season, in general, we recorded higher number of eggs on stem and lateral sites compared to calyx end of fruit. It could be due to higher abundance of microcracks as well as stronger bonding between CM eggs and fruit surfaces on stem and lateral fruit surfaces than calyx end (Al Bitar et al., 2014).

Cultivar, season (early or late), study year and oviposition sites (i.e., calyx, stem, and lateral) on fruits had a significant influence on the oviposition of CM on different apple cultivars. Covariate interactions (except, Site:Season, Site:Cultivar:Season, and Site:Cultivar:Year:Season) were also significant. Oviposition-sites on fruits may vary from one cultivar to another. In the multiple-choice and no-choice tests, CM deposited more eggs on lateral and stem sites than on the calyx site of fruits. Such patterns of egg deposition could be due to the physical characteristics of the apple fruit surface as discussed earlier or due to the 'vertical' placement of fruits, as in all these tests, fruits were vertically placed in oviposition chambers. In contrast, oriental fruit moth adult females preferred to oviposit on the calyx and stem sites of apple fruit, and their oviposition site preferences are also reported to vary between different apple cultivars (Myers et al., 2006b).

Susceptibility to various pest infestations may vary among cultivated varieties as well as wild varieties (e.g., crab apples). In the past, susceptibility of apple cultivars/germplasms to different arthropod pests has been studied using several methods, such as their impact on pest developmental rate and pest survival rate (Mackenzie and Cummins, 1982; Myers et al., 2006b), damage

(in terms of fruit injury) caused by pests (Dean and Chapman, 1973; Goonewardene et al., 1979; Straub, 2003; Hogmire and Miller, 2005) and the occurrence of pests (Goonewardene et al., 1976; Straub, 2003; Hogmire and Miller, 2005; Myers et al., 2007). However, using a standardized oviposition-based susceptibility index of apple cultivars as developed in this study reveals important information about the relative susceptibility of cultivars when evaluated under different seasons and times during the season. The newly developed CM oviposition susceptibility index for apple cultivars showed that susceptibility is linked to the oviposition preferences of CM, as female moths least preferred 'Pristine,' 'Sunrise,' 'Arlet,' and 'Honeycrisp' (less susceptible cultivars) for oviposition than 'Golden Delicious' (highly susceptible cultivar). Similarly, Hogmire and Miller (2005) reported that 'Golden Delicious' was significantly more susceptible than 'Pristine,' 'Honeycrisp,' 'Arlet,' and 'Sunrise' to injury by CM in the field environment. Straub (2003) studied the relative susceptibility of some new apple cultivars in New York to different orchard pests, and found that cultivars such as 'Sunrise,' 'Pristine,' 'McIntosh' (Pioneer), and 'Honeycrisp' were comparatively resistant to CM larval damage compared to 'Golden Delicious'. The CM oviposition susceptibility index could be useful to researchers/research extension workers and fruit growers in IPM decision-making in apple orchards.

To summarize, CM preferred to oviposit on later maturing cultivars 'Golden Delicious,' 'Stayman,' 'York Imperial,' 'Fuji,' and 'Delicious' (preferred cultivars) than early maturing cultivars, *viz.*, 'Pristine,' 'Honeycrisp,' 'Arlet,' 'Sunrise,' and 'Gala' (less preferred cultivars) in the majority of the multiple-choice tests. In the no-choice tests, CM deposited more eggs on these preferred cultivars than the less preferred cultivars. Regardless of choice test type and season, CM deposited significantly more eggs on 'Golden Delicious' over other cultivars, *viz.*, 'Pristine,' 'Honeycrisp,' 'Arlet,' and 'Sunrise.' In both types of tests, more eggs were laid on lateral and stem sites than the calyx site of fruits across different cultivars. In terms of a CM oviposition susceptibility index, 'Golden Delicious' was the most susceptible cultivar to oviposition, while 'Pristine,' 'Honeycrisp,' 'Arlet,' and 'Sunrise' were least susceptible. From an integrated pest management perspective, the newly developed susceptibility index can assist fruit growers and consultants select the most appropriate cultivar(s) for monitoring and detecting the initial signs of fruit injury from this pest. For instance, 'Golden Delicious' is the most preferred cultivar for oviposition, therefore it should be the cultivar of choice for monitoring CM injury in mixed-cultivar planted orchards. If it is not present in a block/orchard, then the next preferred cultivar for oviposition should be selected for examining CM injury or oviposition. In addition, results of these studies would be helpful in breeding programs, particularly in developing CM resistant apple varieties. As previously discussed, oviposition by CM is likely stimulated by fruit volatiles, and variations in the production and release of these volatiles from different apple cultivars may result in different oviposition preferences. Further investigations are needed to understand the biochemical as well as physical aspects of fruits and other factors involved in determining apple cultivar susceptibility for CM oviposition.

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REFERENCES

- Al Bitar, L., Gorb, S. N., Zebitz, C. P. W., and Voigt, D. (2014). Egg adhesion of the codling moth *Cydia pomonella* L. (Lepidoptera, Tortricidae) to various substrates: II. Fruit surfaces of different apple cultivars. *Arthropod Plant Interact.* 8, 57–77. doi: 10.1007/s11829-013-9288-6
- Al Bitar, L., Voigt, D., Zebitz, C. P. W., and Gorb, S. N. (2009). Tarsal morphology and attachment ability of the codling moth *Cydia pomonella* L. (Lepidoptera, Tortricidae) to smooth surfaces. *J. Insect Physiol.* 55, 1029–1038. doi: 10.1016/j.jinsphys.2009.07.008
- Al Bitar, L., Voigt, D., Zebitz, C. P. W., and Gorb, S. N. (2010). Attachment ability of the codling moth *Cydia pomonella* L. to rough surfaces. *J. Insect Physiol.* 56, 1966–1972. doi: 10.1016/j.jinsphys.2010.08.021
- Barnes, M. M. (1991). “Codling moth occurrence, host race formation, and damage,” in *Tortricid Pests: Their Biology, Natural Enemies and Control. World Crop Pests*, Vol. 5, eds L. P. S. van der Geest and H. H. Evenhuis (Amsterdam: Elsevier), 313–328.
- Belding, R. D., Blankenship, S. M., Young, E., and Leidy, R. B. (1998). Composition and variability of epicuticular waxes in apple cultivars. *J. Am. Soc. Hortic. Sci.* 123, 348–356.
- Bengtsson, M., Backmann, A. C., Liblikas, I., Ramirez, M. I., Karlsson, A. K. B., Ansebo, L., et al. (2001). Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J. Agric. Food Chem.* 49, 3736–3741. doi: 10.1021/jf0100548
- Bezemer, T. M., and Mills, N. J. (2001). Walnut development affects chemical composition and codling moth performance. *Agric. For. Entomol.* 3, 191–199. doi: 10.1046/j.1461-9555.2001.00101.x
- Blomfield, T. L., Pringle, K. L., and Sadie, A. (1997). Field observation on oviposition of codling moth, *Cydia pomonella* (Linnaeus) (Lepidoptera: olethreutidae), in an unsprayed apple orchard in South Africa. *Afr. Entomol.* 5, 319–336.
- Chew, F. S., and Robbins, R. K. (1984). Egg-laying in butterflies. *Symp. R. Entomol. Soc. Lond.* 11, 65–79.
- Dean, R. W. (1989). Biology of the codling moth in Hudson Valley orchards. *Search Agric.* 36, 1–28.
- Dean, R. W., and Chapman, P. J. (1973). Bionomics of the apple maggot in eastern New York. *Search Agric.* 3, 1–58.
- Gehring, R. D., and Madsen, H. F. (1963). Some aspects of the mating and oviposition behavior of the codling moth, *carpocapsa pomonella*. *J. Econ. Entomol.* 56, 140–143. doi: 10.1093/jee/56.2.140
- Geier, P. W. (1963). The life history of codling moth, *Cydia pomonella* (L.) (Lepidoptera: Tortricidae), in the Australian Capital Territory. *Aust. J. Zool.* 11, 323–367. doi: 10.1071/ZO9630323
- Goonewardene, H. F., Kwolek, W. F., Mouzin, T. E., and Williams, E. B. (1979). A ‘no choice’ study for evaluating resistance of apple fruits to four insect pests. *HortScience* 14, 165–166.
- Goonewardene, H. F., Williams, E. B., Kwolek, W. F., and McCabe, L. D. (1976). Resistance to European red mite, *Panonychus ulmi* (Koch), in apple. *J. Am. Soc. Hortic. Sci.* 101, 532–537.
- Hern, A., and Dorn, S. (1999). Sexual dimorphism in the orientation of adult *Cydia pomonella* in response to α -farnesene. *Entomol. Exp. Appl.* 92, 63–72. doi: 10.1046/j.1570-7458.1999.00525.x
- Hogmire, H. W., and Miller, S. S. (2005). Relative susceptibility of new apple cultivars to arthropod pests. *HortScience* 40, 2071–2075.
- Howell, J. F., Hutt, R. B., and Hill, W. B. (1978). Codling moth: mating behavior in the laboratory. *Ann. Entomol. Soc. Am.* 71, 891–895. doi: 10.1093/esa/71.6.891
- Iseley, D. (1943). Early maturing varieties in codling moth control. *J. Econ. Entomol.* 36, 757–759. doi: 10.1093/jee/36.5.757
- Jackson, D. M. (1979). Codling moth egg distribution on unmanaged apple tree. *Ann. Entomol. Soc. Am.* 72, 361–368. doi: 10.1093/esa/72.3.361
- Joshi, N. K. (2011). *Codling Moth, Cydia pomonella (L.) Ecology and Phenology Model Development for Pennsylvania Apple Orchards*. Ph.D. dissertation, Pennsylvania State University, University Park, PA.
- Joshi, N. K., Hull, L. A., Myers, C. T., Krawczyk, G., and Rajotte, E. G. (2007). “Oviposition preference of Oriental fruit moth [*Grapholita molesta* (Busck), Lepidoptera: tortricidae] for apple cultivars,” in *Proceedings of the 16th International Plant Protection Congress*, Glasgow, 308–309.
- Joshi, N. K., Hull, L. A., Myers, C. T., Rajotte, E. G., and Krawczyk, G. (2009). Studies on oviposition of codling moth, *Cydia pomonella* (L.) in apple orchards of Pennsylvania. *Pa. Fruit News* 89, 39–45.
- Lombarkia, N., and Derridj, S. (2002). Incidence of apple fruit and leaf surface metabolites on *Cydia pomonella* oviposition. *Entomol. Exp. Appl.* 104, 79–87. doi: 10.1046/j.1570-7458.2002.00993.x
- Mackenzie, J. D., and Cummins, J. N. (1982). Differentiation of *Malus* clones into resistance classes by their effects on the biology of *Eriosoma lanigerum* Hausmann. *J. Am. Soc. Hortic. Sci.* 107, 737–740.
- Madsen, H. F., and Borden, A. D. (1954). Codling moth and orange tortix control on apricots in California, 1948–1953. *J. Econ. Entomol.* 47, 161–165. doi: 10.1093/jee/47.1.161
- Mattheis, J. P., Fellman, J. K., Chen, P. M., and Patterson, M. E. (1991). Changes in headspace volatiles during physiological development of Bisbee Delicious apple fruit. *J. Agric. Food Chem.* 39, 1902–1906. doi: 10.1021/jf0011a002
- Myers, C. T., Hull, L. A., and Krawczyk, G. (2006a). Seasonal and cultivar associated variation in the Oviposition behavior of Oriental fruit moth, (Lepidoptera: tortricidae) adults and feeding behavior of neonate larvae in apples. *J. Econ. Entomol.* 99, 349–358. doi: 10.1093/jee/99.2.349
- Myers, C. T., Hull, L. A., and Krawczyk, G. (2006b). Effects of orchard host plants on the oviposition preference of the Oriental fruit moth (Lepidoptera: tortricidae). *J. Econ. Entomol.* 99, 1176–1183. doi: 10.1093/jee/99.4.1176
- Myers, C. T., Joshi, N. K., Hull, L. A., and Glenn, D. M. (2007). Observation and evaluation of exotic and domestic apple germplasm for resistance to attack from Oriental fruit moth and codling moth. *Pa. Fruit News* 87, 51–56.
- Olson, W. H. (1977). Walnut varieties differ in susceptibility to codling moth damage. *Calif. Agric.* 31, 14–15.
- Pashely, D. P., and Bush, G. L. (1979). “The use of allozymes in studying insect movement with special reference to the codling moth, *Laspeyresia pomonella* (L.) (Olethreutidae),” in *Movement of Highly Mobile Insects: Concepts and Methodology in Research*, eds R. L. Rabb and G. G. Kennedy (Raleigh, NC: North Carolina State University), 333–341.
- Phillips, P. A., and Barnes, M. M. (1975). Host race formation among sympatric apple, walnut, and plum populations of the codling moth, *Laspeyresia pomonella*. *Ann. Entomol. Soc. Am.* 68, 1053–1060. doi: 10.1093/esa/68.6.1053
- Putman, W. L. (1963). The codling moth, *Carpocapsa pomonella* (L.) (Lepidoptera: Tortricidae): a review with special reference to Ontario. *Proc. Entomol. Soc. Ont.* 93, 22–60.
- R Development Core Team (2005). *R: A Language and Environment for Statistical Computing*. Vienna: R Development Core Team, R Foundation for Statistical Computing.

- Reed, H. C., and Landolt, P. J. (2002). Attraction of mated female codling moths (Lepidoptera: tortricidae) to apples and apple odor in a flight tunnel. *Fla. Entomol.* 85, 324–329. doi: 10.1653/0015-4040(2002)085[0324:AOMFCM]2.0.CO;2
- Renwick, J. A. A. (1989). Chemical ecology of oviposition in phytophagous insects. *Experientia* 45, 223–228. doi: 10.1007/BF01951807
- Shelford, V. E. (1927). *An Experimental Investigation of the Relations of the Codling Moth to Weather and Climate*, Vol. XVI. Urbana, IL: Division of the Natural History Survey, 315–445.
- Shelton, M. D., and Anderson, J. L. (1990). Walnut cultivars: evidence for differential susceptibility to insect pests. *Fruit Varieties J.* 44, 179–182.
- Straub, D. (2003). Susceptibility of new apple cultivars to various arthropod pests. *N. Y. Fruit Q.* 11, 25–28.
- Summerland, S. A., and Steiner, L. F. (1943). Codling moth oviposition and fate of the eggs. *J. Econ. Entomol.* 36, 72–75. doi: 10.1093/jee/36.1.72
- Sutherland, O. R. W., Hutchins, R. F. N., and Wearing, C. H. (1974). “The role of the hydrocarbon -farnesene in the behavior of codling moth larvae and adults,” in *Experimental Analysis of Insect Behavior*, ed. L. B. Browne (Berlin: Springer), 249–263.
- Sutherland, O. R. W., Wearing, C. H., and Hutchins, R. F. N. (1977). Production of α -farnesene, an attractant and oviposition stimulant for codling moth, by developing fruit of ten varieties of apple. *J. Chem. Ecol.* 3, 625–631. doi: 10.1007/BF00988062
- Tulecke, W., and McGranahan, G. (1994). *The Walnut Germplasm Collection of the University of California, Davis. A Description of the Collection and A History of the Breeding Program of Eugene F. Serr and Harold I. Forde*. Report No. 13. Davis, CA: University of California Conservation Program.
- Van Leeuwen, E. R. (1929). *Life History of the Codling moth in Northern Georgia*. Washington, DC: U.S. Department of Agriculture.
- Verardo, G., Pagani, E., Geatti, P., and Martinuzzi, P. (2003). A thorough study of the surface wax of apple fruits. *Anal. Bioanal. Chem.* 376, 659–667. doi: 10.1007/s00216-003-1945-7
- Wearing, C. H., Connor, P. J., and Ambler, K. D. (1973). Olfactory stimulation of oviposition and flight activity of the codling moth *Laspeyresia pomonella*, using apples in an automated olfactometer. *N. Z. J. Sci.* 16, 697–710.
- Wearing, C. H., and Hutchins, R. F. (1973). Alpha-Farnesene, a naturally occurring oviposition stimulant for the codling moth, *Laspeyresia pomonella*. *J. Insect Physiol.* 19, 1251–1256. doi: 10.1016/0022-1910(73)90208-4
- Wildbolz, T. (1958). Über die Orientierung des Apfelwicklers bei der Eiablage. *Mitt. Schweiz. Entomol. Ges.* 31, 25–34.
- Witzgall, P., Stelinski, L., Gut, L., and Thomson, D. (2008). Codling moth management and chemical ecology. *Annu. Rev. Entomol.* 53, 503–522. doi: 10.1146/annurev.ento.53.103106.093323
- Zar, J. H. (1999). *Biostatistical Analysis*, 4th Edn. Upper Saddle River, NJ: Prentice Hall.

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Plant–Aphid Interactions Under Elevated CO₂: Some Cues from Aphid Feeding Behavior

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Although the increasing concentration of atmospheric carbon dioxide (CO₂) accelerates the accumulation of carbohydrates and increases the biomass and yield of C3 crop plants, it also reduces their nitrogen concentration. The consequent changes in primary and secondary metabolites affect the palatability of host plants and the feeding of herbivorous insects. Aphids are phloem feeders and are considered the only feeding guild that positively responds to elevated CO₂. In this review, we consider how elevated CO₂ modifies host defenses, nutrients, and water-use efficiency by altering concentrations of the phytohormones jasmonic acid, salicylic acid, ethylene, and abscisic acid. We will describe how these elevated CO₂-induced changes in defenses, nutrients, and water status facilitate specific stages of aphid feeding, including penetration, phloem-feeding, and xylem absorption. We conclude that a better understanding of the effects of elevated CO₂ on aphids and on aphid damage to crop plants will require research on the molecular aspects of the interaction between plant and aphid but also research on aphid interactions with their intra- and inter-specific competitors and with their natural enemies.

Keywords: elevated CO₂, aphid, nitrogen metabolism, plant defenses, water potential, legumes

INTRODUCTION

Since the industrial revolution, atmospheric CO₂ concentrations have increased from 280 ppm to approximately 400 ppm due to anthropogenic effects, i.e., deforestation and fossil fuel combustion. These increases in atmospheric CO₂ concentrations have serious implications for global warming and climate change (Stocker et al., 2013). Although changes in climate have been anticipated to greatly affect agricultural ecosystems (Fuhrer, 2003), increases in atmospheric CO₂ concentration alone can also be very important because they can directly affect plant physiology and indirectly alter interactions between plants and herbivores and plant pathogens (Robinson et al., 2012). These altered interactions may then lead to more severe and frequent outbreaks of pest insects and plant diseases in agricultural ecosystems (Percy et al., 2002).

To understand how elevated concentrations of atmospheric CO₂ could increase pest problems, we must first recognize that increases in CO₂ tends to increase the growth of plants by enhancing their photosynthetic rate, resulting in higher yields for most C3 crops (Ainsworth and Rogers, 2007). Under elevated CO₂, however, C3 crop plants exhibit decreases in nitrogen (N) and other trace elements, i.e., zinc and iron (Bloom et al., 2010). These decreases reduce the nutritional value for herbivorous insects and may therefore change their feeding behaviors (Myers et al., 2014). For

those insects that chew leaves, a reduction in the N concentration in crop tissue and the resulting increase in the carbon/nitrogen ratio (C:N ratio) under elevated CO₂ could cause these insect pests to consume more leaves to meet their N needs (Bezemer and Jones, 1998; Sun and Ge, 2011). In addition, leaves grown under elevated CO₂ decrease their ability to produce jasmonic acid (JA), a hormone that contributes to plant defenses against chewing insects (Zavala et al., 2008).

Elevated CO₂ may also increase the damage to crops caused by phloem-sucking insects including aphids. Aphids feed exclusively on the phloem sap and are very sensitive to changes in plant quality caused by climate change (Pritchard et al., 2007). Recent meta-analysis result shows that aphids tend to perform better under elevated CO₂ on average (Robinson et al., 2012). The conclusions from many statistically significant researches, however, exhibit idiosyncratic responses of aphids in terms of population abundance, fecundity as well as survival (summarized in Table 1). Although predictions are difficult, it is nevertheless useful to determine why some aphids are more fit while others are less fit under elevated CO₂. A mechanistic understanding can help make sense of these contradictory results. Previous study demonstrates that the effect of elevated CO₂ on plant, which includes C and N assimilation, secondary metabolism, plant stomatal conductance as well as leaf temperature, could in turn affect aphid population numbers and growth (Ainsworth et al., 2006; May et al., 2013). Furthermore, the feeding behavior of aphids and their interaction with host plant under elevated CO₂ are largely ignored but should be crucial to the understanding of idiosyncratic responses. The aim of this review is to highlight overlooked processes and new discoveries that how elevated CO₂ affects the components of plant leaves and how these effects alter the different feeding phases of aphids. We also suggest some possible molecular mechanisms underlying the interactions between aphids and their host plants under elevated CO₂.

APHID FEEDING BEHAVIOR

Recent advances indicate that complex molecular interactions occur when aphids feed on plants. Unlike chewing insects that remove large pieces of plant tissues, aphids use their flexible and long stylets to obtain nutrients from the phloem sap and only inflict slight physical damage (Jaouannet et al., 2014). The specialized feeding behavior of aphids can be detected with electrical penetration graph (EPG) methods, i.e., EPG methods can be used to determine the locations and activities of aphid stylets, including pooled pathway phase activities, probing, salivation into sieve elements, passive uptake of the phloem sap, and xylem absorption (Tjallingii and Esch, 1993). Data on the initiation and duration of these feeding phases provide valuable cues regarding aphid activities and plant responses (Alvarez et al., 2006). Rather than simply withdrawing food from hosts, aphids can change their feeding location to avoid plant defenses or can secrete ‘effector’ proteins to suppress plant defenses (Hogenhout and Bos, 2011). To enhance their feeding,

aphids can also alter host physiological traits, e.g., they can induce changes in host primary metabolism and in stomatal movement, and suppress the plant defenses (Giordanengo et al., 2010). Thus, a better understanding of aphid feeding behavior, its effects on hosts, and host responses is critical for understanding how elevated CO₂ is likely to affect plant–aphid interactions.

APHID PROBING AND PENETRATION STAGE AND ITS RELATION TO PLANT RESISTANCE

Influence of Plant Physical Barriers

Once they have arrived on a plant leaf, aphids must conquer host physical defenses including trichomes and waxes before they can insert their stylets into the host (Wang et al., 2004). Surface resistance is the first barrier of plant defense against aphid attack. The time that aphids spend between arriving on a leaf and making their first probe mainly reflects the physical barriers of the leaf surface including trichomes, repellent volatiles, and a thick or tough leaf surface (van Helden and Tjallingii, 1993). Plants can deter aphid attack by releasing secondary metabolites such as glucose esters and sesquiterpenes from glandular trichomes (Avé et al., 1987; Goffreda et al., 1989; Neal et al., 1990). Furthermore, a specifically expressed gene, *NtLTP1*, in the glandular trichomes of *Nicotiana tabacum* could enhance the plant’s defense against aphids (Choi et al., 2012). The changes in trichome density in response to CO₂ are idiosyncratic. For example, trichome density increased in *Brassica rapa* and *Medicago truncatula* (Karowe and Grubb, 2011; Guo et al., 2014a) but decreased in *Arabidopsis* and wheat under elevated CO₂ (Masle, 2000; Bidart-Bouzat et al., 2005; Lake and Wade, 2009). In the legume *M. truncatula* under elevated CO₂, the increased density of non-glandular and glandular trichomes caused aphids to spend more time before they made their first probe and to experience a prolonged pathway phase (Guo et al., 2014a). CO₂ concentrations may affect trichome development by affecting the levels of gibberellic acid (GA), JA, and the microRNA molecule miR156. Elevated CO₂ tends to increase plant GA content and decrease plant JA content (Teng et al., 2006; Zavala et al., 2008) and to decrease expression of miR156 (May et al., 2013). Additional research is needed, however, to clarify whether the effects of elevated CO₂ on glandular trichome development and surface resistance to aphids is due to changes in GA, JA, and miR156.

Phytohormone-Mediated Defenses

When the aphid stylet penetrates the plant epidermis and mesophyll, it forms a channel that permits the delivery of saliva into the phloem (Jaouannet et al., 2014). On the one hand, elicitors in aphid saliva could trigger the formation of reactive oxygen species (ROS), which in turn could induce plant defenses (Giordanengo et al., 2010). On the other hand, “effectors” in aphid saliva could suppress plant resistance and manipulate host cell processes to favor aphid feeding and colonization (Bos

TABLE 1 | Potential mechanisms regarding aphid performance respond to elevated CO₂

Potential mechanism	Aphid-host plant system	Response	Parameter	Reference
Alters absorption of foliar amino acid or changes the sap flow of plant	<i>Acyrthosiphon pisum</i> – <i>Medicago sativa</i>	Positive	Population abundance	Ryalls et al., 2015
	<i>Acyrthosiphon pisum</i> – <i>Medicago truncatula</i>	Positive	Population abundance, feeding efficiency	Guo et al., 2013
	<i>Aphis gossypii</i> – <i>Gossypium hirsutum</i> <i>Rhopalosiphum padi</i> – <i>Hordeum vulgare</i>	Unchanged	Growth rate	Sun et al., 2009
		Positive	Population abundance, intrinsic rate of population	Ryan et al., 2015
	<i>Aphis fabae</i> – <i>Cardamine pratensis</i>	Positive	Population abundance	Salt et al., 1996
	<i>Myzus persicae</i> – <i>Solanum dulcamara</i>	Positive	Population abundance	Salt et al., 1996
	<i>Acyrthosiphon pisum</i> – <i>Medicago sativa</i>	Depend on plant genotypes	Population abundance	Johnson et al., 2014
Changes of nitrogen concentration or whole plant quality of host plant	<i>Myzus persicae</i> – <i>Bell pepper</i>	Negative	Pre-reproductive period, fecundity	Dáder et al., 2016
	<i>Phylaphis fagi</i> – <i>Fagus sylvatica</i>	Negative	Fecundity, nymph weight, nymph weight	Docherty et al., 1997
	<i>Rhopalosiphum padi</i> – <i>Triticum aestivum</i>	Positive	Weight, relative growth rate, life span	Oehme et al., 2013
	<i>Myzus persicae</i> – <i>Brassica napus</i>	Negative	Weight, relative growth rate, life span	Oehme et al., 2013
	<i>Rhopalosiphum maidis</i> – <i>Hordeum vulgare</i>	Positive	Developmental duration, fecundity	Xie et al., 2014
Increase of photosynthesis	<i>Myzus persicae</i> – four plant species (<i>Careamine hirsute</i> , <i>Poa annua</i> , <i>Senecio vulgar</i> , <i>Spergula arvensis</i>)	Positive	Population abundance	Bezemer et al., 1998
Plant endophyte induced resistance	<i>Rhopalosiphum padi</i> – <i>Festuca arundinacea</i>	Negative	Population abundance, aphid density	Newman et al., 1999; Ryan et al., 2014a,b
Decrease of phytohormone resistance	<i>Myzus persicae</i> – <i>Arabidopsis</i>	Positive	Population abundance	Sun et al., 2013
	<i>Acyrthosiphon pisum</i> – <i>Medicago truncatula</i>	Positive	Mean relative growth rate; feeding efficiency	Guo et al., 2014a,b
R-gene mediated resistance decreased	<i>Amphorophora idaei</i> – <i>Rubus idaeus</i>	Positive	Population abundance, adult mass	Martin and Johnson, 2011
Increase of leaf temperature	<i>Aphis glycines</i> – <i>Glycine max</i>	Positive	Population abundance	O'Neill et al., 2011
Decrease of stomatal aperture	<i>Acyrthosiphon pisum</i> – <i>Medicago truncatula</i>	Positive	Population abundance, feeding efficiency	Sun et al., 2015
Sensitivity to alarm pheromone	<i>Amphorophora idaei</i> – <i>Rubus idaeus</i>	Negative	Escape response to predator	Hentley et al., 2014
	<i>Sitobion avenae</i> – <i>Triticum aestivum</i>	Negative	Sensitivity to (E)-β-farnesene	Sun et al., 2010

et al., 2010a,b; McLellan et al., 2013; Gimenez-Ibanez et al., 2014; King et al., 2014). Parameters of aphid feeding behavior revealed by EPG could reflect the intensity of plant resistance; these parameters include the minimum duration of pathway phase activity, the number of test probes, and the total time before phloem ingestion begins (Alvarez et al., 2006). In the *M. truncatula*-pea aphid system, elevated CO₂ increased the number of test probes but decreased the total time before phloem ingestion began (Guo et al., 2014a). The inconsistent effects of elevated CO₂ on aphid feeding parameters may result from the contrasting effects of elevated CO₂ on the defense signaling pathways involving the phytohormones JA, salicylic acid (SA), and ethylene (ET) (Guo et al., 2014a). Elevated CO₂ tends to enhance SA-dependent defense but reduce JA- and ET-dependent defenses in plants (Zavala et al., 2009; Guo et al., 2012; Sun et al., 2013). Furthermore, the enhanced SA signaling pathway under elevated CO₂ caused aphids to spend more time before

the first probe and reduced aphid fitness (Casteel et al., 2012; Guo et al., 2014a). The suppression of the JA signaling pathway under elevated CO₂, however, reduces the time required by aphids to reach the phloem. In addition, elevated CO₂ down-regulates the expression of the ET signaling pathway genes ACC, SKL, and ERF in *M. truncatula* under attack by the pea aphid system; this downregulation, decreases the accumulation of H₂O₂ and the activities of key enzymes related to ROS (Guo et al., 2014b).

Moreover, elevated CO₂ potentially disrupts the homeostatic cross-talk between SA and JA/ET pathways by directly activating the *NPR1* (NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1) gene (DeLucia et al., 2012; Zavala et al., 2013). *NPR1*-mediated suppression of JA signaling is regulated by glutathione biosynthesis (Spoel and Loake, 2011). Elevated CO₂ changes the expression of genes that encode thioredoxins and glutathione S-transferase, which may activate the expression

of *NPR1* (DeLucia et al., 2012). However, Sun et al. (2013) found that when the *NPR1* gene was knocked down, the JA-dependent defenses of *Arabidopsis* were not enhanced by elevated CO₂, suggesting that the activation of *NPR1* may not explain the response of SA, JA, and ET signaling pathways to elevated CO₂. Clearly, the upstream network regulating plant immunity against aphids is complex. The elicitors secreted from aphid salivary glands were recognized by the host co-receptor BRI-ASSOCIATED RECEPTOR KINASE 1 (*BAK1*) which subsequently phosphorylates *BOTRYTIS* INDUCED KINASE1 (*BIK1*). The *BAK1* and the *BIK1* complexes could jointly modulate the downstream phytohormone-mediated defense signaling pathway (Chaudhary et al., 2014; Lei et al., 2014; Prince et al., 2014). In addition to *BAK/BIK*, other kinases such as mitogen protein kinases (MAPKs) are also important for regulating plant defense responses against insect herbivores (Hettenhausen et al., 2015). A number of studies reported that MAPKs could regulate the JA, SA, and ET signaling pathways by activating WRKY genes (Zavala et al., 2013). It is still unknown whether elevated CO₂ affects the JA- and SA-dependent signaling pathways by regulating upstream *BAK/BIK* or MAPK signaling. Thus, additional research is needed to determine how elevated CO₂ affects these regulatory molecules in phytohormone signaling networks.

Secondary Metabolite-Mediated Resistance

Many plant secondary metabolites may help plants resist aphid attack by negatively affecting the penetration pathway stage of aphid feeding. These secondary metabolites include alkaloids, steroids, foliar phenolic esters (rutin, cholorogenic acid, etc.), terpenoids, cyanogenic glycosides, glucosinolates, saponins, flavonoids, and pyrethrins (Sharma et al., 2000; Urbanska et al., 2002). For example, aphids that fed on high-saponin lines of alfalfa required a prolonged time to penetrate the epidermis and mesophyll (pattern C wave) and showed a significant reduction in phloem sap ingestion (Golawska, 2007). Furthermore, different phenolic compounds seem to have different effects on the feeding parameters of aphids. Caffeic and gallic acids in cereals, for example, drastically shortened the probing phase of the grain aphid, whereas catechin prolonged the pathway phase and also decreased the number of probes by the grain aphid (Urbanska et al., 2002). On average, elevated CO₂ increases the total phenolics in plants by an average of 19%, condensed tannins by 22%, and flavonoids by 27% (Robinson et al., 2012). The excess of secondary metabolites in plants may help explain the increased epidermis and mesophyll resistance of plants during pathway and probing feeding stages of aphids under elevated CO₂ (Guo et al., 2014a). Despite of increasing tannin content and phenolic compounds in whole host plant leaves, the bird cherry-oat aphid *Rhopalosiphum padi* performed better under elevated CO₂ (Bezemer and Jones, 1998; Zhang et al., 2003). This result suggested that the tricky feeding strategy of the aphid allows it to avoid some potential defensive components. Thus, it is hard to predict

the impact on aphid fitness only through surface or pathway effects.

Phenylalanine ammonialyase (PAL) and polyphenol oxidase (PPO) are two key enzymes involved in the synthesis of phenolic compounds that may be absorbed by the salivary sheath of the aphid stylet. The further polymerization of phenolic compounds causes browning of cells in contact with the saliva; such browning was associated with aphid probing activity during penetration of the epidermal and mesophyll tissues (Jiang and Miles, 1993; Urbanska et al., 1998; Han et al., 2009). PAL and PPO activities are changed by elevated CO₂. For example, elevated CO₂ tends to increase PAL activity but decrease PPO activity in *M. truncatula*. However, it is still unknown how changes in PPO and PAL activities under elevated CO₂ affect the penetration phase of aphid feeding (Guo et al., 2014b).

Resistance Expressed in the Phloem

After overcoming defenses associated with the plant epidermis and mesophyll, the aphid stylet may finally reach the phloem, but plants have ways to prevent or reduce the ingestion of phloem sap. Phloem sap contains carbohydrates, proteins, and amino acids that are essential for plant development (Gündüz and Douglas, 2009). If the phloem is impaired, plants could suffer loss of nutrients, disturbance of translocation, and increased vulnerability to infection by microbial pathogens (Dinant et al., 2010). Therefore, plants have evolved a range of defenses to inhibit phloem feeding by aphids (Will et al., 2013). The most common defense involves the occlusion of sieve tubes by the plugging of sieve pores (Knoblauch and van Bel, 1998). Two groups of sieve-tube occlusion mechanisms can be found in plants: callose deposition and protein plugging (e.g., Will and van Bel, 2006; Furch et al., 2007). The Ca²⁺ signaling pathway in plants plays a key role in sieve-tube occlusion during aphid penetration. When the stylet penetrates the sieve membrane, the high concentration gradient of Ca²⁺ between the apoplast and the sieve element lumen leads to an influx of Ca²⁺ into the sieve element lumen, which induces occlusion (Knoblauch and van Bel, 1998). When this occurs, aphids must secrete watery saliva into the phloem; the saliva contains proteins that bind Ca²⁺ and counteract sieve element occlusion. Thus, the time spent during salivary secretion into sieve elements reflects the defenses located in the phloem (Will et al., 2013).

Phloem resistance against aphids may be affected by elevated CO₂. The key gene involved in callose biosynthesis is up-regulated in *Arabidopsis* under elevated CO₂ (Li et al., 2008). Furthermore, cytosolic free Ca²⁺ is increased by elevated CO₂ in *Commelina communis* (Webb et al., 1996). The increased production of callose and free Ca²⁺ in cells may cause aphids to spend more time in overcoming phloem resistance. EPG data consistently showed that elevated CO₂ increased the time of salivary secretion into sieve elements when pea aphids fed on *M. truncatula* (Guo et al., 2013). Still, there is no direct evidence confirming that elevated CO₂ increases the phloem resistance against pea aphids because of increases in callose deposition and in the Ca²⁺ signaling pathway.

Aphid Phloem Ingestion and Its Relation to Plant Nutrition

The efficiency with which aphids feed on phloem sap is determined by the nutritional composition of the sap (Douglas, 2003). Sucrose is the dominant organic compound in the phloem sap and is a crucial C source for aphids (Fisher and Cash-Clark, 2000; Douglas, 2003). Sucrose is the principal energy source for aphids and also provides the C skeleton for lipid, amino acid, and protein synthesis (Rhodes et al., 1996; Febvay et al., 1999). In potato, a mutation of the sucrose transporter *StSUT1* gene reduced the phloem sucrose content and simultaneously reduced the performance of the potato aphid (Pescod et al., 2007). Nevertheless, high concentrations of soluble carbohydrates in plant tissues often reduce aphid performance because they dilute other nutrients such as amino acids and proteins; as a consequence, the aphids must increase their consumption of phloem sap and excrete the excess sucrose as honeydew (Wilkinson et al., 1997). In contrast to carbohydrate-based nutrients, N nutrition is a limiting factor for aphid growth. The phloem sap ingested by aphids has a protein/carbohydrate ratio (w/w) as low as 0.1 while the leaf tissue ingested by chewing insects has a protein/carbohydrate ratio ranging from 0.8 to 1.5 (Behmer, 2008).

Increases in atmospheric CO₂ accelerate photosynthesis and synthesizes and transport of sucrose into the phloem, which dilutes the N concentration and increases the C:N ratio in the phloem of non-legumes (Barbehenn et al., 2004). The decreased nitrogen concentration of plants could prolong the pre-reproductive period and decrease the fecundity of some aphids under elevated CO₂ (Dáder et al., 2016). However, Sun et al. (2009) found that although amino acid relative concentration in the phloem of cotton plants was lower under elevated CO₂ than under ambient CO₂, higher amounts of free amino acids were found in cotton aphids fed on cotton grown in elevated CO₂. These results suggested that cotton aphids under elevated CO₂ will ingest increased quantities of phloem sap to satisfy their nutritional requirements. Moreover, the relative concentrations of predominantly essential amino acids in the phloem of barley are increased under elevated CO₂ (Ryan et al., 2015). The latter result is consistent with the large increases in the levels of minor amino acids (most of which are considered essential) in tobacco seedlings under elevated CO₂ (Geiger et al., 1998). These results suggest that although the total N concentration of plants is decreased, amino acids biosynthesis and translation in some non-legumes may increase under elevated CO₂. Moreover, the mathematic model constructed by Newman et al. (2003) predicted that aphid populations tend to be larger under elevated CO₂ if host plants have higher N supplementation, that the nitrogen requirement of aphids is low and that the density-dependent response is weak. Thus, a general explanation for the species-specific responses of aphids to elevated CO₂ remains to be elucidated.

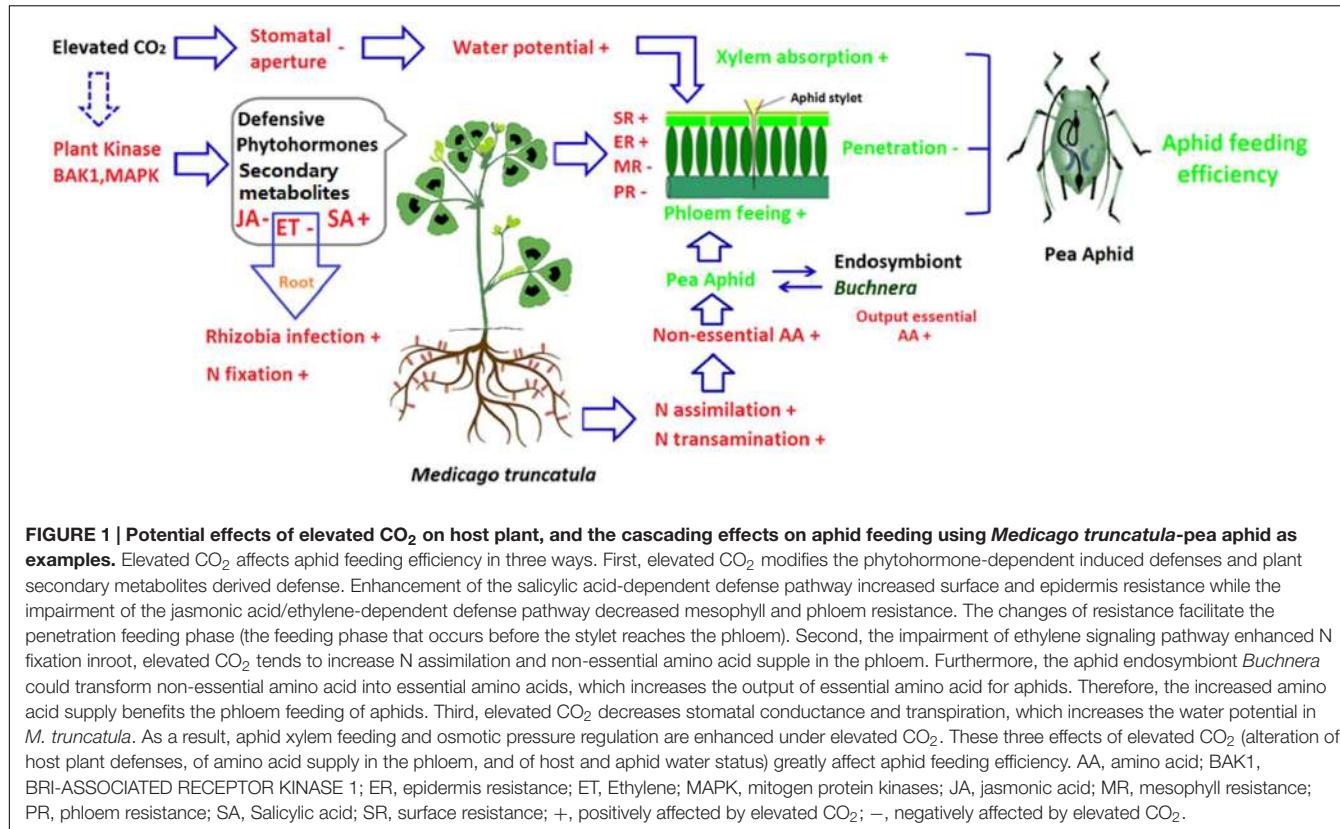
In legumes, elevated CO₂ leads to a 38% increase in the quantity of N fixed from the atmosphere, which can compensate for decreases in plant N under elevated CO₂ and cause

the legumes to maintain a C:N ratio similar to that under ambient CO₂ (Lam et al., 2012). When *M. truncatula* was infested by pea aphids, elevated CO₂ significantly increased the concentration of total amino acids in leaves and of most individual amino acids in the phloem by enhancing the enzyme activities of N transamination (Guo et al., 2013). The increased amino acids, however, are mostly nonessential, and require the aphid endosymbiont *Buchnera* to convert them into essential amino acids (Nikoh et al., 2010). When the N-fixation ability was reduced by artificially induced mutation, the individual amino acid relative concentration in the phloem of *M. truncatula* was decreased such that *Buchnera* could no longer convert the nonessential amino acids into essential amino acids (Guo et al., 2013). These results with legumes suggest that elevated CO₂ may increase the phloem feeding time of the pea aphid by altering amino acid metabolism, and that this response depends on a functional N fixation system. Responses of different cultivars, varieties, or genotypes of the same species to elevated CO₂, however, can also vary. For example, Johnson et al. (2014) found elevated CO₂ increased 86% and 56% essential amino acid concentrations and pea aphid colonization success on the high resistant cultivar 'Sequel' of *M. sativa*. However, elevated CO₂ decreased 53% and 33% essential amino acid concentrations and aphid colonization on the moderate resistant cultivar 'Genesis'. This result suggested some cultivars may become more or less susceptible to aphid attack under climate change conditions, an important consideration for determining future outcomes (McKenzie et al., 2013).

The ability to fix N is regulated by several hormone signaling pathways including the ET signaling pathway (Ma et al., 2002; Penmetsa et al., 2008). When the key gene *Mtskl* in the ET-perception pathway was mutated in *M. truncatula*, the nitrogenase activity was increased about two times (Penmetsa and Cook, 1997). Previous study has shown that elevated CO₂ decreases the ET signaling pathway in *Arabidopsis* (Sun et al., 2013). The suppression of the ET signaling pathway in *M. truncatula* increased nodulation and N fixation ability, which thereby satisfied the increased demand for N by plants growing under elevated CO₂. The down-regulation of the ET signaling pathway, however, is accompanied by decreased ET-mediated host resistance against the pea aphid (Guo et al., 2014b). This result suggested that in the *M. truncatula*-pea aphid system under elevated CO₂, both nutritional and resistance effects would increase the fitness of the pea aphid by suppressing the ET signaling pathway (Figure 1).

APHID XYLEM ABSORPTION AND ITS RELATION TO PLANT WATER STATUS

Aphids occasionally ingest xylem to increase their phloem feeding efficiency (Tjallingii and Esch, 1993; Douglas, 2006). Because the sugar-enriched phloem sap has an osmotic pressure that is as much as 4 to 5 times greater than that of the aphid's haemolymph, continuously passive uptake of the phloem sap could result in aphid dehydration. To avoid self-dehydration and



osmotic stress in the haemolymph during the phloem-feeding phase (i.e., to balance haemolymph osmolarity), aphids must consume a certain amount of xylem sap, which has a lower osmolarity than phloem sap (Pompon et al., 2011). This xylem-feeding behavior requires that the host plant has a relatively high plant water potential because the feeding is passive, i.e., fluid moves from plant to aphid because of a water potential gradient (Huberty and Denno, 2004; Daniels et al., 2009; Nalam et al., 2012). Aphids, like pathogens, can trigger stomatal closure, decrease leaf transpiration, and maintain the water content of the host plant by up-regulating the ABA signaling pathway. This manipulation of host stomata helps aphids absorb water from the xylem to neutralize phloem osmotic pressure (Sun et al., 2015).

Under elevated CO₂, plants also exhibit reduced stomatal apertures and stomatal conductance. In FACE experiments, elevated CO₂ has decreased stomatal conductance by an average of 22% for five functional plant groups that include 285 plant species (Ainsworth and Rogers, 2007). As noted, the decreased stomatal conductance reduces water loss from plants and increases plant water potential and water content (Wullschleger et al., 2002; Pritchard et al., 2007). Sun et al. (2015) found that the decreases in stomatal aperture and increases in plant water potential induced by elevated CO₂ facilitated xylem feeding by aphids and thereby decreased aphid haemolymph osmolarity, which indicated a decreased cost of osmoregulation in aphids under elevated CO₂.

CONCLUSION AND PERSPECTIVES

Recent studies have provided evidence that elevated CO₂ alters plant resistance, nutritional value, and water status and that these changes affect certain feeding stages of aphids (Figure 1). The evidence also indicates that such changes and effects could be mediated by the phytohormones JA, SA, ET, and ABA (Guo et al., 2013, 2014a,b; Sun et al., 2015). In these and related studies, elevated CO₂ stimulated the SA signaling pathway and thereby increased the epidermis and mesophyll resistance of plants. However, elevated CO₂ decreased JA and ET signaling pathways, which reduced the total time required by aphids to reach the phloem. The decreased ET signaling pathway also increased the N fixation ability of legumes and thereby increased their synthesis of amino acids, which in turn increased amino acid acquisition by aphids (Guo et al., 2013). Moreover, elevated CO₂ decreased stomatal aperture and increased plant water potential, which thereby increased aphid xylem absorption and enhanced aphid osmoregulation (Sun et al., 2015). Nevertheless, transcriptomic evidence shows that elevated CO₂ has a wide range of effects on plant metabolism (including C and N assimilation, secondary metabolism, and transportation), all of which may affect aphid performance (Ainsworth et al., 2006; May et al., 2013). Thus, the effects of elevated CO₂ on the interaction between plants and aphids cannot be understood by simply relating one aspect of plant quality to a specific feeding phase of the aphid. Because the responses to elevated CO₂ differ among plant species, it is currently difficult to generalize about

how further increases in concentrations in atmospheric CO₂ affects aphid feeding and damage. An increased understanding of the molecular mechanisms underlying the recognition and interactions between plants and aphids should increase our ability to predict aphid damage under elevated CO₂.

In addition to changes in aphid feeding behavior, changes in aphid physiology must be considered to understand how aphid performance is affected by elevated CO₂. Some studies have reported increases in aphid growth rate and fecundity under elevated CO₂, which suggests that elevated CO₂ increases aphid fitness and increases the probability of aphid outbreaks. At present, we have some understanding of what happens but we do not know how it happens. Like chewing insects, aphids could sense and respond to nutritional changes in host plants by regulating a complex regulatory network involving the insulin-related peptides, the target of rapamycin (TOR), ecdysteroids, and juvenile hormone (Badisco et al., 2013). For example, TOR acts as a central regulator of protein synthesis by sensing and integrating signals from amino acid nutrition, while the insulin signaling pathway is responsible for sensing carbohydrate-derived nutrients (Grewal, 2009). Thus, research is needed on how these two nutrient-sensing and regulatory pathways in aphids affect vitellogenins and juvenile hormone/ecdysone when aphids feed on plants with increased C:N ratios under elevated CO₂.

Herbivorous insects can be affected by environmental change via changes in host physiology and chemical composition or via changes in competitors or natural enemies (Awmack and Leather, 2002). Elevated CO₂ affects aphid performance from the level of individual physiology or even molecular function to the level of the ecosystem (Sun and Ge, 2011). The effects of elevated CO₂ on individual plants and aphids may differ from the effects involving the entire ecosystem and multiple trophic levels because responses to elevated CO₂ may differ among trophic levels. It is well known that elevated CO₂ has bottom-up effects on the feeding behavior and population size of aphids, but the situation becomes more complicated when aphid-aphid interactions or top-down effects involving natural enemies are considered. For example, aphid species, or even different genotypes within the same species, differ in their responses to elevated CO₂ (Mondor et al., 2005), and these differences might affect the outcome of intra- or inter-specific competition between aphid species or genotypes (Stacey and Fellowes, 2002; Sun et al., 2009). Furthermore, some reports indicate that parasitoids and predators are more abundant or

effective under elevated CO₂ (Percy et al., 2002; Chen et al., 2005) and that aphids are less sensitive to alarm pheromones under elevated CO₂ (Awmack et al., 1997; Mondor et al., 2004). It seems that enhanced top-down effects on aphids under elevated CO₂ may strongly alter the effects of aphids on host plants (Hentley et al., 2014).

The different feeding strategies evident in aphid responses to environmental changes are possibly driven by synchronous adaptation to host and environment. Because it directly affects herbivorous only weakly, elevated CO₂ mainly influences herbivorous insect by altering the host plant (Yin et al., 2010). Thus, understanding plant-aphid interactions is likely to be central to understanding how aphids respond to elevated CO₂. We suggest that molecular tools be used to better understand how the host plant ‘recognizes’ the aphid and vice versa; this research might focus on salivary secretions (the most obvious ‘signal’ available), which could trigger various molecular responses in the host that then affect the aphid in various ways. Although the knowledge from literatures shows that aphids may have species-specific molecular interaction with the hosts, it is believed that the genetics and physiology governing plant-aphid interactions have many commonalities rooted in their phylogenies so that understanding the complexity of interaction will provide meaningful insights into aphids acting on different kinds of plants and aid us in using them to our best advantage. Given increasing concentrations of atmospheric CO₂ and climate change, new crop varieties will be needed that can produce sustainable yields in spite of the changing environment and the potential for increased pressure from aphids and other pests. The development of such crop varieties will be facilitated by a better understanding of the interactions between plants and aphids at molecular, community, and ecosystem levels.

AUTHOR CONTRIBUTIONS

YS and HG wrote this article, FG revised it.

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REFERENCES

- Ainsworth, E. A., and Rogers, A. (2007). The response of photosynthesis and stomatal conductance to rising [CO₂]: mechanisms and environmental interactions. *Plant Cell Environ.* 30, 258–270. doi: 10.1111/j.1365-3040.2007.01641.x
- Ainsworth, E. A., Rogers, A., Vodkin, L. O., Walter, A., and Schurr, U. (2006). The effects of elevated CO₂ concentration on soybean gene expression. An analysis of growing and mature leaves. *Plant Physiol.* 142, 135–147.
- Alvarez, A. E., Tjallingii, W. F., Garzo, E., Vleeshouwers, V., Dicke, M., and Vosman, B. (2006). Location of resistance factors in the leaves of potato and wild tuber-bearing *Solanum* species to the aphid *Myzus persicae*. *Entomol. Exp. Appl.* 121, 145–157. doi: 10.1111/j.1570-8703.2006.00464.x
- Avé, D. A., Gregory, P., and Tingey, W. M. (1987). Aphid repellent sesquiterpenes in glandular trichomes of *Solanum berthaultii* and *S. tuberosum*. *Entomol. Exp. Appl.* 44, 131–138. doi: 10.1111/j.1570-7458.1987.tb01057.x
- Awmack, C., Harrington, R., and Leather, S. (1997). Host plant effects on the performance of the aphid *Aulacorthum solani* (Kalt.) (Homoptera: Aphididae) at ambient and elevated CO₂. *Glob. Chang. Biol.* 3, 545–549. doi: 10.1046/j.1365-2486.1997.t01-1-00087.x
- Awmack, C. S., and Leather, S. R. (2002). Host plant quality and fecundity in herbivorous insects. *Annu. Rev. Entomol.* 47, 817–844. doi: 10.1146/annurev.ento.47.091201.145300

- Badisco, L., Van Wielendaele, P., and Broeck, J. V. (2013). Eat to reproduce: a key role for the insulin signaling pathway in adult insects. *Front. Physiol.* 4:202. doi: 10.3389/fphys.2013.00202
- Barbehenn, R. V., Chen, Z., Karowe, D. N., and Spickard, A. (2004). C3 grasses have higher nutritional quality than C4 grasses under ambient and elevated atmospheric CO₂. *Glob. Chang. Biol.* 10, 1565–1575. doi: 10.1111/j.1365-2486.2004.00833.x
- Behmer, S. T. (2008). Insect herbivore nutrient regulation. *Ann. Rev. Entomol.* 54, 165–187. doi: 10.1146/annurev.ento.54.110807.090537
- Bezemer, T. M., and Jones, T. H. (1998). Plant-insect herbivore interactions in elevated atmospheric CO₂: quantitative analyses and guild effects. *Oikos* 82, 212–222. doi: 10.2307/3546961
- Bezemer, T. M., Jones, T. H., and Knight, K. J. (1998). Long-term effects of elevated CO₂ and temperature on populations of the peach potato aphid *Myzus persicae* and its parasitoid *Aphidius matricariae*. *Oecologia* 116, 128–135. doi: 10.1007/s004420050571
- Bidart-Bouzat, M. G., Mithen, R., and Berenbaum, M. R. (2005). Elevated CO₂ influences herbivory-induced defense responses of *Arabidopsis thaliana*. *Oecologia* 145, 415–424. doi: 10.1007/s00442-005-0158-5
- Bloom, A. J., Burger, M., Asensio, J. S. R., and Cousins, A. B. (2010). Carbon dioxide enrichment inhibits nitrate assimilation in wheat and *Arabidopsis*. *Science* 328, 899–903. doi: 10.1126/science.1186440
- Bos, J. I. B., Armstrong, M. R., Gilroy, E. M., Boevink, P. C., Hein, I., Taylor, R. M., et al. (2010a). Phytophthora infestans effector AVR3a is essential for virulence and manipulates plant immunity by stabilizing host E3 ligase CMPG1. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9909–9914. doi: 10.1073/pnas.09144 08107
- Bos, J. I. B., Prince, D. C., Pitino, M., Maffei, M. E., Win, J., and Hogenhout, S. A. (2010b). A functional genomics approach identifies candidate effectors from the aphid species *Myzus persicae* (Green Peach Aphid). *PLoS Genet.* 6:e1001216. doi: 10.1371/journal.pgen.1001216
- Casteel, C. L., Segal, L. M., Niziolek, O. K., Berenbaum, M. R., and DeLucia, E. H. (2012). Elevated carbon dioxide increases salicylic acid in *Glycine max*. *Environ. Entomol.* 41, 1435–1442. doi: 10.1603/EN12196
- Chaudhary, R., Atamian, H. S., Shen, Z., Briggs, S. P., and Kaloshian, I. (2014). GroEL from the endosymbiont *Buchnera aphidicola* betrays the aphid by triggering plant defense. *Proc. Natl. Acad. Sci. U.S.A.* 111, 8919–8924. doi: 10.1073/pnas.1407687111
- Chen, F. J., Ge, F., and Parajulee, M. N. (2005). Impact of elevated CO₂ on tri-trophic interaction of *Gossypium hirsutum*, *Aphis gossypii*, and *Leis axyridis*. *Environ. Entomol.* 34, 37–46. doi: 10.1603/0046-225X-34.1.37
- Choi, Y. E., Lim, S., Kim, H. J., Han, J. Y., Lee, M. H., Yang, Y., et al. (2012). Tobacco NtLTP1, a glandular-specific lipid transfer protein, is required for lipid secretion from glandular trichomes. *Plant J.* 70, 480–491. doi: 10.1111/j.1365-313X.2011.04886.x
- Dáder, B., Fereres, A., Moreno, A., and Trębicki, P. (2016). Elevated CO₂ impacts bell pepper growth with consequences to *Myzus persicae* life history, feeding behaviour and virus transmission ability. *Sci. Rep.* 6:19120. doi: 10.1038/srep19120
- Daniels, M., Bale, J. S., Newbury, H. J., Lind, R. J., and Pritchard, J. (2009). A sublethal dose of thiamethoxam causes a reduction in xylem feeding by the bird cherry-oat aphid (*Rhopalosiphum padi*), which is associated with dehydration and reduced performance. *J. Insect Physiol.* 55, 758–765. doi: 10.1016/j.jinsphys.2009.03.002
- DeLucia, E. H., Nabity, P. D., Zavala, J. A., and Berenbaum, M. R. (2012). Climate change: resetting plant-insect interactions. *Plant Physiol.* 160, 1677–1685. doi: 10.1104/pp.112.204750
- Dinant, S., Bonnemain, J. L., Girousse, C., and Kehr, J. (2010). Phloem sap intricacy and interplay with aphid feeding. *C. R. Biol.* 333, 504–515. doi: 10.1016/j.crvi.2010.03.008
- Docherty, M., Wade, F., Hurst, D., Whittaker, J., and Lea, P. (1997). Responses of tree sap-feeding herbivores to elevated CO₂. *Glob. Chang. Biol.* 3, 51–59. doi: 10.1046/j.1365-2486.1997.00096.x
- Douglas, A. E. (2003). The nutritional physiology of aphids. *Adv. Insect Physiol.* 31, 73–140. doi: 10.1016/S0065-2806(03)31002-1
- Douglas, A. E. (2006). Phloem-sap feeding by animals: problems and solutions. *J. Exp. Bot.* 57, 747–754. doi: 10.1093/jxb/erj067
- Febvay, G., Rahbé, Y., Rynkiewicz, M., Guillaud, J., and Bonnot, G. (1999). Fate of dietary sucrose and neosynthesis of amino acids in the pea aphid, *Acyrrhosiphon pisum*, reared on different diets. *J. Exp. Biol.* 202, 2639–2652.
- Fisher, D. B., and Cash-Clark, C. E. (2000). Sieve tube unloading and post-phloem transport of fluorescent tracers and proteins injected into sieve tubes via severed aphid stylets. *Plant Physiol.* 123, 125–138. doi: 10.1104/pp.123.1.125
- Fuhrer, J. (2003). Agroecosystem responses to combinations of elevated CO₂, ozone, and global climate change. *Agric. Ecosyst. Environ.* 97, 1–20. doi: 10.1016/S0167-8809(03)00125-7
- Furch, A. C. U., Hafke, J. B., Schulz, A., and van Bel, A. J. E. (2007). Ca²⁺ mediated remote control of reversible sieve tube occlusion in *Vicia faba*. *J. Exp. Bot.* 58, 2827–2838. doi: 10.1093/jxb/erm143
- Geiger, M., Walch-Liu, P., Engels, C., Harnecker, J., Schulze, E.-D., Ludewig, F., et al. (1998). Enhanced carbon dioxide leads to a modified diurnal rhythm of nitrate reductase activity in older plants, and a large stimulation of nitrate reductase activity and higher levels of amino acids in young tobacco plants. *Plant Cell Environ.* 21, 253–268. doi: 10.1046/j.1365-3040.1998.00277.x
- Gimenez-Ibanez, S., Boter, M., Fernández-Barbero, G., Chini, A., Rathjen, J. P., and Solano, R. (2014). The bacterial effector HopX1 targets JAZ transcriptional repressors to activate jasmonate signaling and promote infection in *Arabidopsis*. *PLOS Biol.* 12:e1001792. doi: 10.1371/journal.pbio.1001792
- Giordanengo, P., Brunissen, L., Rusterucci, C., Vincent, C., Van Bel, A., Dinant, S., et al. (2010). Compatible plant-aphid interactions: how aphids manipulate plant responses. *C. R. Biol.* 333, 516–523. doi: 10.1016/j.crvi.2010.03.007
- Goffreda, J. C., Mutschler, M. A., Avé, D. A., Tingey, W. M., and Steffens, J. C. (1989). Aphid deterrence by glucose esters in glandular trichome exudate of the wild tomato, *Lycopersicon pennellii*. *J. Chem. Ecol.* 15, 2135–2147. doi: 10.1007/BF01207444
- Goławska, S. (2007). Deterrence and toxicity of plant saponins for the pea aphid *Acyrrhosiphon pisum* Harris. *J. Chem. Ecol.* 33, 1598–1606. doi: 10.1007/s10886-007-9333-y
- Grewal, S. S. (2009). Insulin/TOR signaling in growth and homeostasis: a view from the fly world. *Int. J. Biochem. Cell Biol.* 41, 1006–1010. doi: 10.1016/j.biocel.2008.10.010
- Gündüz, E. A., and Douglas, A. E. (2009). Symbiotic bacteria enable insect to use a nutritionally inadequate diet. *Proc. R. Soc. Lond. B Biol. Sci.* 276, 987–991. doi: 10.1098/rspb.2008.1476
- Guo, H., Sun, Y., Li, Y., Liu, X., Wang, P., Zhu-Salzman, K., et al. (2014a). Elevated CO₂ alters the feeding behaviour of the pea aphid by modifying the physical and chemical resistance of *Medicago truncatula*. *Plant Cell Environ.* 37, 2158–2168. doi: 10.1111/pce.12306
- Guo, H., Sun, Y., Li, Y., Liu, X., Zhang, W., and Ge, F. (2014b). Elevated CO₂ decreases the response of the ethylene signaling pathway in *Medicago truncatula* and increases the abundance of the pea aphid. *New Phytol.* 201, 279–291. doi: 10.1111/nph.12484
- Guo, H., Sun, Y., Li, Y., Tong, B., Harris, M., Zhu-Salzman, K., et al. (2013). Pea aphid promotes amino acid metabolism both in *Medicago truncatula* and bacteriocytes to favor aphid population growth under elevated CO₂. *Glob. Chang. Biol.* 19, 3210–3223. doi: 10.1111/gcb.12260
- Guo, H., Sun, Y., Ren, Q., Zhu-Salzman, K., Kang, L., Wang, C., et al. (2012). Elevated CO₂ reduces the resistance and tolerance of tomato plants to *Helicoverpa armigera* by suppressing the JA signaling pathway. *PLoS ONE* 7:e41426. doi: 10.1371/journal.pone.0041426
- Han, Y., Wang, Y., Bi, J. L., Yang, X. Q., Huang, Y., Zhao, X., et al. (2009). Constitutive and induced activities of defense-related enzymes in aphid-resistant and aphid-susceptible cultivars of wheat. *J. Chem. Ecol.* 35, 176–182. doi: 10.1007/s10886-009-9589-5
- Hentley, W. T., Vanbergen, A. J., Hails, R. S., Jones, T. H., and Johnson, S. N. (2014). Elevated atmospheric CO₂ impairs aphid escape responses to predators and conspecific alarm signals. *J. Chem. Ecol.* 40, 1110–1114. doi: 10.1007/s10886-014-0506-1
- Hettenhausen, C., Schuman, M. C., and Wu, J. (2015). MAPK signaling: a key element in plant defense response to insects. *Insect Sci.* 22, 157–164. doi: 10.1111/1744-7917.12128
- Hogenhout, S. A., and Bos, J. I. (2011). Effector proteins that modulate plant-insect interactions. *Cur. Opin. Plant Biol.* 14, 422–428. doi: 10.1016/j.pbi.2011.05.003

- Huberty, A. F., and Denno, R. F. (2004). Plant water stress and its consequences for herbivorous insects: a new synthesis. *Ecology* 85, 1383–1398. doi: 10.1890/03-0352
- Jouannet, M., Rodriguez, P. A., Thorpe, P., Lenoir, C. J., MacLeod, R., Escudero-Martinez, C., et al. (2014). Plant immunity in plant-aphid interactions. *Front. Plant Sci.* 5:663. doi: 10.3389/fpls.2014.00663
- Jiang, Y., and Miles, P. W. (1993). Responses of a compatible lucerne variety to attack by spotted alfalfa aphid: changes in the redox balance in affected tissues. *Entomol. Exp. Appl.* 67, 263–274. doi: 10.1111/j.1570-7458.1993.tb01677.x
- Johnson, S. N., Ryalls, J. M. W., and Karley, A. J. (2014). Global climate change and crop resistance to aphids: contrasting responses of lucerne genotypes to elevated atmospheric carbon dioxide. *Ann. Appl. Biol.* 165, 62–72. doi: 10.1111/aab.12115
- Karowe, D. N., and Grubb, C. (2011). Elevated CO₂ increases constitutive phenolics and trichomes, but decreases inducibility of phenolics in *Brassica rapa* (Brassicaceae). *J. Chem. Ecol.* 37, 1332–1340. doi: 10.1007/s10886-011-0044-z
- King, S. R. F., McLellan, H., Boevink, P. C., Armstrong, M. R., Bukharova, T., Sukarta, O., et al. (2014). Phytophthora infestans RXLR effector PexRD2 interacts with host MAPKKK_e to suppress plant immune signaling. *Plant Cell* 26, 1345–1359. doi: 10.1105/tpc.113.120055
- Knoblauch, M., and van Bel, A. J. E. (1998). Sieve tubes in action. *Plant Cell* 10, 35–50. doi: 10.1105/tpc.10.1.35
- Lake, J. A., and Wade, R. N. (2009). Plant-pathogen interactions and elevated CO₂: morphological changes in favour of pathogens. *J. Exp. Bot.* 60, 3123–3131. doi: 10.1093/jxb/erp147
- Lam, S. K., Chen, D., Norton, R., and Armstrong, R. (2012). Does phosphorus stimulate the effect of elevated [CO₂] on growth and symbiotic nitrogen fixation of grain and pasture legumes? *Crop Pasture Sci.* 63, 53–62. doi: 10.1071/CP11296
- Lei, J., Finlayson, S. A., Salzman, R. A., Shan, L., and Zhu-Salzman, K. (2014). BOTRYTIS-INDUCED KINASE1 modulates *Arabidopsis* resistance to green peach aphids via PHYTOALEXIN DEFICIENT4. *Plant Physiol.* 165, 1657–1670. doi: 10.1104/pp.114.242206
- Li, P., Ainsworth, E. A., Leakey, A. D., Ulanov, A., Lozovaya, V., Ort, D. R., et al. (2008). *Arabidopsis* transcript and metabolite profiles: ecotype-specific responses to open air elevated [CO₂]. *Plant Cell Environ.* 31, 1673–1687. doi: 10.1111/j.1365-3040.2008.01874.x
- Ma, W., Penrose, D. M., and Glick, B. R. (2002). Strategies used by rhizobia to lower plant ethylene levels and increase nodulation. *Can. J. Microbiol.* 48, 947–954. doi: 10.1139/w02-100
- Martin, P., and Johnson, S. N. (2011). Evidence that elevated CO₂ reduces resistance to the European large raspberry aphid in some raspberry cultivars. *J. Appl. Entomol.* 135, 237–240. doi: 10.1111/j.1439-0418.2010.01544.x
- Masle, J. (2000). The effects of elevated CO₂ concentrations on cell division rates, growth patterns, and blade anatomy in young wheat plants are modulated by factors related to leaf position, vernalization, and genotype. *Plant Physiol.* 122, 1399. doi: 10.1104/pp.122.4.1399
- May, P., Liao, W., Wu, Y., Shuai, B., McCombie, W. R., Zhang, M. Q., et al. (2013). The effects of carbon dioxide and temperature on microRNA expression in *Arabidopsis* development. *Nat. Commun.* 4:2145. doi: 10.1038/ncomms3145
- McKenzie, S. W., Hentley, W. T., Hails, R. S., Jones, T. H., Vanbergen, A. J., and Johnson, S. N. (2013). Global climate change and above-ground insect herbivore interactions. *Front. Plant Sci.* 4:412. doi: 10.3389/fpls.2013.00412
- McLellan, H., Boevink, P. C., Armstrong, M. R., Pritchard, L., Gomez, S., Morales, J., et al. (2013). An RxLR effector from *Phytophthora infestans* prevents localisation of two plant NAC transcription factors from the endoplasmic reticulum to the nucleus. *PLoS Pathog.* 9:e1003670. doi: 10.1371/journal.ppat.1003670
- Mondor, E. B., Tremblay, M., Awmack, C. S., and Lindroth, R. L. (2004). Divergent pheromone-mediated insect behaviour under global atmospheric change. *Glob. Chang. Biol.* 10, 1820–1824. doi: 10.1111/j.1365-2486.2004.00838.x
- Mondor, E. B., Tremblay, M. N., Awmack, C. S., and Lindroth, R. L. (2005). Altered genotypic and phenotypic frequencies of aphid populations under enriched CO₂ and O₃ atmospheres. *Glob. Chang. Biol.* 11, 1990–1996.
- Myers, S. S., Zanobetti, A., Kloog, I., Huybers, P., Leakey, A. D. B., Bloom, A. J., et al. (2014). Increasing CO₂ threatens human nutrition. *Nature* 510, 139–143. doi: 10.1038/nature13179
- Nalam, V. J., Keeretawee, J., Sarowar, S., and Shah, J. (2012). Root-derived oxylipins promote green peach aphid performance on *Arabidopsis* foliage. *Plant Cell* 24, 1643–1653. doi: 10.1105/tpc.111.094110
- Neal, J. J., Tingey, W. M., and Steffens, J. C. (1990). Sucrose esters of carboxylic acids in glandular trichomes of *Solanum berthaultii* deter settling and probing by green peach aphid. *J. Chem. Ecol.* 16, 487–497. doi: 10.1007/BF01021780
- Newman, J. A., Gibson, D. J., Hickam, E., Lorenz, M., Adams, E., Bybee, L., et al. (1999). Elevated carbon dioxide results in smaller populations of the bird cherry-oat aphid *Rhopalosiphum padi*. *Ecol. Entomol.* 24, 486–489. doi: 10.1046/j.1365-2311.1999.00210.x
- Newman, J. A., Gibson, D. J., Parsons, A. J., and Thornley, J. H. M. (2003). How predictable are aphid population responses to elevated CO₂? *J. Anim. Ecol.* 72, 556–566. doi: 10.1046/j.1365-2656.2003.00725.x
- Nikoh, N., McCutcheon, J. P., Kudo, T., Miyagishima, S. Y., Moran, N. A., and Nakabachi, A. (2010). Bacterial genes in the aphid genome: absence of functional gene transfer from *Buchnera* to its host. *PLoS Genet.* 6:e1000827. doi: 10.1371/journal.pgen.1000827
- Oehme, V., Högy, P., Zebitz, C. P., and Fangmeier, A. (2013). Effects of elevated atmospheric CO₂ concentrations on phloem sap composition of spring crops and aphid performance. *J. Plant Interact.* 8, 74–84. doi: 10.1080/17429145.2012.736200
- O'Neill, B. F., Zangerl, A. R., DeLucia, E. H., Casteel, C., Zavala, J. A., and Berenbaum, M. R. (2011). Leaf temperature of soybean grown under elevated CO₂ increases *Aphis glycines* (Hemiptera: Aphididae) population growth. *Insect Sci.* 18, 419–425. doi: 10.1111/j.1744-7917.2011.01420.x
- Penmetsa, R. V., and Cook, D. R. (1997). A legume ethylene-insensitive mutant hyperinfected by its rhizobial symbiont. *Science* 275, 527–530. doi: 10.1126/science.275.5299.527
- Penmetsa, R. V., Uribe, P., Anderson, J., Lichtenzveig, J., Gish, J. C., Nam, Y. W., et al. (2008). The *Medicago truncatula* ortholog of *Arabidopsis* EIN2, sickle, is a negative regulator of symbiotic and pathogenic microbial associations. *Plant J.* 55, 580–595. doi: 10.1111/j.1365-313X.2008.03531.x
- Percy, K. E., Awmack, C. S., Lindroth, R. L., Kubiske, M. E., Koppen, B. J., Isebrands, J. G., et al. (2002). Altered performance of forest pests under atmospheres enriched by CO₂ and O₃. *Nature* 420, 403–407. doi: 10.1038/nature01028
- Pescod, K. V., Quick, W. P., and Douglas, A. E. (2007). Aphid responses to plants with genetically manipulated phloem nutrient levels. *Physiol. Entomol.* 32, 253–258. doi: 10.1111/j.1365-3032.2007.00577.x
- Pompon, J., Quiring, D., Goyer, C., Giordanengo, P., and Pelletier, Y. (2011). A phloem-sap feeder mixes phloem and xylem sap to regulate osmotic potential. *J. Insect Physiol.* 57, 1317–1322. doi: 10.1016/j.jinsphys.2011.06.007
- Prince, D. C., Drury, C., Zipfel, C., and Hogenhout, S. A. (2014). The leucine-rich repeat receptor-like kinase BRASSINOSTEROID INSENSITIVE1-ASSOCIATED KINASE1 and the cytochrome P450 PHYTOALEXIN DEFICIENT3 contribute to innate immunity to aphids in *Arabidopsis*. *Plant Physiol.* 164, 2207–2219. doi: 10.1104/pp.114.235598
- Pritchard, J., Griffiths, B., and Hunt, E. J. (2007). Can the plant-mediated impacts on aphids of elevated CO₂ and drought be predicted? *Glob. Chang. Biol.* 13, 1616–1629. doi: 10.1111/j.1365-2486.2007.01401.x
- Rhodes, J., Croghan, P., and Dixon, A. (1996). Uptake, excretion and respiration of sucrose and amino acids in the pea aphid *Acyrtosiphon pisum*. *J. Exp. Biol.* 199, 1269–1276.
- Robinson, E. A., Ryan, G. D., and Newman, J. A. (2012). A meta-analytical review of the effects of elevated CO₂ on plant-arthropod interactions highlights the importance of interacting environmental and biological variables. *New Phytol.* 194, 321–336. doi: 10.1111/j.1469-8137.2012.04074.x
- Ryalls, J. M., Moore, B. D., Riegler, M., Gherlenda, A. N., and Johnson, S. N. (2015). Amino acid-mediated impacts of elevated carbon dioxide and simulated root herbivory on aphids are neutralized by increased air temperatures. *J. Exp. Bot.* 66, 613–623. doi: 10.1093/jxb/eru439
- Ryan, G. D., Emiljanowicz, L., Haerri, S. A., and Newman, J. A. (2014a). Aphid and host-plant genotype × genotype interactions under elevated CO₂. *Ecol. Entomol.* 39, 309–315. doi: 10.1111/een.12101
- Ryan, G. D., Shukla, K., Rasmussen, S., Shelp, B. J., and Newman, J. A. (2014b). Phloem phytochemistry and aphid responses to elevated CO₂, nitrogen fertilization and endophyte infection. *Agri. For. Entomol.* 16, 273–283. doi: 10.1111/afe.12055

- Ryan, G. D., Sylvester, E. V. A., Shelp, B. J., and Newman, J. A. (2015). Towards an understanding of how phloem amino acid composition shapes elevated CO₂-induced changes in aphid population dynamics. *Ecol. Entomol.* 40, 247–257. doi: 10.1111/een.12181
- Salt, D. T., Fenwick, P., and Whittaker, J. B. (1996). Interspecific herbivore interactions in a high CO₂ environment: root and shoot aphids feeding on Cardamine. *Oikos* 77, 182–237. doi: 10.2307/3546072
- Sharma, H. C., Sharma, K. K., Seetharama, N., and Ortiz, R. (2000). Prospects for using transgenic resistance to insects in crop improvement. *Electron. J. Biotechnol.* 3, 21–22. doi: 10.2225/vol3-issue2-fulltext-3
- Spoel, S. H., and Loake, G. J. (2011). Redox-based protein modifications: the missing link in plant immune signalling. *Cur. Opin. Plant Biol.* 14, 358–364. doi: 10.1016/j.pbi.2011.03.007
- Stacey, D. A., and Fellowes, M. E. (2002). Influence of elevated CO₂ on interspecific interactions at higher trophic levels. *Glob. Chang. Biol.* 8, 668–678. doi: 10.1046/j.1365-2486.2002.00506.x
- Stocker, T. F., Qin, D., Plattner, G. K., Tignor, M., Allen, S. K., Boschung, J., et al. (2013). *IPCC, 2013: Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*. Cambridge, MA: Cambridge University Press.
- Sun, Y., and Ge, F. (2011). How do aphids respond to elevated CO₂? *J. Asia-Pac. Entomol.* 14, 217–220. doi: 10.1016/j.aspen.2010.08.001
- Sun, Y., Guo, H., Yuan, L., Wei, J., Zhang, W., and Ge, F. (2015). Plant stomatal closure improves aphid feeding under elevated CO₂. *Glob. Chang. Biol.* 21, 2739–2748. doi: 10.1111/gcb.12858
- Sun, Y., Guo, H., Zhu-Salzman, K., and Ge, F. (2013). Elevated CO₂ increases the abundance of the peach aphid on *Arabidopsis* by reducing jasmonic acid defenses. *Plant Sci.* 210, 128–140. doi: 10.1016/j.plantsci.2013.05.014
- Sun, Y., Su, J. W., and Ge, F. (2010). Elevated CO₂ reduces the response of *Sitobion avenae* (Homoptera: Aphididae) to alarm pheromone. *Agric. Ecosyst. Environ.* 135, 140–147. doi: 10.1016/j.agee.2009.09.011
- Sun, Y. C., Jing, B. B., and Ge, F. (2009). Response of amino acid changes in *Aphis gossypii* (Glover) to elevated CO₂ levels. *J. Appl. Entomol.* 133, 189–197. doi: 10.1111/j.1439-0418.2008.01341.x
- Teng, N., Wang, J., Chen, T., Wu, X., Wang, Y., and Lin, J. (2006). Elevated CO₂ induces physiological, biochemical and structural changes in leaves of *Arabidopsis thaliana*. *New Phytol.* 172, 92–103. doi: 10.1111/j.1469-8137.2006.01818.x
- Tjallingii, W. F., and Esch, T. H. (1993). Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiol. Entomol.* 18, 317–328. doi: 10.1111/j.1365-3032.1993.tb00604.x
- Urbanska, A., Leszczynski, B., Tjallingii, W. F., and Matok, H. (2002). Probing behaviour and enzymatic defence of the grain aphid against cereal phenolics. *Electron. J. Polish Agric. Univ. Biol.* 5,
- Urbanska, A., Tjallingii, W. F., Dixon, A. F. G., and Leszczynski, B. (1998). Phenol oxidising enzymes in the grain aphid's saliva. *Entomol. Exp. Appl.* 86, 197–203. doi: 10.1046/j.1570-7458.1998.00281.x
- van Helden, M., and Tjallingii, W. F. (1993). Tissue localisation of lettuce resistance to the aphid *Nasonovia ribisnigri* using electrical penetration graphs. *Entomol. Exp. Appl.* 68, 269–278. doi: 10.1111/j.1570-7458.1993.tb01713.x
- Wang, E., Hall, J. T., and Wagner, G. J. (2004). Transgenic *Nicotiana tabacum* L. with enhanced trichome exudate cembratrieneols has reduced aphid infestation in the field. *Mol. Breed.* 13, 49–57. doi: 10.1023/B:MOLB.0000012328.04974.fb
- Webb, A. A. R., McAinsh, M. R., Mansfield, T. A., and Hetherington, A. M. (1996). Carbon dioxide induces increases in guard cell cytosolic free calcium. *Plant J.* 9, 297–304. doi: 10.1046/j.1365-313X.1996.09030297.x
- Wilkinson, T., Ashford, D., Pritchard, J., and Douglas, A. (1997). Honeydew sugars and osmoregulation in the pea aphid *Acyrthosiphon pisum*. *J. Exp. Biol.* 200, 2137–2143.
- Will, T., Furch, A. C., and Zimmermann, M. R. (2013). How phloem-feeding insects face the challenge of phloem-located defenses. *Front. Plant Sci.* 4:336. doi: 10.3389/fpls.2013.00336
- Will, T., and van Bel, A. J. E. (2006). Physical and chemical interactions between aphids and plants. *J. Exp. Bot.* 57, 729–737. doi: 10.1093/jxb/erj089
- Wullschleger, S. D., Tschaplinski, T. J., and Norby, R. J. (2002). Plant water relations at elevated CO₂-implications for water-limited environments. *Plant Cell Environ.* 25, 319–331. doi: 10.1046/j.1365-3040.2002.00796.x
- Xie, H., Zhao, L., Wang, W., Wang, Z., Ni, X., Cai, W., et al. (2014). Changes in life history parameters of *Rhopalosiphum maidis* (Homoptera: Aphididae) under four different elevated temperature and CO₂ combinations. *J. Econ. Entomol.* 107, 1411–1418. doi: 10.1603/EC13302
- Yin, J., Sun, Y., Wu, G., and Ge, F. (2010). Effects of elevated CO₂ associated with maize on multiple generations of the cotton bollworm, *Helicoverpa armigera*. *Entomol. Exp. Appl.* 136, 12–20. doi: 10.1111/j.1570-7458.2010.00998.x
- Zavala, J. A., Casteel, C. L., DeLucia, E. H., and Berenbaum, M. R. (2008). Anthropogenic increase in carbon dioxide compromises plant defense against invasive insects. *Proc. Natl. Acad. Sci. U.S.A.* 105, 5129–5133. doi: 10.1073/pnas.0800568105
- Zavala, J. A., Casteel, C. L., Nabity, P. D., Berenbaum, M. R., and DeLucia, E. H. (2009). Role of cysteine proteinase inhibitors in preference of Japanese beetles (*Popillia japonica*) for soybean (*Glycine max*) leaves of different ages and grown under elevated CO₂. *Oecologia* 161, 35–41. doi: 10.1007/s00442-009-1360-7
- Zavala, J. A., Nabity, P. D., and DeLucia, E. H. (2013). An emerging understanding of mechanisms governing insect herbivory under elevated CO₂. *Annu. Rev. Entomol.* 58, 79–97. doi: 10.1146/annurev-ento-120811-153544
- Zhang, J., Xing, G. M., Liao, J. X., Hou, Z. D., Wang, G. X., and Wang, Y. F. (2003). Effects of different atmospheric CO₂ concentrations and soil moistures on the populations of bird cherry-oat aphid (*Rhopalosiphum padi*) feeding on spring wheat. *Eur. J. Entomol.* 100, 521–530. doi: 10.14411/eje.2003.080

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Root Herbivores Drive Changes to Plant Primary Chemistry, but Root Loss Is Mitigated under Elevated Atmospheric CO₂

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Above- and belowground herbivory represents a major challenge to crop productivity and sustainable agriculture worldwide. How this threat from multiple herbivore pests will change under anthropogenic climate change, via altered trophic interactions and plant response traits, is key to understanding future crop resistance to herbivory. In this study, we hypothesized that atmospheric carbon enrichment would increase the amount (biomass) and quality (C:N ratio) of crop plant resources for above- and belowground herbivore species. In a controlled environment facility, we conducted a microcosm experiment using the large raspberry aphid (*Amphorophora idaei*), the root feeding larvae of the vine weevil (*Otiorrhynchus sulcatus*), and the raspberry (*Rubus idaeus*) host-plant. There were four herbivore treatments (control, aphid only, weevil only and a combination of both herbivores) and an ambient (aCO₂) or elevated (eCO₂) CO₂ treatment (390 versus 650 ± 50 μmol/mol) assigned to two raspberry cultivars (cv Glen Ample or Glen Clova) varying in resistance to aphid herbivory. Contrary to our predictions, eCO₂ did not increase crop biomass or the C:N ratio of the plant tissues, nor affect herbivore abundance either directly or via the host-plant. Root herbivory reduced belowground crop biomass under aCO₂ but not eCO₂, suggesting that crops could tolerate attack in a CO₂ enriched environment. Root herbivory also increased the C:N ratio in leaf tissue at eCO₂, potentially due to decreased N uptake indicated by lower N concentrations found in the roots. Root herbivory greatly increased root C concentrations under both CO₂ treatments. Our findings confirm that responses of crop biomass and biochemistry to climate change need examining within the context of herbivory, as biotic interactions appear as important as direct effects of eCO₂ on crop productivity.

Keywords: aphid, vine weevil, carbon, nitrogen, plant productivity, aboveground, belowground

INTRODUCTION

Root herbivory is very damaging to plants, especially when combined with multiple biotic and abiotic stresses (Zvereva and Kozlov, 2012) that can lead to substantial losses of crop yields (Villani and Wright, 1990; Blossey and Hunt-Joshi, 2003; Blackshaw and Kerry, 2008). Crop traits such as compensatory growth are key to crop survival and primary productivity in the face of herbivore

pest pressure (Strauss and Agrawal, 1999; Watts et al., 2011; Huang et al., 2012; Robert et al., 2014). Plants, however, generally are less able to compensate for root herbivory compared to shoot herbivory (Johnson et al., 2016a). Moreover, even in simple agroecosystems insect herbivores occur as part of an above–belowground community (Megías and Müller, 2010; Soler et al., 2012). Consequently, the direct and indirect (mediated by host-plant plasticity) interactions among plants and herbivores occupying different guilds or niches, are key to understanding crop resistance and resilience to herbivory (Johnson et al., 2009; Huang et al., 2013; McKenzie et al., 2013; Hagenbucher et al., 2014).

Environmental stressors such as drought, elevated atmospheric CO₂ (eCO₂) and temperature can modify these trophic interactions (Johnson et al., 2011b; Stevnak et al., 2012; Ryalls et al., 2013; Johnson et al., 2016a,b). Atmospheric CO₂ concentrations are predicted to continue increasing during the 21st century and this is likely to affect plant productivity directly (Ainsworth and Long, 2005; Leakey et al., 2009; IPCC, 2013). For instance, greater accrual of plant biomass or altered biochemistry is one outcome of eCO₂ (e.g., Hentley et al., 2014; Dáder et al., 2016). However, such effects may vary greatly due to intrinsic differences between plant species or the presence of other environmental stressors such as water stress or herbivory (Ainsworth and Long, 2005; Bader et al., 2009; Kohler et al., 2009; Johnson et al., 2011a; Johnson and Riegler, 2013). Changes to plant productivity has the potential to affect the performance of herbivores via changes in the quality (e.g., altered C and N content) of their plant food resource (DeLucia et al., 2012; Robinson et al., 2012). For example, in an eCO₂ environment concentrations of N typically decrease by 17% in leaves and by 7% in roots (Robinson et al., 2012). This results in higher C:N ratios in plant tissues which generally reduces host plant quality for herbivores (Luo et al., 2006; Dáder et al., 2016), but this is a far from universal response. Many insect taxa respond idiosyncratically depending on species (e.g., aphids: Bezemer et al., 1999; Newman et al., 2003; Sun and Ge, 2011; Dáder et al., 2016; Ryalls and Harrington, 2016; Trebicki et al., 2016) or empirical information is so scarce for other groups (e.g., Staley and Johnson, 2008) that we cannot generalize either way. Moreover, while plant biomass or nutrient levels may alter in an eCO₂ environment this may be moderated by the effects of herbivory. For instance, Johnson and Riegler (2013) showed concomitant increases in root herbivory in *Eucalyptus* seedlings, reversed several of the effects of elevated CO₂ on plant growth and chemistry.

Herbivores shape plant primary productivity either by manipulating chemistry directly (e.g., aphid induced changes in source–sink relations; Crawley, 1989) or causing the plant to mobilize resources away from sites of attack (e.g., induced resource sequestration; Orians et al., 2011). Induced resource sequestration is thought to be a tolerance strategy to relocate resources temporarily away from the attacker (Kaplan et al., 2008; Schultz et al., 2013). This has traditionally focussed on plant attack aboveground, with photoassimilate transported to the roots for storage following shoot herbivory. Whether plants

translocate primary compounds in the reverse direction in response to root herbivory has been subject to recent debate (Johnson et al., 2016a,b). Evidence is limited, but Robert et al. (2014) showed that maize plants infested with root herbivores allocated carbon to the stems as a prelude to root regrowth. Similarly, nitrogen reallocated from roots to shoots in knapweed (Newingham et al., 2007) and the stems in milkweed (Tao and Hunter, 2013) following root attack. It has been suggested, however, that root herbivores may manipulate their hosts to allocate primary metabolites belowground to improve host plant quality (Erb et al., 2013). Indeed, there is evidence that root herbivory causes increases in root carbon (Pierre et al., 2012; Robert et al., 2014) and blackcurrant (*Ribes nigrum*) plants attacked by root-feeding vine weevils had 72% lower concentrations of foliar phosphorus, with a concomitant increase of 56% in the roots (Johnson et al., 2013). In the present study, we term this ‘feeding-induced resource accumulation.’

It is clear that herbivores have the capacity to moderate plant primary chemistry and these impacts may vary at different CO₂ concentrations. In this study we investigate how eCO₂ influences plant (red raspberry *Rubus idaeus* L.) growth and primary chemistry when under attack from an aboveground (large raspberry aphid – *Amphorophora idaei* Börner) and belowground (vine weevil larvae – *Otiorrhynchus sulcatus* F.) herbivore. Moreover, these two herbivores are thought to influence one another positively when sharing a host plant (McKenzie et al., 2013). In this study, we hypothesized that atmospheric carbon enrichment would alter the amount and quality of resources for herbivore species thus altering crop susceptibility to herbivory. Specifically we predicted that:

- (i) eCO₂ would cause an increase in plant biomass and the C:N ratio of above and belowground plant tissues,
- (ii) the CO₂ driven increase in host-plant biomass would result in greater herbivore abundance, above and belowground, but this may be negated by high C:N reducing host-plant quality
- (iii) root herbivory will impede crop biomass gains under eCO₂ and alter plant primary chemistry, via one or more mechanisms including impaired uptake of N, induced resource sequestration or feeding-induced resource accumulation.

MATERIALS AND METHODS

Experimental Design

A microcosm experiment was carried out with 192 individual raspberry plants challenged with multifactorial combinations of herbivore, cultivar, and CO₂ treatments. The experiment was performed in three runs (64 plants × 3 occasions) to avoid pseudoreplication and with CO₂ treatments switched between different chambers per run to avoid any potential influence of chamber identity on the experiment. Each experimental run was of 10-weeks duration so the whole experiment spanned in total the period November 2011 – November 2012. Two cultivars (Glen Ample or Glen Clova), which varied in resistance to insect

herbivory (Glen Clova was selectively bred for resistance to aphid herbivory), were exposed to an herbivore treatment comprising four levels: (i) herbivore-free control, (ii) aphid only, (iii) weevil only, and (iv) both herbivores present (12 plant replicates each). These herbivore \times cultivar combinations were further challenged by exposure to either ambient ($390 \pm 50 \mu\text{mol/mol}$) or elevated ($650 \pm 50 \mu\text{mol/mol}$) atmospheric CO₂ concentrations ($n = 96$), with the latter based on Climate Change (2007) predictions of atmospheric CO₂ concentrations by 2100. Individual plant replicates were assigned to randomized blocks within four controlled environment chambers ($\sim 4 \text{ m} \times 9 \text{ m}$) of the GroDome™ climate change research facility at the Centre for Ecology and Hydrology (CEH), Wallingford, UK. A CO₂ sensor (GMW22; Vaisala, Finland) in every chamber and was connected to a controller unit (AL2-24MR-D micro-controller, Mitsubishi, Japan). If CO₂ levels fell below the treatment level (390 and $650 \mu\text{mol/mol}$, respectively), CO₂ gas (BOC, UK) was injected for 1 s, followed by a 30 s delay, repeating until the required atmospheric concentration was reached.

Individual plants were grown for 10-weeks from rootstock in the CO₂ treatment chambers to which they were assigned. Photoperiod was maintained at 16:8 h (light:dark) with additional lighting provided by halide bulbs (400W) when photosynthetically active radiation (PAR) dropped below $400 \mu\text{mol/s/m}^2$, and a controlled daytime temperature of 18°C ($\pm 2^\circ\text{C}$) and minimum night temperature of 10°C ($\pm 2^\circ\text{C}$). Weevil eggs collected from cultures maintained at 18°C were added (20 per replicate) to the soil of appropriate replicates (weevil only and both herbivore treatment) in Week 4, with egg hatch occurring some 2 weeks later (Son and Lewis, 2005). Three adult large raspberry aphids were added to the upper-most unfurled leaf of the appropriate plants (aphid only and both herbivore treatment) in Week 8. The chronological sequence of weevil and aphid colonization of host-plants simulated in this experiment mimics the natural phenology of these organisms observed in the field (Moorhouse et al., 1992; McMenemy et al., 2009).

Plant and Insect Sampling

After 10 weeks, aphid population sizes were determined by counts and removal of individuals. Vine weevil larvae were extracted from the soil for 24 h with Tullgren funnels and counted. Plants were carefully removed from the soil, roots washed and a random sample of leaves and roots was taken and snap-frozen in liquid nitrogen for analysis of plant primary chemistry. The remainder of the aboveground (stems, leaves) and belowground (root) plant biomass was then oven-dried (80°C for 24 h) and weighed (g). After being snap-frozen the roots and shoot samples were freeze dried for 24 h, then the tissue samples ($\leq 5 \text{ mg}$) were ball-milled to a fine powder for subsequent C:N analysis. Chemical analysis of carbon and nitrogen concentrations of leaf and root tissue was undertaken at the Centre for Ecology and Hydrology (Lancaster), using an Exeter Analytical Elemental Analyser (EAI, Coventry, UK).

Statistical Analysis

Co-linearity amongst parameters of plant biomass and biochemistry was initially assessed with Pearson

correlation coefficients (proc CORR in SAS version 9.3). Subsequently, the response of plant biometrics (above- and belowground biochemistry and biomass) and herbivore abundance (aphid and weevil counts) to experimental treatments were analyzed with generalized linear mixed effects models (proc GLIMMIX). Categorical experimental treatments were: 'herbivore' (herbivore-free control, aphid only, weevil only, both herbivores), 'Cultivar' (Glen Ample or Glen Clova) and 'CO₂ regime' (aCO₂ or eCO₂). For models of insect herbivore abundance, 'herbivore treatment' was replaced by continuous predictors: above- or belowground plant dry weight, % concentration of C, N, or C:N ratio of leaves or roots. Plant responses were modeled with Gaussian distribution and an identity link function, plant biomass was log transformed to meet the assumption that residuals were normally distributed with homogeneity of variance. Aphid and weevil counts were modeled with a Poisson distribution and a log link function.

Random effects were fitted to all models to account for different chambers used during the three experimental runs (chamber nested within run) and the randomized block design (block). Over-dispersion of count data in herbivore abundance models was accounted for with an observation-level parameter 'plant replicate' fitted as an additional random effect (Elston et al., 2001). The full model (experimental treatments and their pairwise interactions) was simplified through backward stepwise elimination of the least significant term (interactions before main effects) until a minimum adequate model was obtained. F-ratios and *p*-values reported are adjusted (SAS type III) for the other significant parameters retained in the final reduced model. Statistical significance of main effects are always reported, whereas two-way interactions are reported only where $P < 0.05$. Degrees of freedom were estimated using the Satterthwaite approximation (Littell et al., 1996). Least square means (with Bonferroni adjusted *p*-values) were plotted to show the effect of the significant explanatory variables conditional on other effects in the final models.

RESULTS

Crop Biomass

Above- and belowground biomass were positively correlated (0.67 ; $p < 0.0001$). In contrast to our prediction, eCO₂ concentrations did not increase crop biomass overall, either aboveground ($F_{1,4} = 1.78$, $p = 0.2544$) or belowground ($F_{1,4} = 3.54$, $p = 0.1345$). There was, however, an interaction between CO₂ treatment and crop cultivar ($F_{1,175} = 4.52$, $p = 0.0349$), explained by cv. Glen Ample accruing greater aboveground biomass than cv. Glen Clova at eCO₂ levels (Bonferroni adjusted $p = 0.0252$).

Although there was no indication of any effect of herbivore treatment on aboveground biomass ($F_{3,173} = 0.44$ $p = 0.7275$), root herbivory consistently reduced root biomass with treatments where weevil larvae were present (weevil only, both herbivore species) yielding significantly less root biomass than treatments without weevils (control and aphid only; $F_{3,172} = 5.88$,

$p = 0.0008$, **Figure 1**). Root biomass was also affected by the significant interaction between the herbivore and CO₂ treatments ($F_{3,172} = 4.66$, $p = 0.0037$, **Figure 1**). While under aCO₂ conditions root biomass was significantly reduced by treatments including root-feeding weevils (weevils only and both herbivore species), this effect dissipated under eCO₂ (**Figure 1**), suggesting a mitigation of herbivory on roots.

The identity of the crop cultivar also had an influence on above- and belowground crop biomass. Aboveground biomass was greatest in the cultivar (Glen Clova) selectively bred to be most resistant to aphid herbivory (Glen Clova LS mean = -0.32 ± 0.17 ; Glen Ample LS mean = -0.19 ± 0.17 ; $F_{1,175} = 3.93$, $p = 0.0349$). Whereas, belowground biomass was significantly greater in the cultivar (Glen Ample) that was less resistant to aphid herbivory (Glen Clova LS mean = -0.03 ± 0.16 ; Glen Ample LS mean = -0.23 ± 0.16 ; $F_{1,171} = 4.17$, $p = 0.0427$).

Crop Biochemistry

Correlation analysis revealed the intimately connected balance of C and N within the crop plant and these relationships are shown in Supplementary Material (Appendix S1).

As with aboveground crop biomass, and contrary to prediction, the experimental eCO₂ treatment had little overall impact on plant tissue biochemistry. There was only a slight increase in percent leaf C (LS mean: ambient = 42.27, elevated = 42.98 ± 0.1658 ; $F_{1,4} = 9.24$, $p = 0.0388$), with little overall effect on leaf N ($F_{1,4} = 6.26$, $p = 0.0672$) and hence the C:N ratio of leaves ($F_{1,4} = 6.47$, $p = 0.0666$). The CO₂ treatment had no effect on the percent C ($F_{1,4} = 0.00$, $p = 0.9968$), percent N ($F_{1,4} = 0.50$, $p = 0.5207$) or the C:N ratio ($F_{1,4} = 0.59$, $p = 0.4909$) of roots.

There was no evidence that the herbivore treatment affected the overall percent content of C ($F_{3,177} = 0.98$, $p = 0.4019$) or N ($F_{3,174} = 1.82$, $p = 0.1452$) or the C:N ratio ($F_{3,169} = 2.00$,

$p = 0.1158$, **Figure 3**) of leaf tissues. While root herbivory did not significantly affect belowground N content ($F_{3,174} = 2.24$, $p = 0.0851$), it did greatly increase the C content of root tissues relative to control and aphid treatments ($F_{3,171} = 30.99$, $p < 0.0001$, **Figure 2**). This herbivore effect was reflected in a higher C:N ratio ($F_{3,174} = 4.68$, $p = 0.0036$) in roots where belowground herbivory was present, relative to the aphid-only herbivore treatment (**Figure 3**).

Furthermore, similar to the effect of root herbivory on belowground biomass (see above), the interaction between the herbivore and CO₂ treatments affected percentage N ($F_{3,174} = 4.02$, $p = 0.0085$) and C:N ratio ($F_{3,169} = 3.01$, $p = 0.0319$) of leaves. At aCO₂ conditions, the leaf N content (**Figure 4A**) and C:N ratio (**Figure 4B**) was unaffected by root-feeding weevils or foliar-feeding aphids. Under eCO₂ conditions, however, root-feeding weevils generally decreased N content (**Figure 4A**) and hence increased the aboveground C:N ratio (**Figure 4B**).

Crop cultivar affected the C content of above- and belowground tissues. Leaf C content was generally greater in cultivar Glen Clova (LS mean = 42.89 ± 0.13) than Gl. Ample (LS mean = 42.36 ± 0.13 ; $F_{1,180} = 15.83$, $p = 0.0001$). Root C content was similarly higher in Glen Clova (LS mean = 445.38 ± 0.55) than Glen Ample (LS mean = 44.33 ± 0.55 ; $F_{1,169} = 24.62$, $p < 0.0001$). The interaction between the CO₂ treatment and cultivar also affected crop biochemistry, with the greatest effects in aboveground tissues (**Table 1**). The C content of Glen Clova leaves was increased significantly by exposure to an eCO₂ environment, whereas Glen Ample was largely unaffected (**Table 1**). While the impact on root C content was generally lower, there was a significant difference in the response of the cultivars to eCO₂ with Glen Clova allocating more C to roots (**Table 1**). Similarly, leaf N content was lowered by CO₂ treatment in both cultivars, but was most pronounced in the Glen Clova cultivar, while root N was largely unaffected by this

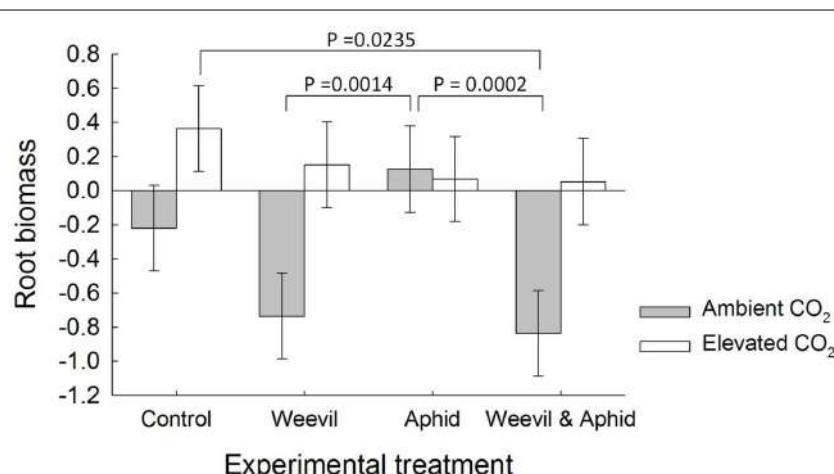


FIGURE 1 | The effect on raspberry root biomass of CO₂ treatment (dark bars = ambient $390 \pm 50 \mu\text{mol/mol}$; light bars = elevated $650 \pm 50 \mu\text{mol/mol}$) and herbivore treatments (herbivore-free control, root-feeding weevil only, foliar-feeding aphid only, both herbivores). Data are least square means \pm SE derived from final GLMM accounting for variation due to other treatments. Difference among treatments following Bonferroni adjustment for multiple comparisons indicated with solid lines ($p < 0.05$).

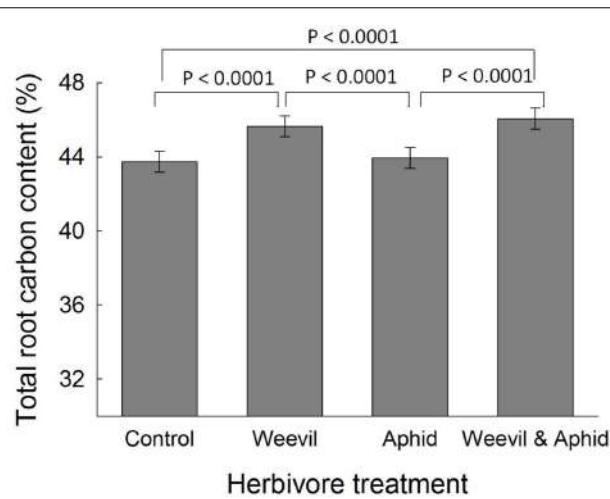


FIGURE 2 | The effect of herbivore treatment (herbivore-free control, root-feeding weevil only, foliar-feeding aphid only, both herbivores) on the carbon content (%) of raspberry roots. Data are least square means \pm SE derived from final GLMM accounting for variation due to other treatments. Difference among treatments following Bonferroni adjustment for multiple comparisons indicated with solid lines ($p < 0.05$).

interaction (Table 1). These shifts in the crop biochemical balance translated into a highly significant increase in the aboveground C:N ratio following exposure to an eCO₂ environment, largely driven by the cultivar most resistant to herbivory (Glen Clova; Table 1).

Insect Herbivore Responses

Aphid abundance was weakly but positively related to leaf C content (Figure 5; $F_{1,77} = 4.47$, $p = 0.0378$). There was no statistically significant evidence that aphid abundance was related

to either aboveground crop biomass ($F_{1,69} = 2.77$, $p = 0.0770$), leaf N content ($F_{1,81} = 3.44$, $p = 0.0674$) or the leaf C:N ratio ($F_{1,74} = 1.16$, $p = 0.2860$). Weevil abundance was positively related to root C content (Figure 5; $F_{1,76} = 5.56$, $p = 0.0210$), but not root N ($F_{1,83} = 0.41$, $p = 0.5253$) or belowground biomass ($F_{1,71} = 1.80$, $p = 0.1838$) or the root C:N ratio ($F_{1,80} = 0.160$, $p = 0.6862$).

Despite bred resistance to aphid herbivory (cv. Glen Clova), there was no significant differences in insect herbivore abundance between the cultivars (aphid: $F_{1,69} = 0.48$, $p = 0.4894$; weevil: $F_{1,68} = 0.63$, $p = 0.4311$) nor was there any direct effect of the CO₂ treatments on herbivore abundance (aphid: $F_{1,4} = 3.58$, $p = 0.4957$; weevil: $F_{1,4} = 0.55$, $p = 0.4996$).

There was no evidence that the abundance of each herbivore was influenced by the abundance of the other species (weevil: $F_{1,37} = 3.01$, $p = 0.0911$; aphid: $F_{1,26} = 2.44$, $p = 0.1305$), and hence no indication of a positive or negative plant-mediated herbivore interaction in this study.

DISCUSSION

Contrary to our first prediction, eCO₂ did not directly increase crop biomass or the C:N ratio of the plant tissues. Enhanced growth rates in response to eCO₂ are common (Hentley et al., 2014; Dáder et al., 2016), especially in C3 plant species that at current CO₂ concentrations operate below the maximum capacity of the carboxylating plant enzyme Rubisco (Ainsworth and Long, 2005; Leakey et al., 2009). These gains in biomass, however, range between 0 and 20% depending on plant species or functional type, for instance tree species typically accrue greater biomass than cereal crops or many wild herbaceous species (Ainsworth and Long, 2005; Ainsworth and Rogers, 2007; DeLucia et al., 2012). Furthermore, plant growth can even decrease in response to eCO₂ according to the presence

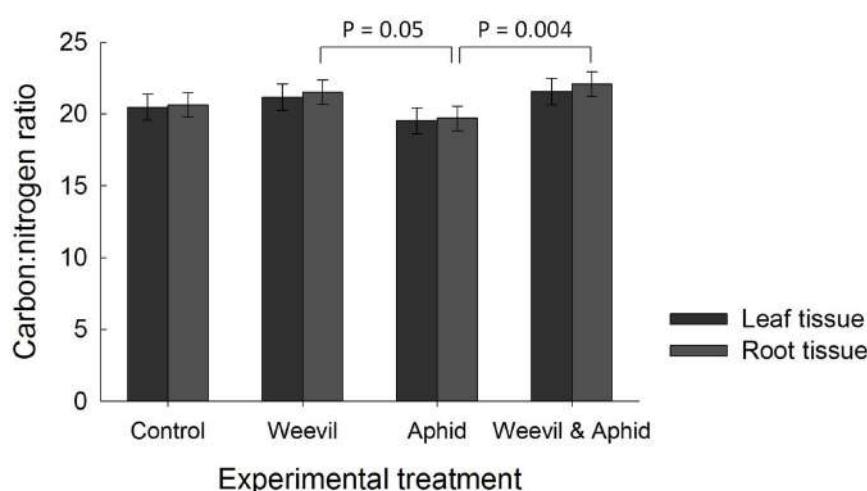


FIGURE 3 | The effect of herbivore treatment (herbivore-free control, root-feeding weevil only, foliar-feeding aphid only, both herbivores) on the ratio of carbon to nitrogen (C:N) in raspberry leaf (dark bars) and root (light bars) tissues. Data are least square means \pm SE derived from final GLMM accounting for variation due to other treatments. Difference among treatments following Bonferroni adjustment for multiple comparisons indicated with solid lines ($p < 0.05$).

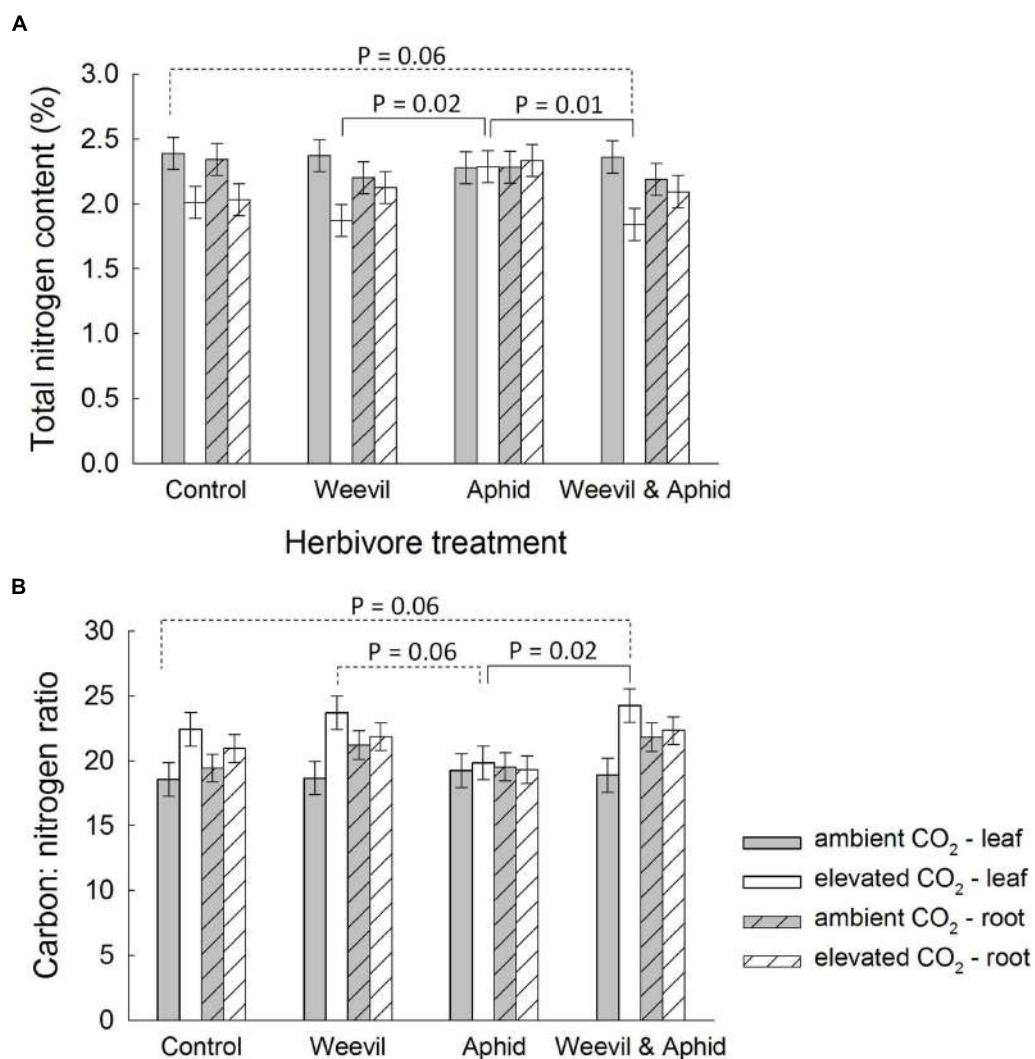


FIGURE 4 | The effect on raspberry (A) carbon to nitrogen (C:N) ratio and (B) nitrogen content (%) of the interaction between CO₂ (dark bars = ambient 390 ± 50 µmol/mol; light bars = elevated 650 ± 50 µmol/mol) and herbivore treatments (herbivore-free control, root-feeding weevil only, foliar-feeding aphid only, both herbivores). Data are least square means ± SE derived from final GLMM accounting for variation due to other treatments. Difference among treatments following Bonferroni adjustment for multiple comparisons indicated with dashed (marginally non-significant) or solid lines ($p < 0.05$).

TABLE 1 | The effect on crop primary biochemistry of the interaction between crop cultivar and experimental CO₂ treatment.

Cultivar	Glen Clova		Glen Ample		F(df)	P
CO ₂ regime	390 µmol/mol	650 µmol/mol	390 µmol/mol	650 µmol/mol		
Leaf						
Nitrogen (%)	2.49 ± 0.11	1.96 ± 0.11	2.21 ± 0.11	2.04 ± 0.11	8.38 (1, 174)	0.0043
Carbon (%)	42.34 ± 0.19	43.44 ± 0.19	42.20 ± 0.19	42.52 ± 0.19	8.55 (1, 180)	0.0039
C:N	17.75 ± 1.13	23.34 ± 1.13	19.91 ± 1.13	21.74 ± 1.13	8.90 (1, 169)	0.0033
Root						
Nitrogen (%)	2.34 ± 0.11	2.14 ± 0.11	2.17 ± 0.11	2.15 ± 0.11	3.78 (1, 173)	0.0535
Carbon (%)	45.16 ± 0.77	45.59 ± 0.77	44.54 ± 0.77	44.11 ± 0.77	4.20 (1, 169)	0.0420
C:N	19.78 ± 1.09	21.96 ± 1.09	21.08 ± 1.09	21.11 ± 1.09	4.99 (1, 173)	0.0268

Data are least-square means and F & P values derived from final GLMM for each crop parameter.

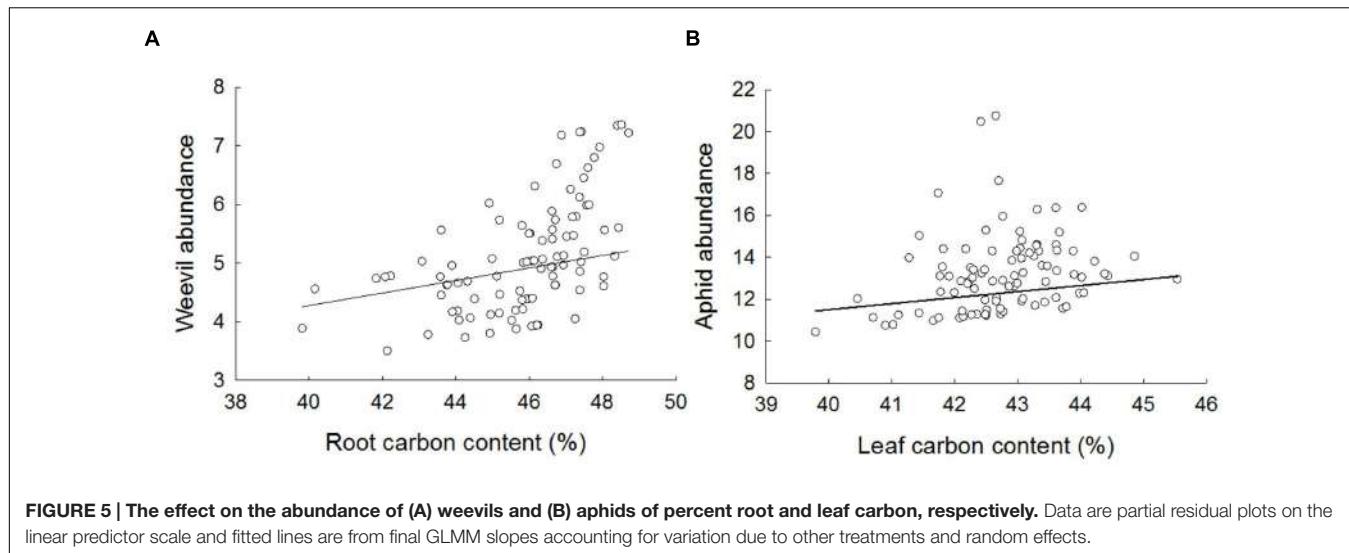


FIGURE 5 | The effect on the abundance of (A) weevils and (B) aphids of percent root and leaf carbon, respectively. Data are partial residual plots on the linear predictor scale and fitted lines are from final GLMM slopes accounting for variation due to other treatments and random effects.

of other environmental stressors, such as water availability (Bader et al., 2009; Kohler et al., 2009). Herbivores can also offset any plant biomass gain due to eCO₂ by compensating for lower host-plant quality (e.g., reduced N content) by increasing or maintaining feeding rates through behavioral or physiological plasticity (Barbehenn et al., 2004; Johnson et al., 2011a).

Aphid and weevil abundance were independent of atmospheric CO₂ concentrations, therefore there was also no evidence to support our second prediction that eCO₂ would increase insect herbivore abundance. This finding fits among the many examples of aphids showing positive, negative or neutral responses to CO₂ treatments (Bezemer et al., 1999; Newman et al., 2003; Sun and Ge, 2011; Dáder et al., 2016; Trébicki et al., 2016). Elsewhere, the nitrogen status (e.g., C:N ratio) of plant tissues has been shown to be intimately related to life-history or population performance of other aphid species under eCO₂ (e.g., *Myzus persicae* Sulzer – Dáder et al., 2016; *Rhopalosiphum padi* L. – Trébicki et al., 2016). For instance, eCO₂ decreased the foliar N content, but not the C content, in pepper plants (*Capsicum annuum* L.) leading to longer individual development and lower fecundity of *Myzus persicae* due to an unfavorable nutritional quality of the host-plant (Dáder et al., 2016). In this experiment, the comparatively weak effects of eCO₂ on the nitrogen balance in these raspberry cultivars offer a potential explanation for the lack of an effect on the aphid or weevil herbivore. Although unquantified here, this lack of a profound eCO₂ effect on the C–N balance implies it was unlikely to have modified the herbivore nutrients (e.g., essential amino acids) or the physical (e.g., cuticular waxes) or secondary (i.e., salicylic acid signaling pathway) defenses governing crop-herbivore interactions (Sun and Ge, 2011).

To understand better crop performance in eCO₂ environments more work is clearly needed to unravel the interplay between, biochemical state, insect nutrition and performance in different crop varieties. In agreement with our study, Hentley et al. (2014) showed *A. idaei* did not respond

to eCO₂ when reared on these same raspberry cultivars (Glen Ample and Glen Clova) in the absence of the competing belowground herbivore. Similarly, Martin and Johnson (2011) also reported that *A. idaei* was unaffected by eCO₂ on two other raspberry cultivars (Glen Rosa and Malling Jewel). However, aphid performance improved under eCO₂ on other raspberry cultivars (Glen Lyon in Martin and Johnson, 2011; cv. Octavia – Hentley et al., 2014). These different outcomes among experiments and cultivars may point to the pre-dominance of the host-plant and insect identity over climate effects for herbivore performance, or just simply to experimental artifacts. Nonetheless, further experimental information on the role of different cultivars in shaping herbivory under climate change should continue to be an important avenue of research.

In terms of insect interactions, this experiment did not find evidence for the previously observed reciprocal feeding facilitation between these two spatially separated herbivores at aCO₂ (McKenzie et al., 2013). Different crop growing conditions, use of different climate controlled facilities, and the fact that the current experiment was performed over a longer time-period (three 10-week runs over a calendar year vs. single run of 10 weeks) could explain this difference between these two studies.

Root herbivory affected root biomass and the C:N ratio of above- and belowground crop tissues and this was modified by the level of atmospheric CO₂ that the crop experienced. In accord with our third prediction, root herbivory reduced belowground biomass significantly under aCO₂ conditions, however, this impact dissipated under eCO₂. This suggests a mitigation of herbivory on roots, potentially via impacts on herbivore performance at the individual or population level in an enriched CO₂ atmosphere (Johnson et al., 2011a).

The most likely mechanism explaining the nullification of root herbivory is that increased concentrations of atmospheric carbon enable enhanced compensatory root re-growth, therefore

lessening the net root loss. The net effect of the combination of root herbivory and eCO₂ was similar to that found by Johnson and Riegler (2013), where the same combination produced root biomass at levels similar to those at aCO₂ concentrations in the absence of herbivory. A notable difference is that Johnson and Riegler (2013) showed eCO₂ to increase root biomass, which was subsequently reduced by herbivory; whereas here loss of biomass by root-herbivory under aCO₂ conditions was mitigated by increased root production at eCO₂. The net effect, however, remains the same with the abiotic and biotic pressures balancing one another.

Mirroring the change in crop biomass, the leaf C:N ratio was increased by root herbivory at eCO₂, but not aCO₂ conditions. This finding is consistent with our third prediction that root herbivores would cause changes in primary chemistry. We suggest that damage to roots from herbivory would restrict the uptake of nitrogen from the soil, as evidenced by the lower N concentrations in roots, and this likely shifted the C:N ratio in leaves (Zvereva and Kozlov, 2012). We found no support for induced resource sequestration (i.e., movement of C or N to the shoots) as a result of root herbivory, since foliar concentrations were not affected by either herbivore. On the contrary, we found evidence that root herbivores increased C concentrations in the roots. This may reflect ‘feeding-induced resource accumulation’ either because the herbivore is manipulating the plant for its own benefit, or the plant is mobilizing resources for root regrowth.

This study emphasizes the importance of understanding crop biomass and biochemical responses to climate change in the context of herbivory. In this system, biotic interactions appear as important as direct effects of climate change on crop productivity. Experimental work should continue to test how increasing the

REFERENCES

- Ainsworth, E. A., and Long, S. P. (2005). What have we learned from 15 years of free-air CO₂ enrichment (FACE)? A meta-analytic review of the responses of photosynthesis, canopy properties and plant production to rising CO₂. *New Phytol.* 165, 351–372. doi: 10.1111/j.1469-8137.2004.01224.x
- Ainsworth, E. A., and Rogers, A. (2007). The responses of photosynthesis and stomatal conductance to rising [CO₂]: mechanisms and environmental interactions. *Plant Cell Environ.* 30, 258–270. doi: 10.1111/j.1365-3040.2007.01641.x
- Bader, M., Hiltbrunner, E., and Körner, C. (2009). Fine root responses of mature deciduous forest trees to free air carbon dioxide enrichment (FACE). *Funct. Ecol.* 23, 913–921. doi: 10.1111/j.1365-2435.2009.01574.x
- Barbehenn, R., Karowe, D., and Chen, Z. (2004). Performance of a generalist grasshopper on a C3 and a C4 grass: compensation for the effects of elevated CO₂ on plant nutritional quality. *Oecologia* 140, 96–103. doi: 10.1007/s00442-004-1555-x
- Bezemer, T., Knight, K. J., Newington, J. E., and Jones, T. H. (1999). How general are aphid responses to elevated atmospheric CO₂? *Ann. Entomol. Soc. Am.* 92, 724–730. doi: 10.1093/esa/92.5.724
- Blackshaw, R., and Kerr, B. (2008). *Root Herbivory in Agricultural Ecosystems. Root Feeders: An Ecosystem Perspective*. Wallingford: CAB International, 35–53.
- Blossey, B., and Hunt-Joshi, T. R. (2003). Belowground herbivory by insects: influence on plants and aboveground herbivores. *Annu. Rev. Entomol.* 48, 521–547. doi: 10.1146/annurev.ento.48.091801.112700
- Climate Change (2007). “Climate change 2007: the physical science basis,” in *Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change*, eds S. Solomon, D. Qin, M. Manning, Z. Chen, M. Marquis, K. B. Averyt, et al. (Cambridge: Cambridge University Press), 996.
- Crawley, M. J. (1989). Insect herbivores and plant population dynamics. *Annu. Rev. Entomol.* 34, 531–562. doi: 10.1146/annurev.en.34.010189.002531
- Dáder, B., Fereres, A., Moreno, A., and Trébicki, P. (2016). Elevated CO₂ impacts bell pepper growth with consequences to *Myzus persicae* life history, feeding behaviour and virus transmission ability. *Sci. Rep.* 6, 19120. doi: 10.1038/srep19120
- DeLucia, E. H., Nabity, P. D., Zavala, J. A., and Berenbaum, M. R. (2012). Climate change: resetting plant-insect interactions. *Plant Physiol.* 160, 1677–1685. doi: 10.1104/pp.112.204750
- Elston, D. A., Moss, R., Boulinier, T., Arrowsmith, C., and Lambin, X. (2001). Analysis of aggregation, a worked example: numbers of ticks on red grouse chicks. *Parasitology* 122, 563–569. doi: 10.1017/S0031182001007740
- Erb, M., Huber, M., Robert, C. A., Ferrier, A. P., Machado, R. A., and Arce, C. C. (2013). The role of plant primary and secondary metabolites in root-herbivore behaviour, nutrition and physiology. *Adv. Insect Physiol.* 45, 53–95. doi: 10.1016/B978-0-12-417165-7.00002-7
- Hagenbucher, S., Wäckers, F. L., and Romeis, J. (2014). Indirect multi-trophic interactions mediated by induced plant resistance: impact of caterpillar feeding on aphid parasitoids. *Biol. Lett.* 10, 20130795. doi: 10.1098/rsbl.2013.0795
- Hentley, W. T., Hails, R. S., Johnson, S. N., Jones, T. H., and Vanbergen, A. J. (2014). Top-down control by *Harmonia axyridis* mitigates the impact of elevated atmospheric CO₂ on a plant-aphid interaction. *Agric. For. Entomol.* 16, 350–358. doi: 10.1111/afe.12065

trophic complexity of the crop system affects species interactions and crop performance in a carbon-enriched world (Soler et al., 2012; Hentley et al., 2014; Dáder et al., 2016; Trébicki et al., 2016).

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SM helped design and run the experiment, carried out measurements and contributed to analysis and paper preparation. THJ and RH helped conceive and design the experiment and contributed to the writing of the paper. AV helped conceive, design the experiment, analyzed the data, and prepared the paper. SJ helped conceive and design the experiment and prepared the paper. NO conceived and oversaw biochemical analysis of the experiment and contributed to the writing of the paper.

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- Huang, J., Liu, M., Chen, F., Griffiths, B. S., Chen, X., Johnson, S. N., et al. (2012). Crop resistance traits modify the effects of an aboveground herbivore, brown planthopper, on soil microbial biomass and nematode community via changes to plant performance. *Soil Biol. Biochem.* 49, 157–166. doi: 10.1016/j.soilbio.2012.02.022
- Huang, W., Siemann, E., Yang, X., Wheeler, G. S., and Ding, J. (2013). Facilitation and inhibition: changes in plant nitrogen and secondary metabolites mediate interactions between above-ground and below-ground herbivores. *Proc. R. Soc. B Biol. Sci.* 280, 20131318. doi: 10.1098/rspb.2013.1318
- IPCC (2013). “Summary for policymakers,” in *Climate Change 2013: The Physical Science Basis. Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change*, eds D. Q. T. F. Stocker, G.-K. Plattner, M. Tignor, S. K. Allen, J. Boschung, A. Nauels, et al. (Cambridge: Cambridge University Press).
- Johnson, S. N., Barton, A. T., Clark, K. E., Gregory, P. J., McMenemy, L. S., and Hancock, R. D. (2011a). Elevated atmospheric carbon dioxide impairs the performance of root-feeding vine weevils by modifying root growth and secondary metabolites. *Glob. Change Biol.* 17, 688–695. doi: 10.1111/j.1365-2486.2010.02264.x
- Johnson, S. N., Erb, M., and Hartley, S. E. (2016a). Roots under attack: contrasting plant responses to below- and aboveground insect herbivory. *New Phytol.* 210, 413–418. doi: 10.1111/nph.13807
- Johnson, S. N., Hawes, C., and Karley, A. J. (2009). Reappraising the role of plant nutrients as mediators of interactions between root- and foliar-feeding insects. *Funct. Ecol.* 23, 699–706. doi: 10.1111/j.1365-2435.2009.01550.x
- Johnson, S. N., Mitchell, C., McNicol, J. W., Thompson, J., and Karley, A. J. (2013). Downstairs drivers – root herbivores shape communities of above-ground herbivores and natural enemies via changes in plant nutrients. *J. Anim. Ecol.* 82, 1021–1030. doi: 10.1111/1365-2656.12070
- Johnson, S. N., and Riegler, M. (2013). Root damage by insects reverses the effects of elevated atmospheric CO₂ on eucalypt seedlings. *PLoS ONE* 8:e79479. doi: 10.1371/journal.pone.0079479
- Johnson, S. N., Ryalls, J. M. W., and Staley, J. T. (2016b). “Impacts of climate and atmospheric change on aboveground-belowground invertebrate interactions,” in *Global Climate Change and Terrestrial Invertebrates*, eds S. N. Johnson and T. H. Jones (Oxford: Wiley).
- Johnson, S. N., Staley, J. T., McLeod, F. A. L., and Hartley, S. E. (2011b). Plant-mediated effects of soil invertebrates and summer drought on above-ground multitrophic interactions. *J. Ecol.* 99, 57–65. doi: 10.1111/j.1365-2745.2010.01748.x
- Kaplan, I., Halitschke, R., Kessler, A., Rehill, B. J., Sardanelli, S., and Denno, R. F. (2008). Physiological integration of roots and shoots in plant defense strategies links above- and belowground herbivory. *Ecol. Lett.* 11, 841–851. doi: 10.1111/j.1461-0248.2008.01200.x
- Kohler, J., Caravaca, F., del Mar Alguacil, M., and Roldán, A. (2009). Elevated CO₂ increases the effect of an arbuscular mycorrhizal fungus and a plant-growth-promoting rhizobacterium on structural stability of a semiarid agricultural soil under drought conditions. *Soil Biol. Biochem.* 41, 1710–1716. doi: 10.1016/j.soilbio.2009.05.014
- Leakey, A. D. B., Ainsworth, E. A., Bernacchi, C. J., Rogers, A., Long, S. P., and Ort, D. R. (2009). Elevated CO₂ effects on plant carbon, nitrogen, and water relations: six important lessons from FACE. *J. Exp. Bot.* 60, 2859–2876. doi: 10.1093/jxb/erp096
- Littell, R. C., Milliken, G. A., Stroup, W. W., and Wolfinger, R. D. (1996). *SAS System for Mixed Models*. Cary, NC: SAS Institute Inc.
- Luo, Y., Hui, D., and Zhang, D. (2006). Elevated CO₂ stimulates net accumulations of carbon and nitrogen in land ecosystems: a meta-analysis. *Ecology* 87, 53–63. doi: 10.1890/04-1724
- Martin, P., and Johnson, S. N. (2011). Evidence that elevated CO₂ reduces resistance to the European large raspberry aphid in some raspberry cultivars. *J. Appl. Entomol.* 135, 237–240. doi: 10.1111/j.1439-0418.2010.01544.x
- McKenzie, S. W., Vanbergen, A. J., Hails, R. S., Jones, T. H., and Johnson, S. N. (2013). Reciprocal feeding facilitation between above- and below-ground herbivores. *Biol. Lett.* 9, 20130341. doi: 10.1098/rsbl.2013.0341
- McMenemy, L. S., Mitchell, C., and Johnson, S. N. (2009). Biology of the European large raspberry aphid (*Amphorophora idaei*): its role in virus transmission and resistance breakdown in red raspberry. *Agric. For. Entomol.* 11, 61–71. doi: 10.1111/j.1461-9563.2008.00409.x
- Megias, A. G., and Müller, C. (2010). Root herbivores and detritivores shape above-ground multitrophic assemblage through plant-mediated effects. *J. Anim. Ecol.* 79, 923–931. doi: 10.1111/j.1365-2656.2010.01681.x
- Moorhouse, E. R., Charnley, A. K., and Gillespie, A. T. (1992). A review of the biology and control of the vine weevil, *Otiorrhynchus sulcatus* (Coleoptera: Curculionidae). *Ann. Appl. Biol.* 121, 431–454. doi: 10.1111/j.1744-7348.1992.tb03455.x
- Newingham, B., Callaway, R., and BassiriRad, H. (2007). Allocating nitrogen away from a herbivore: a novel compensatory response to root herbivory. *Oecologia* 153, 913–920. doi: 10.1007/s00442-007-0791-2
- Newman, J. A., Gibson, D. J., Parsons, A. J., and Thornley, J. H. M. (2003). How predictable are aphid population responses to elevated CO₂? *J. Anim. Ecol.* 72, 556–566. doi: 10.1046/j.1365-2656.2003.00725.x
- Orians, C. M., Thorn, A., and Gómez, S. (2011). Herbivore-induced resource sequestration in plants: why bother? *Oecologia* 167, 1–9. doi: 10.1007/s00442-011-1968-2
- Pierre, P. S., Dugravot, S., Cortesero, A.-M., Poinsot, D., Raaijmakers, C. E., Hassan, H. M., et al. (2012). Broccoli and turnip plants display contrasting responses to belowground induction by *Delia radicum* infestation and phytohormone applications. *Phytochemistry* 73, 42–50. doi: 10.1016/j.phytochem.2011.09.009
- Robert, C. A. M., Ferrieri, R. A., Schirmer, S., Babst, B. A., Schueller, M. J., Machado, R. A. R., et al. (2014). Induced carbon reallocation and compensatory growth as root herbivore tolerance mechanisms. *Plant Cell Environ.* 37, 2613–2622. doi: 10.1111/pce.12359
- Robinson, E. A., Ryan, G. D., and Newman, J. A. (2012). A meta-analytical review of the effects of elevated CO₂ on plant–arthropod interactions highlights the importance of interacting environmental and biological variables. *New Phytol.* 194, 321–336. doi: 10.1111/j.1469-8137.2012.04074.x
- Ryalls, J., and Harrington, R. (2016). “Climate and atmospheric change impacts on aphids as vectors of plant diseases,” in *Global Climate Change and Terrestrial Invertebrates*, eds S. N. Johnson and T. H. Jones (Oxford: Wiley).
- Ryalls, J. M. W., Riegler, M., Moore, B. D., Lopaticki, G., and Johnson, S. N. (2013). Effects of elevated temperature and CO₂ on aboveground-belowground systems: a case study with plants, their mutualistic bacteria and root / shoot herbivores. *Front. Plant Sci.* 4:445. doi: 10.3389/fpls.2013.00445
- Schultz, J. C., Appel, H. M., Ferrieri, A. P., and Arnold, T. M. (2013). Flexible resource allocation during plant defense responses. *Front. Plant Sci.* 4:324. doi: 10.3389/fpls.2013.00324
- Soler, R., Putten, W., Harvey, J., Vet, L. M., Dicke, M., and Bezemer, T. M. (2012). Root herbivore effects on aboveground multitrophic interactions: patterns, processes and mechanisms. *J. Chem. Ecol.* 38, 755–767. doi: 10.1007/s10886-012-0104-z
- Son, Y., and Lewis, E. E. (2005). Modelling temperature-dependent development and survival of *Otiorrhynchus sulcatus* (Coleoptera: Curculionidae). *Agric. For. Entomol.* 7, 201–209. doi: 10.1111/j.1461-9555.2005.00260.x
- Staley, J. T., and Johnson, S. N. (2008). “Climate change impacts on root herbivores,” in *Root Feeders: An Ecosystem Perspective*, eds S. N. Johnson and P. J. Murray (Wallingford: CABI), 192–215.
- Stenvbak, K., Scherber, C., Gladbach, D. J., Beier, C., Mikkelsen, T. N., and Christensen, S. (2012). Interactions between above- and belowground organisms modified in climate change experiments. *Nat. Clim. Chang.* 2, 805–808. doi: 10.1038/nclimate1544
- Strauss, S. Y., and Agrawal, A. A. (1999). The ecology and evolution of plant tolerance to herbivory. *Trends Ecol. Evol.* 14, 179–185. doi: 10.1016/S0169-5347(98)01576-6
- Sun, Y., and Ge, F. (2011). How do aphids respond to elevated CO₂? *J. Asia-Pac. Entomol.* 14, 217–220. doi: 10.1016/j.aspen.2010.08.001
- Tao, L., and Hunter, M. D. (2013). Allocation of resources away from sites of herbivory under simultaneous attack by aboveground and belowground herbivores in the common milkweed, *Asclepias syriaca*. *Arthropod Plant Interact.* 7, 217–224. doi: 10.1007/s11829-012-9235-y
- Trębicki, P., Vandemeer, R. K., Bosque-Pérez, N. A., Powell, K. S., Dader, B., Freeman, A. J., et al. (2016). Virus infection mediates the effects of

- elevated CO₂ on plants and vectors. *Sci. Rep.* 6, 22785. doi: 10.1038/srep22785
- Villani, M. G., and Wright, R. J. (1990). Environmental influences on soil macroarthropod behavior in agricultural systems. *Annu. Rev. Entomol.* 35, 249–269. doi: 10.1146/annurev.en.35.010190.001341
- Watts, S. M., Dodson, C. D., and Reichman, O. J. (2011). The roots of defense: plant resistance and tolerance to belowground herbivory. *PLoS ONE* 6:e18463. doi: 10.1371/journal.pone.0018463
- Zvereva, E., and Kozlov, M. (2012). Sources of variation in plant responses to belowground insect herbivory: a meta-analysis. *Oecologia* 169, 441–452. doi: 10.1007/s00442-011-2210-y

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Integration of Plant Defense Traits with Biological Control of Arthropod Pests: Challenges and Opportunities

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Crop plants exhibit a wide diversity of defensive traits and strategies to protect themselves from damage by herbivorous pests and disease. These defensive traits may be naturally occurring or artificially selected through crop breeding, including introduction via genetic engineering. While these traits can have obvious and direct impacts on herbivorous pests, many have profound effects on higher trophic levels, including the natural enemies of herbivores. Multi-trophic effects of host plant resistance have the potential to influence, both positively and negatively, biological control. Plant defense traits can influence both the numerical and functional responses of natural enemies; these interactions can be semiochemically, plant toxin-, plant nutrient-, and/or physically mediated. Case studies involving predators, parasitoids, and pathogens of crop pests will be presented and discussed. These diverse groups of natural enemies may respond differently to crop plant traits based on their own unique biology and the ecological niches they fill. Genetically modified crop plants that have been engineered to express transgenic products affecting herbivorous pests are an additional consideration. For the most part, transgenic plant incorporated protectant (PIP) traits are compatible with biological control due to their selective toxicity to targeted pests and relatively low non-target impacts, although transgenic crops may have indirect effects on higher trophic levels and arthropod communities mediated by lower host or prey number and/or quality. Host plant resistance and biological control are two of the key pillars of integrated pest management; their potential interactions, whether they are synergistic, complementary, or disruptive, are key in understanding and achieving sustainable and effective pest management.

Keywords: host plant resistance, tritrophic interactions, transgenic crops, biological control, herbivore-induced plant volatiles (HIPVs)

INTRODUCTION TO KEY CONCEPTS

The worldwide population is growing, with projections of 9–10 billion people living on Earth by 2050 (United Nations, 2004; Lutz and Samir, 2010). Global food demands are increasing concomitantly, with a need for heightened food security, increased agricultural productivity and improved water use efficiency of crops. In a global review of factors contributing to losses for eight

major food and cash crops, animal pests came in second only to weeds, causing potential yield losses of 17.6% (Oerke and Dehne, 2004). Clearly, crop pests are responsible for significant losses to agricultural commodities worldwide despite profound efforts at management. Identification and promotion of sustainable solutions to these agricultural threats are essential for meeting future needs. The concepts of **Integrated Pest Management** (IPM), first championed by Stern et al. (1959), support practical efforts to achieve sustainable pest management. IPM has been described as “the harmonious use of multiple methods to control” pests, using “a set of decision rules based on ecological principles and economic and social considerations” (Kogan, 1998). Ideally, IPM incorporates the use of economic thresholds (Higley and Peterson, 2009) and a variety of control tactics (mechanical, physical, cultural, chemical, biological, and host plant resistance) making it essential to understand the interactions between different control tactics. Two key approaches for sustainable pest management have been (1) **host plant resistance**, the selection or development (via traditional breeding or genetic modification) and use of crop plants that possess defensive traits against herbivores and disease, and (2) **biological control**, the use of living organisms that are natural enemies of crop pests.

The concept of breeding plants to select for heritable traits that reduce pest impacts has been a part of agricultural production for over 100 years (Painter, 1951; Smith, 2005) and can be separated into tolerance and resistance mechanisms (Stout, 2013). **Tolerance** allows plants to withstand pest injury while **resistance** is conferred by plant traits that reduce the extent of pest injury and can be divided into constitutive or inducible and direct or indirect plant defenses (Stout, 2013). A **constitutive defense** is expressed in a plant regardless of whether it has been attacked by an herbivore, whereas an **inducible defense** is only expressed (or expressed to a greater degree) after attack. **Direct defenses** affect the herbivore without a mediating factor, whereas **indirect defenses** act via the actions of natural enemies. While indirect resistance may have the most obvious implications for biological control, other forms of resistance and tolerance also impact pest control by natural enemies. Holistic consideration of all these mechanisms is critical for their successful integration into pest control schemes.

Biological control programs use natural enemies (predators, parasitoids, and pathogens) of targeted pests to keep populations below the economic threshold. **Classical biological control** is the importation and establishment of natural enemies to control exotic pests while **augmentation biological control** incorporates the supplemental release of natural enemies. **Conservation biological control** involves modification of the environment or existing agronomic practices to protect and enhance specific natural enemies already present in the ecosystem (e.g., Landis et al., 2000; Eilenberg et al., 2001). The maintenance of natural enemy populations via conservation biological control can be a practical and sustainable option for low-value and high-acreage commodities, such as maize and other annual field crops (Thorbek et al., 2004; Naranjo et al., 2015). The responses of natural enemies to pest population changes are critically important and these can be classified as **numerical** (changes in

natural enemy abundance due to reproduction or aggregation) or **functional** (changes in natural enemy behavior) (Hajek, 2004). Seminal work on functional responses of predators to their prey items by Holling (1966) demonstrated that rate of prey discovery, search time, handling time, and predator hunger were important factors in determining functional response. In the years since Holling's research, studies in pest management have frequently examined how predators respond to prey, documenting the existence of functional responses in the context of biological control (e.g., De Clercq et al., 2000; Lee and Kang, 2004; Rutledge and O'Neil, 2005). Interestingly, some studies also describe variable responses of predators on different plants using plant-based defenses such as glandular trichomes and allelochemical production (De Clercq et al., 2000). These variable responses therefore highlight the need for careful consideration of the effects of different plant traits on pest suppression.

The interactions between plants, herbivores, and their natural enemies are referred to as **tritrophic interactions** and this multi-trophic exchange is key to understanding the interactions between host plant resistance and biological control. Natural enemies can be considered an extension of plant defense if plant traits, such as release of herbivore-induced plant volatiles (HIPVs), draw in these natural enemies. The literature is replete with examples of natural enemies acting in a top-down fashion, reducing herbivore populations, thereby providing plant defense.

The intention of this section is to provide a general introduction to the key concepts that provide context for the remainder of this review article. For more in-depth discussion of these topics, please refer to the many texts that review these topics (i.e., Painter, 1951; Panda and Khush, 1995; Kogan, 1998; Bellows et al., 1999; Agrawal, 2000a; Landis et al., 2000; Hajek, 2004; Smith, 2005; Heil, 2008; Radcliffe et al., 2009; van Lenteren, 2012; Stout, 2013; Pedigo and Rice, 2014). This review will focus on the interactions between biological control and host plant resistance, addressing the mechanisms and potential outcomes of interactions, with special attention to genetically modified insect-resistant crops and case studies for application of host plant resistance and biological control in cropping systems.

IMPACT OF PLANT TRAITS ON BIOLOGICAL CONTROL

The mechanisms by which plant defensive traits can affect biological control can be divided into four major categories: semiochemically, plant toxin-, plant nutrient-, and physically mediated interactions. These have been widely recognized as the major mechanisms by which the three trophic levels interact (Price, 1986; Thomas and Waage, 1996; Agrawal, 2000a) and will be reviewed in detail here. Their integration (see Discussion) into biological control programs is critical as we develop sustainable solutions for pest management.

Semiochemically Mediated Interactions

Plants produce a wide range of volatile compounds that are the predominant signals used by arthropod herbivores to

locate suitable host plants (Schoonhoven et al., 2005). These volatile profiles can change both quantitatively and qualitatively following herbivory (Dicke, 1999; Páre and Tumlinson, 1999; Heil and Ton, 2008), dramatically altering their attractiveness (or repellency) to herbivores and their natural enemies (Heil, 2014). Feeding, especially by chewing herbivores, results in mechanical damage to plant tissues eliciting a wound response thereby creating electrical, hydraulic, and chemical signals (e.g., systemin; Kessler and Baldwin, 2002). This action results in local and systemic release of linolenic acid from plant cell membranes and is converted by the enzyme lipoxygenase (LOX) to 13-hydroperoxide, which enters one of two pathways (Walling, 2000; Kessler and Baldwin, 2002). In one pathway, 13-hydroperoxide may be hydrolyzed by hydroperoxide lyase to yield 'green leaf volatiles' (GLVs; e.g., C₆ alcohols and aldehydes) and these, and other volatiles such as terpenoids, are often considered indirect defenses because they attract natural enemies. Alternatively, 13-hydroperoxide can enter the octadecanoid pathway, resulting in the production of jasmonic acid (JA), ultimately producing an array of anti-herbivore defenses including proteinase inhibitors (anti-digestive proteins), polyphenol oxidases (anti-nutritive enzymes), and a bewildering diversity of plant-specific toxins (Walling, 2000; Kessler, 2015; see Plant Toxin-Mediated Interactions). These inducible defensive chemicals are generally termed direct defenses in that they directly deter or inhibit feeding by herbivores.

Yet, plant responses to herbivory are more complex than simple wound responses to mechanical damage, which cannot explain the specificity of some plant responses to herbivores. In addition to physical damage, herbivores secrete substances that may modify plant responses. Collectively, these substances are referred to as herbivore-associated molecular patterns (HAMPs; Felton and Tumlinson, 2008; Mithöfer and Boland, 2008) and include substances such as regurgitants and salivary secretions (Alborn et al., 1997; Musser et al., 2002; Schäfer et al., 2011; Tian et al., 2012; Louis et al., 2013), and even frass production (Ray et al., 2015). Behavioral interactions, too, modify plant volatile production with walking on leaf surfaces (Tooker et al., 2010) and oviposition (Hilker and Meiners, 2006; Kim et al., 2012; Hilfiker et al., 2014) having profound effects. It is therefore unsurprising that plants respond to herbivory in specific ways that provide informative semiochemical-based information for both herbivores and their natural enemies. Plants emit different suites of volatiles, attracting different parasitoid complexes, depending on the species of herbivore attacking the plant. Clearly, there is abundant evidence that HAMPs and behavioral interactions of herbivores with host plants alter plant defensive responses beyond that of simple mechanical damage (e.g., Dicke, 1999; Reymond et al., 2000; Kessler and Baldwin, 2002). This highlights a cautionary note when interpreting findings of the large number of ecological studies using artificial leaf clippings and hole punches as a proxy for herbivore damage.

As discussed above, plant volatiles that attract natural enemies are considered indirect defenses (Vet and Dicke, 1992; Kessler and Baldwin, 2002; Turlings and Wäckers, 2004;

Wäschke et al., 2013). These GLVs, and others produced via different pathways such as volatile terpenoids (Kessler and Baldwin, 2002; Dudareva et al., 2013; Kessler, 2015), play a crucial role in signaling specific information for parasitoids regarding the status of herbivores and their natural enemies. The information conveyed in HIPVs can provide information on the species of herbivore present, the level of herbivory damage sustained, the developmental stage of the host, and even whether the herbivore has been previously parasitized. For instance, tomato plants attacked by tobacco budworm *Heliothis virescens* (F.) (Lepidoptera: Noctuidae), but not the closely related tomato fruitworm *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), emit a volatile profile that is highly attractive to the specialist parasitoid of the tobacco budworm, *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae) (De Moraes et al., 1998). Such information conveyed to natural enemies has profound consequences for the biological control services afforded by them and maximizes the top-down effect of such species on herbivorous pests. The quantity of HIPVs released may reflect the level of herbivory and determine the level of attractiveness to parasitoids. In studies of *Cotesia glomerata* (L.) (Hymenoptera: Braconidae) attacking *Pieris rapae* (L.) (Lepidoptera: Pieridae), plants attacked by more herbivores or induced with higher concentrations of JA (simulating higher levels of herbivory) were more attractive to *C. glomerata* (Geervliet et al., 1998; Bruinsma et al., 2009). Yet, HIPV production may also influence the plant's attractiveness to herbivores. In an interesting study of two chrysomelid beetles (*Gynandrobrotica guerreroensis* (Jacoby) and *Cerotoma ruficornis* Olivier) attacking wild lima beans [*Phaseolus lunatus* L. (Fabales: Fabaceae)], female beetles were repelled by HIPVs produced by induced plants regardless of level of induction (possibly reflecting competition and a lack of enemy-free space) whereas males were attracted by weakly induced plants (possibly indicating the presence of a mate) but repelled by strongly induced plants (Ballhorn et al., 2013). The effect of such changes in herbivore densities on parasitoid foraging decisions is unexplored. Furthermore, parasitoid species identity may also influence plant volatile production. Cabbage [*Brassica oleracea* L. (Brassicales: Brassicaceae)] produced similar HIPV profiles when attacked by imported cabbageworm *Pieris rapae* (L.) or large cabbage white *P. brassicae* (L.) (Lepidoptera: Pieridae) (Poelman et al., 2011). Yet, intriguingly, herbivore regurgitant characteristics were strongly influenced by the species of parasitoid developing within the herbivore, which differentially expressed genes within the plant's JA-signaling pathway. Even hyperparasitoids use HIPVs to locate their parasitoid hosts; the hyperparasitoid *Lysibia nana* Gravenhorst (Hymenoptera: Ichneumonidae) was more attracted to *P. rapae* hosts attacked by *C. glomerata* than those attacked by *C. rubecula* or unparasitized hosts. Field surveys showed hosts parasitized by *C. glomerata* are more likely to be hyperparasitized than *C. rubecula*-parasitized hosts and this preference was due to differences in HIPV profiles elicited by the oral secretions of *P. rapae* (Poelman et al., 2012). The sheer complexity of such semiochemically mediated interactions demonstrates the need for consideration of the multitude of

factors influencing pest control, rather than single elements acting along.

Case study: Maize Volatiles, Western Corn Rootworm, and Entomopathogenic Nematodes

Domestication can inadvertently alter the volatile profiles of many crop plants, affecting rates of parasitism. One example is the production of the sesquiterpene (E)- β -caryophyllene ($E\beta C$) in maize. $E\beta C$ is emitted in response to above- (Turlings et al., 1998) and below-ground injury (Rasmann et al., 2005). It serves as an attractant for natural enemies of maize pests (Rasmann et al., 2005; Köllner et al., 2008) and provides protection from herbivores with different modes and sites of attack (Köllner et al., 2008). Unfortunately, $E\beta C$ production has been unintentionally bred out of commercially available North American maize hybrids, but it is still present in European maize lines and teosinte (*Zea mays* ssp. *parviflora*) (Degen et al., 2004; Rasmann et al., 2005). $E\beta C$ production can be reintroduced by insertion of a gene from oregano, *Origanum vulgare* L. (Lamiaceae) (Degenhardt et al., 2009), demonstrating the ability to genetically enhance crops to increase natural enemy control of insect pests.

The most challenging belowground pest of maize production in North America and Europe is the western corn rootworm (WCR) *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae). Upon injury to the roots, European maize hybrids induce a strong production of $E\beta C$ locally and a weak systemic response throughout root tissues (Hiltbold et al., 2011). $E\beta C$ released into the rhizosphere recruits the entomopathogenic nematode (EPN) *Heterorhabditis megidis* Poinar, Jackson and Klein (Rhabditida: Heterorhabditidae). In field studies, maize hybrids producing $E\beta C$ had significantly higher rates of *H. megidis* infection in WCR larvae and reduced rootworm adult emergence than non- $E\beta C$ -emitting hybrids; non- $E\beta C$ -emitting maize varieties do not recruit *H. megidis* when attacked by the WCR (Rasmann et al., 2005).

Numerous studies have shown the potential of EPNs to suppress WCR populations (Wright et al., 1993; Jackson, 1996; Toepfer et al., 2005, 2008; Kurtz et al., 2009; Hiltbold et al., 2012) but not all EPN species and strains that attack WCR larvae are attracted to $E\beta C$ (Hiltbold et al., 2010c; Anbesse and Ehlers, 2013; Laznik and Trdan, 2013). *Heterorhabditis bacteriophora* Poinar (Rhabditida: Heterorhabditidae), for instance, is highly effective against WCR larvae (Jackson, 1996; Toepfer et al., 2008; Pilz et al., 2009) but is not attracted to $E\beta C$ (Hiltbold et al., 2010a,c). Selective breeding of *H. bacteriophora*, however, can increase the attraction of infective juveniles to $E\beta C$ -emitting maize roots, thereby increasing WCR mortality (Hiltbold et al., 2010a,b).

Maximizing the expression of HIPVs via bioengineering, while increasing EPN responsiveness to volatiles, can help enhance the effectiveness of biological control in crops. However, more studies are needed to assess the costs, viability and potential risks of introducing $E\beta C$ -emitting maize varieties with EPN releases. The WCR has a high propensity for invasion and adaptation (Gray et al., 2009) and has already developed resistance to multiple chemical (Meinke et al., 1998; Ciosi et al., 2009; Pereira et al., 2015), genetic (Gassmann et al., 2011; Wangila et al., 2015),

and cultural (Levine et al., 2002) management tools. Alternative control strategies, such as recruitment of entomopathogens using plant volatiles, must be explored in order to sustainably manage this critical pest.

Plant Toxin-Mediated Interactions

Of the more than 100,000 identified plant secondary metabolites, many play roles in direct defense against herbivorous insects through anti-nutritive, anti-digestive, or toxic compounds. Many of these defensive chemicals are produced constitutively, regardless of whether a plant is attacked by herbivores; others are often inducible via the JA-based signaling pathway described in Semiochemically Mediated Interactions above (Memelink et al., 2001; Agrawal, 2011; De Geyter et al., 2012). While plant anti-herbivore toxins might be expected to exhibit similar responsiveness as semiochemicals to the damage done by specific herbivores and the presence of their natural enemies, little evidence suggests this is the case. Rather, many secondary compounds are present within only a limited range of plant families (e.g., the glucosinolates are found almost exclusively in plants in the Order Brassicales (Halkier and Gershenson, 2006), furanocoumarins are primarily associated with the families Apiaceae and Rutaceae (Berenbaum, 1983, 1990)). Specificity of plant defensive responses to different herbivores ('specificity of elicitation' *sensu* Stout et al., 1998) seems, for the most part, to be quantitative rather than qualitative. For instance, levels of damage caused by different herbivores (Van Zandt and Agrawal, 2004) or variable damage by unparasitized vs. parasitized herbivores that results in differential feeding by herbivores (Ode et al., 2016) may result in the induction of different plant defensive compounds. While some evidence indicates that different herbivores can differentially induce plant defenses (e.g., Stout et al., 1998; Agrawal, 2000b; Poelman et al., 2008), the effects on higher trophic levels are poorly studied.

Unlike indirect defenses (see Semiochemically Mediated Interactions), direct plant defenses typically have negative effects on parasitoid fitness (Ode, 2006, 2013) and occur through one of three, non-mutually exclusive routes. Plant toxins may: (1) reduce host size, having negative consequences for parasitoids feeding on such hosts, (2) pass unmetabolized through the herbivore's midgut into the hemolymph where they are directly encountered by developing parasitoid larvae (Campbell and Duffey, 1979; McGovern et al., 2006; Lampert et al., 2008), or (3) be sequestered for defense against their own natural enemies (Nishida, 2002; Ode, 2006; Lampert et al., 2011a). For example, the catalpa sphinx moth, *Ceratomia catalpae* (Boisduval) (Lepidoptera: Sphingidae), sequesters the iridoid glycoside catalpol when it feeds on the catalpa plant, *Catalpa bignonioides* Walter (Lamiales: Bignoniaceae) (Lampert et al., 2010). Interestingly, the parasitoid *Cotesia congregata* (Say) (Hymenoptera: Braconidae) appears to be little affected by concentrations of catalpol, which also accumulate in the tissues of the parasitoid suggesting the role of this compound as protection against its own hyperparasitoids (Lampert et al., 2011a).

Whether parasitoids are adversely affected by plant toxins depends in large part on the level of host plant specialization of their herbivorous hosts. The diversity of host plants on

which a given herbivore develops, in part, on its ability to metabolize or avoid plant defensive toxins (Schoonhoven et al., 2005). Herbivores feeding on a broader range of host plants typically possess detoxification enzyme systems capable of metabolizing a broad array of plant toxins (Krieger et al., 1971; Li et al., 2004; Ali and Agrawal, 2012). Conversely, herbivores with specialized diets tend to have more efficient detoxification enzymes that metabolize the narrower range of plant toxins to which they are exposed (Wittstock et al., 2004; Mao et al., 2006). Far less documentation exists regarding the consequences for parasitoids of developing in generalist vs. specialist herbivores because few studies have documented the levels of unmetabolized plant toxins in the hemolymph of herbivores with different diet breadths. In one study, significantly more xanthotoxin was passed unmetabolized into the hemolymph of the cabbage looper, *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae), a generalist herbivore, than was passed in the hemolymph of the parsnip specialist *Depressaria pastinacella* (Geeze) (Lepidoptera: Oecophoridae) (Lampert et al., 2011b). In turn, *Copidosoma floridanum* Ashmead (Hymenoptera: Encyrtidae) (a parasitoid of *T. ni*) suffered increased mortality and reduced clutch sizes relative to *Copidosoma sosares* (Walker) (Hymenoptera: Encyrtidae) (a specialist parasitoid of *D. pastinacella*) even though both herbivore-parasitoid combinations were reared on the same artificial diets (Lampert et al., 2011b). Other studies have documented similar patterns (e.g., Barbosa et al., 1986, 1991). Finally, generalist and specialist herbivores of cruciferous plants are negatively affected by different classes of glucosinolates. Generalist herbivores are typically susceptible to both indole and aliphatic glucosinolates, whereas specialist herbivores are susceptible to just indole glucosinolates (Gols et al., 2008a,b; Müller et al., 2010; Harvey and Gols, 2011). However, some specialists are known to sequester glucosinolates, providing protection against their natural enemies [e.g., the turnip sawfly *Athalia rosae* (Hymenoptera: Tenthredinidae) (Müller et al., 2002) and the specialist aphids *Brevicoryne brassicae* (L.) and *Lipaphis erysimi* Kaltenbach (Hemiptera: Aphididae)] (Francis et al., 2001; Rossiter et al., 2003; Kazana et al., 2007). Interestingly, survivorship and body size of unparasitized *T. ni* were negatively correlated with concentrations of aliphatic glucosinolates whereas survivorship and clutch sizes of *T. ni* parasitized by *C. floridanum* were negatively affected by concentrations of indole (and not aliphatic) glucosinolates (Ode et al., 2016).

Despite long-running discussions about the potential (in)compatibilities of biological control and breeding programs for plant resistance (e.g., Bergman and Tingey, 1979; van Emden, 1991; Bottrell et al., 1998; Cortesero et al., 2000; Poppy and Sutherland, 2004), surprisingly little is known about the severity of these incompatibilities. This is primarily a reflection of the independent paths that host plant resistance and biological control programs have taken; i.e., IPM is rarely practiced in reality. Part of the difficulty lies in the fact that when crop varieties are bred for insect resistance, rarely do we know the exact mechanism involved. Nonetheless, breeding programs likely select for plant defensive toxins in many cases, which likely mediate resistance. When true, we expect that many of

the patterns outlined above will hold. For instance, soybeans, *Glycine max* (L.) (Fabales: Fabaceae), with the *Rag1* gene are resistant to soybean aphid *Aphis glycines* Matsumura (Hemiptera: Aphididae). Compatibility studies between *Rag1* and biological control agents of *A. glycines* have shown that these agents are less effective (e.g., reduced foraging efficiency and survivorship) on soybean varieties containing the resistant *Rag1* gene (Lundgren et al., 2009b; Ghising et al., 2012; Ode and Crompton, 2013).

Case Study: Cotton, Gossypol and Bt Toxins, Herbivores, and Natural Enemies

Cotton, *Gossypium hirsutum* L. (Malvales: Malvaceae), the most important plant-based fiber used by humans worldwide, presents an interesting example of the difficulties in breeding for resistance against multiple insect pests. It is consumed by a large number of insect herbivores including the boll weevil, bollworm, pink bollworm, tobacco budworm, armyworms, cotton aphid, whiteflies, *Lygus* bugs, and thrips (Matthews and Tunstall, 1994; Hagenbucher et al., 2013a). Prior to the introduction of *Bacillus thuringiensis* (Bt) cotton and more effective IPM approaches, insecticides were the primary means of pest control. An array of morphological (e.g., trichomes) and chemical defenses are produced by cotton and of the chemical defenses, terpenoids (especially gossypol and related compounds) are the best studied. Gossypol, present in leaves and seeds, provides resistance to a broad range of lepidopteran pests (Bottger and Patana, 1966). As it is also toxic to humans, breeding efforts have selected for glandless cultivars that produce low gossypol levels, but these cultivars are particularly susceptible to a range of insect pests (Jenkins et al., 1966). Recent efforts using RNAi to produce low gossypol levels in the seeds while maintaining high levels elsewhere have been successful (reviewed in Hagenbucher et al., 2013a), but gossypol also has negative effects on some natural enemies. For instance, *Campoletis sonorensis* (Cameron) (Hymenoptera: Ichneumonidae) experiences reduced body size, reduced survivorship, and increased development time when developing on *H. virescens* that had fed on diets high in gossypol (Gunasena et al., 1989), although this negative effect is by no means universal across species (e.g., Sun et al., 2011). Similar to semiochemically induced effects, responses of organisms to different compounds are specific to the exact plant-insect interaction.

The recent focus in cotton breeding for insect herbivore resistance has centered on the development of Bt transgenic lines expressing Cry-endotoxins that confer resistance against lepidopteran herbivores. In particular, adoption of Bt cotton has been credited with the eradication of the pink bollworm *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) in the southwestern United States (Carrière et al., 2003) and substantial declines of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in China (Wu et al., 2008). The specificity of Cry toxins against lepidopterans and reduced pesticide use after widespread adoption of Bt cotton has provided an environment favorable to natural enemies, allowing increased control of a wide variety of cotton pests (Naranjo, 2011; Lu et al., 2012). However, Bt has not been without its downsides as damage by some pests, for example, mirid bugs (Lu et al., 2010), have been

documented to increase with the widespread use of Bt cotton, presumably because of competitive release from lepidopterans. Another complication involves improved success of the cotton aphid *Aphis gossypii* Glover (Hemiptera: Aphididae) on Bt cotton. Suppression of feeding by lepidopteran herbivores on Bt cotton reduces induction of key defensive terpenoids, such as gossypol, making these plants much more susceptible to aphids, which do not induce terpenoids (Hagenbucher et al., 2013b). Furthermore, induced terpenoids from non-Bt cotton end up in the hemolymph of the aphids, reducing success of attack by the parasitoid *Lysiphlebus testaceipes* (Cresson) (Hymenoptera: Braconidae) (Hagenbucher et al., 2014b). Reduced parasitism was most likely due to reduced parasitoid acceptance of aphids feeding on lepidopteran-infested non-Bt cotton. Finally, as honeydew is an important source of nutrition for foraging parasitoids, the effect of honeydew from lepidopteran-infested Bt and non-Bt cotton on two important parasitoids of cotton pests, *L. testaceipes* and the whitefly parasitoid *Eretmocerus eremicus* Rose and Zolnerowich (Hymenoptera: Aphelinidae) was compared. While gossypol and other terpenoids were significantly higher in the honeydew produced on lepidopteran-infested non-Bt cotton, this did not affect the quality of the honeydew in terms of its effects on parasitoid longevity or fecundity (Hagenbucher et al., 2014a).

Plant Nutrient-Mediated Interactions

The proteins, sugars, lipids, nucleic acids, vitamins, and minerals contained within plant tissue provide the nutrition necessary for growth, development, and survival of many insects. In turn, the nutrients provided by plants to herbivores affect the nutrients subsequently available to their natural enemies. The presence, quantity, quality, and availability of these nutrients varies significantly between plant species and varieties, and can be affected by season, plant phenology, and other biotic and abiotic conditions (Fox et al., 1990; Roth and Lindroth, 1995; Walde, 1995; Stadler and Mackauer, 1996).

A key indirect interaction between host plant nutrition and natural enemies occurs when herbivore growth and development is delayed by suboptimal plant quality, extending the period of time when herbivores are vulnerable to attack (Moran and Hamilton, 1980; Price et al., 1980; Price, 1986; Loader and Damman, 1991; reviewed in Benrey and Denno, 1997). An example of this “slow-growth-high-mortality” hypothesis was reported for the Mexican bean beetle *Epilachna varivestis* Mulsant (Coleoptera: Coccinellidae) feeding on soybean. The spined soldier bug, *Podisus maculiventris* (Say) (Hemiptera: Pentatomidae), was better able to control *E. varivestis* on crop varieties that lowered the herbivore’s growth rate (Price et al., 1980), although the exact resistance mechanism was not known. In addition to a longer period of vulnerability, a slow herbivore growth rate can be advantageous if the natural enemy’s functional response is stronger when consuming smaller prey, as tends to be the case with predators (Price, 1986). Insect pathogens, in particular, are positively associated with the slow-growth-high-mortality hypothesis (Schuster et al., 1983; Hamm and Wiseman, 1986). In one case, *S. frugiperda* feeding on resistant maize plants had reduced growth and vigor, making them

more susceptible to infection with nuclear polyhedrosis virus (NPV) (Hamm and Wiseman, 1986). However, the slow-growth-high-mortality hypothesis does not hold true for all tritrophic interactions. For example, Leather and Walsh (1993) found that pine beauty moth *Panolis flammea* Denis and Schiffermüller (Lepidoptera: Noctuidae) larvae were not more vulnerable to natural enemies when development was delayed by host plant quality. Some natural enemies, such as parasitoids, may actually be at a disadvantage when their hosts are smaller and/or of lower quality, and smaller hosts may also affect the sex ratio and fecundity of parasitoid populations (Kuo, 1986). It is therefore important to examine whether the presence of smaller and lower quality hosts due to suboptimal plant nutrition has a large enough impact on parasitoids as to affect their ability to suppress pest populations.

Many natural enemies also engage in omnivory, supplementing their prey-based diet with plant-provided resources (reviewed in Lundgren, 2009), particularly during periods when prey abundance is low. This can allow for more stable interactions between predators and prey (Agrawal, 2000a) and may facilitate early season colonization of crop fields and better pest suppression due to this “lying in wait” of natural enemies prior to arrival of the pest species (Settle et al., 1996; Eubanks and Denno, 1999; Athey et al., 2016). Therefore, good quality plant hosts in the case of omnivorous natural enemies is essential for a positive relationship between plant and biocontrol. Plants expressing herbivore defense traits can have direct impacts on facultatively phytophagous predators but the literature is lacking in how these interactions will impact the compatibility of host plant resistance with biological control (Lundgren, 2009).

Some insects are truly omnivorous, having a flexible trophic strategy that allows them to utilize either plant or prey resources, with the potential to inflict crop damage if engaging in phytophagy. For example, the western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) feeds on plant material and arthropod prey, leading to its role as both a serious pest (Grazia-Tomasini, 1995; Kirk and Terry, 2003) and a biological control agent (Trichilo and Leigh, 1986; Wilson et al., 1996; Agrawal and Karban, 1997; Milne and Walter, 1997). Furthermore, Agrawal et al. (1999) revealed that the presence of prey [eggs of the Pacific spider mite *Tetranychus pacificus* McGregor (Thysanoptera: Tetranychidae)] reduced feeding by *F. occidentalis* on cotton by nearly 50%. However, when cotton plants were first exposed to feeding pressure by spider mites, eliciting systemically induced plant defenses that lower host plant quality, herbivory by *F. occidentalis* was reduced (Agrawal et al., 1999). When both induced host plant defenses and *T. pacificus* egg prey were available, feeding preference shifted to consume half the amount of cotton tissue and twice the number of prey (Agrawal et al., 1999). Thus, host plant quality and prey availability are important factors for arthropods with omnivorous trophic tendencies.

Extrafloral nectaries (EFN) are a plant-provided resource that deserve additional attention because of their role in natural enemy nutrition. It is hypothesized that the main function of extrafloral nectar is to recruit predators and parasitoids for the protection of the plant against herbivores, an example of

indirect host plant resistance (Bentley, 1977; Koptur, 1992; Turlings and Wäckers, 2004). Some EFN emit olfactory signals that are attractive to natural enemies, such as parasitoids (Lewis and Takasu, 1990; Stapel et al., 1997). By providing nutritional resources, the presence of EFN can lead to enhanced herbivore suppression by arthropod natural enemies, such as ants (Bentley, 1977; Smiley, 1986), spiders (Ruhren and Handel, 1999), predatory mites (Bakker and Klein, 1992), coccinellids (Stephenson, 1982) and parasitoids (Lindgren and Lukefahr, 1977). Interestingly, some plants produce a consistent low level of EFN, but increase production in response to herbivory; in this manner, extrafloral nectaries can be considered both constitutive and inducible indirect host plant resistance (Wäckers et al., 2001; Wäckers and Bonifay, 2004; Lundgren, 2009; Heil, 2015). The applied implications of EFN production by crop plants is examined in the case study with cotton below.

Case Study: Extrafloral Nectar-Producing Cotton, Its Herbivores, and Natural Enemies

The ability of extrafloral nectar to attract natural enemies for biological control of cotton pests has long been exploited. Cook (1904, 1905) reported on the practice of indigenous farmers in Guatemala, who purposely cultivated cotton near nests of the tropical ant *Ectatomma tuberculatum* (Olivier) (Hymenoptera: Formicidae). In addition to feeding on EFN, these ants attacked boll weevil *Anthonomus grandis* Boheman (Coleoptera: Curculionidae) adults. Subsequently, plant breeding efforts in the mid 1900's attempted to develop cotton varieties that lacked EFN, due to the observation that both natural enemies and some lepidopteran pests, such as *P. gossypiella*, benefitted from cotton nectaries (Lukefahr and Griffin, 1956; Lukefahr and Rhyne, 1960; Bentley, 1983). However, the benefit of a modest reduction in lepidopteran pests was outweighed by the disadvantage of reduced natural enemy populations, although this conclusion was doubted at the time (Rogers, 1985; Schuster and Calderon, 1986). The population of natural enemies in "nectarless" cotton varieties was up to 35% lower than EFN-producing cotton and the presence of EFN in cotton had positive impacts on the attraction, retention, and efficiency of many predators, including chrysopids, anthocorids, and coccinellids (Schuster et al., 1976). Similarly, the parasitoid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae), which attacks larvae of the bollworm *H. zea*, is stimulated to stay longer and attack a greater number of hosts in the presence of nectar (Stapel et al., 1997). Many other examples exist in the literature, providing clear evidence for widespread benefits of EFN to parasitoids (e.g., Treacy et al., 1987). Another functional group of natural enemies, cursorial wandering spiders such as *Cheiracanthium inclusum* (Hentz) (Araneae: Miturgidae) and *Hibana futilis* (Banks) (Araneae: Anyphaenidae), are important nocturnal predators of lepidopterous pest eggs in cotton (Pfaffenstiel, 2008) and consume EFN in the field (Taylor and Pfaffenstiel, 2008). Furthermore, *Hibana futilis* responds to olfactory cues from extrafloral nectar and engages in restricted area searching following contact with nectar (Patt and Pfaffenstiel, 2008, 2009) and profound improvements of

survival are evident when provided EFN in the diet (Taylor and Pfaffenstiel, 2009; Pfaffenstiel and Patt, 2012).

The majority of modern cotton varieties now produce EFN, but past breeding efforts illustrate the difficulty in managing plant traits affecting both pests and natural enemies. Rogers (1985) recommended that for the case of nectar-producing cotton, varieties should be developed that produce nectar that is palatable to beneficial species, but not pests. However, the feasibility of this suggestion has not been explored. Recommendations to improve the recruitment of natural enemies to cotton fields include selecting for varieties with enhanced nectar production. For example, most cotton leaves bear a single nectary, but some have three (Cortesero et al., 2000) and a breeding challenge is whether cotton varieties can be developed with a greater number of nectaries. It is evident that plant nutrients are critically important to a diverse array of natural enemies across multiple functional groups. Integration of this resource into biological control programs through selective enhancement or provisioning of additional nectar sources can assist when developing sustainable solutions to pest management. Clearly, challenges exist when selectively breeding for plant defense traits (described here and in other sections), but careful consideration of their integration with biological control can provide synergistic levels of pest control.

Physically Mediated Interactions

Just as some tritrophic interactions involve both semiochemicals and toxins, physically mediated interactions do not always function alone. For example, substances such as resin or latex physically limit herbivores by trapping or immobilizing them, while simultaneously delivering various toxins (Konno, 2011), and glandular trichomes release sticky and toxic compounds serving as a physical and chemical defense against herbivores (Levin, 1973; Southwood, 1986; Cortesero et al., 2000).

Plant architecture affects the dispersion of herbivores on a host plant, which may in turn affect searching behavior and host-finding abilities of natural enemies. For example, the leaves of winter wheat varieties developed for resistance to Russian wheat aphid *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae) remain flat, compared to susceptible varieties whose leaves curl in response to aphid feeding (Hawley et al., 2003), exposing aphids to disturbances such as wind, rain, and predators inducing them to fall from the plant (von Berg et al., 2008). Characteristics that affect falling behavior of herbivores can affect predation rates as they experience vulnerability to ground-dwelling predators and may also face additional challenges from natural enemies as they attempt to recolonize the plant (Sunderland et al., 1986; Winder, 1990; Winder et al., 1994).

The size and morphology of certain plant structures that confer resistance to herbivores can affect biological control by altering where pests feed, how long they are exposed and how apparent or accessible the pests are to natural enemies, particularly if plant morphology can delay internally feeding pests from entering the plant's tissues. An example would be husk tightness and length in sweet corn plants conferring resistance to *H. zea* larvae attempting to enter the ear and feed on developing kernels (Cameron and Anderson, 1966; Wiseman and Davis, 1990). Plant structures may also act to hide the herbivore from its

natural enemies. For example, open-leaf brassica varieties, such as Brussels sprouts, have higher parasitism on *P. rapae* compared to heading varieties, such as cabbage, due to larvae being able to feed in leaf folds protected from parasitoids (Pimentel, 1961). Furthermore, the size of plant structures impacts the ability of parasitoids to oviposit in pests, particularly if larger fruits allow pests to feed deeper than the parasitoid's ovipositor can reach, creating "enemy-free space" and potentially facilitating host switching by pests (Bush, 1974; Price et al., 1980; Jeffries and Lawton, 1984; Bernays and Graham, 1988).

The plant surface is a complex microenvironment playing a critical role in insect–plant interactions, impacting insect behavior (such as attraction, retention, and host choice), feeding (such as attachment and accessibility of nutrients), and dispersal (by impeding insect movement) (Chapman, 1977; Southwood, 1986). Leaf surface structures that defend the plant from herbivores, such as leaf toughness, cuticle thickness, epicuticular waxes, trichomes and spines, can have direct and indirect effects on natural enemies. An indirect effect can occur if physical defense traits, such as leaf toughness, delay the development of herbivores. The extended period of vulnerability to natural enemies can thereby enhance biological control (slow-growth–high-mortality hypothesis, see Plant Nutrient-Mediated Interactions). A common example of direct effects is when trichomes are physically disruptive to natural enemy movement. In general, trichomes have more harmful than beneficial effects on predators, although most of these effects are sublethal (Riddick and Simmons, 2014a,b). The functional response or attack rate of predators and parasitoids is typically lower when their prey or hosts are found on plants with greater trichome density (e.g., Krips et al., 1999; Kumar et al., 1999; De Clercq et al., 2000; Stavrinides and Skirvin, 2003; Madadi et al., 2007; Jalalizand et al., 2012), although the opposite has been found as well (Koveos and Broufas, 2000). These interactions have significant implications for pest management; for example, biological control is possible on glabrous cucumber varieties, but is seriously hindered on those with dense trichomes due to the reduction in searching efficiency by the parasitoid *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae) attacking greenhouse whiteflies *Trialeurodes vaporariorum* Westwood (Hemiptera: Aleyrodidae) (Hulspas-Jordaan and van Lenteren, 1978). Clusters of trichomes on the underside of plant leaves can form domatia, commonly used by predatory arthropods for shelter (O'Dowd and Willson, 1991; Walter, 1996; Agrawal and Karban, 1997); the positive impact of domatia on biological control has been well-documented for predatory phytoseiid mites (reviewed in Schmidt, 2014). In general, arthropods need to be either quite large (Rabb and Bradley, 1968; Obrycki and Tauber, 1984) or very small (Krips et al., 1999) to move along a leaf surface unimpeded by physical plant defense structures. The effect of trichome density on natural enemy movement can be a function of the relationship between natural enemy size and trichome spacing (Buitenhuis et al., 2014).

This myriad of physical plant traits clearly has an important effect on the feeding efficiency of herbivores. However, integration of plant physical traits with biological control is a complex issue with characteristics hindering herbivore damage

also affecting (positively and negatively) the ability of natural enemies to attack pest species. This trade-off is evident in many examples of physically mediated interactions. In addition to trichomes, another plant surface characteristic that can impact natural enemies is the presence and composition of epicuticular waxes, which will be discussed in the following section.

Case Study: Plant Epicuticular Waxes, the Diamondback Moth, and Its Predators

Plant epicuticular waxes primarily serve to control water, gas and solute exchange (Riederer and Müller, 2006). In addition, these waxes mediate other ecological functions including host plant resistance against pathogens (Reina-Pinto and Yephremov, 2009) and herbivores (Eigenbrode et al., 1991b; Müller, 2008). The interactions between *B. oleracea* (cabbage, broccoli, cauliflower, kale, and others), *Plutella xylostella* (L.) (Lepidoptera: Plutellidae), and its predators highlight the interface between plant waxes and herbivore resistance. Gene mutations yield *B. oleracea* cultivars with altered chemical structures and different crystallization patterns of epicuticular lipids (Macey and Barber, 1970; Netting et al., 1972; Baker, 1974). As a consequence, mutants usually have decreased epicuticular waxes and produce a "glossy" phenotype instead of their normal wax "glaucous" phenotype (Eigenbrode and Espelie, 1995). Although information is limited (Verkerk and Wright, 1996), evidence suggests that glossy plants exhibit resistance against neonate *P. xylostella* larvae (Lin et al., 1983; Eigenbrode and Shelton, 1990; Eigenbrode et al., 1991a) and that physical and chemical differences influence neonate behavior (Eigenbrode et al., 1991b). Neonates on glossy varieties disperse further and faster, spending less time palpatting, biting, mining, and spinning silk (Eigenbrode and Shelton, 1990; Eigenbrode et al., 1991a). This non-preference behavior causes a lack of establishment, reduced feeding and increased larval mortality (Eigenbrode and Shelton, 1990; Eigenbrode et al., 1991a).

Host plant resistance conferred by the glossy phenotype is also enhanced by predators. Field studies revealed that green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae), insidious flower bug *Orius insidiosus* (Say) (Hemiptera: Anthocoridae), and convergent lady beetle *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae), all generalist predators, significantly increased *P. xylostella* larval mortality in glossy, but not normal wax, varieties (Eigenbrode et al., 1995). The reduction in mining behavior renders the larvae more exposed to predators (Eigenbrode et al., 1995). Predators also walked faster, spent more time walking, and covered more leaf area on glossy leaves compared to normal wax varieties (Eigenbrode et al., 1996). Increased mobility was attributed to increased traction/adhesion of predators on glossy vs. normal wax plants. The crystallization and composition of natural waxes have an impact on how natural enemies, such as *H. convergens* and *Chrysoperla plorabunda* (Fitch) (Neuroptera: Chrysopidae) attach to the leaf surface, thereby affecting their ability to exert biological control (Eigenbrode et al., 1999; Eigenbrode and Jetter, 2002).

In summary, this system has multiple pest suppression factors working together. *Plutella xylostella* neonates are less likely

to accept glossy varieties, which increases their mortality and vulnerability to predation (via decreased mining behavior). Predators on glossy varieties have a greater ability to walk and hence, locate and attack prey, due to increased adhesion to the surface of leaves. Altogether, host plant resistance for *P. xylostella* in glossy varieties increases biological control by natural enemies, and hence overall suppression of this key pest of *Brassica* plants.

Mechanisms of Plant Trait-Mediated Interactions: Summary

Plant traits have a profound (and often complex) array of impacts on herbivores and natural enemies. The examples cited within each section above for semiochemically, plant toxin-, plant nutrient-, and physically mediated interactions show the diversity and gradient of interactions occurring between natural enemies and HPR and how these can interact synergistically or antagonistically to suppress the target pest. For instance, semiochemically mediated traits serve as indirect plant defenses by impacting signaling pathways and attraction/repellency between the members of tritrophic interactions. Conversely, plant toxins act as direct defense against herbivores and this in turn can alter host suitability for natural enemies. Insect host/prey vulnerability via the slow-growth-high-mortality hypothesis can be mediated by plant nutrition. Plant-provided nutritional resources can also be linked to the success of natural enemies due to omnivory by predators and/or parasitoids. Moreover, physically mediated traits are known to function together with other traits to deter herbivory, but physical plant defenses are also responsible for increasing or decreasing herbivores' vulnerability to natural enemies and trichomes can have direct negative impacts on biological control by decreasing natural enemy search efficiency. Manipulation of plant traits through plant breeding or bioengineering, as well as knowledge of the ecology and biology of herbivores and natural enemies, can work together to aid crop protection. In the last two decades, another control tactic, Bt, has become a staple of the agricultural landscape throughout much of the world (although notably less so in Europe). This technology will be discussed below given its importance in pest control programs throughout the world.

GENETICALLY MODIFIED CROPS AND INTERACTIONS WITH BIOLOGICAL CONTROL

Transgenic genetically modified (GM) crops have been engineered to incorporate genes derived from another species that confer nutritional or agronomic benefits, such as resistance to insect pests, viruses, herbicides, or protection from environmental conditions (e.g., low water availability). Among insect-resistant GM crops, *Bacillus thuringiensis* (Bt) crops are the most common and express insecticidal proteins derived from a naturally occurring soil bacterium. The insecticidal mode of action occurs when Bt toxins bind to receptors on the midgut lining of susceptible insects, causing

lysis of epithelial cells on the gut wall, perforations in the midgut lining, cessation of feeding, and death by septicemia. Bt toxins target a narrow spectrum of pest insects that possess specific physiological traits (i.e., gut pH and toxin receptor sites in the midgut), and thus pose less direct toxicity risk to non-target species than broad-spectrum insecticides (Marvier et al., 2007; Wolfenbarger et al., 2008; Naranjo, 2009; Duan et al., 2010; Peterson et al., 2011). Commercialized Bt crops include maize, cotton, and soybeans that are protected against a suite of coleopteran and lepidopteran pests. The planting of Bt crops has increased dramatically since their introduction in the mid-1990's; for example, in the United States, the percentage of Bt maize was only 1% of the total crop grown in 1996 but 81% of all maize grown in 2015 (United States Department of Agriculture National Agricultural Statistics Service, 2015). The ecological interactions between insect-resistant GM crops and biological control are complex and have been addressed in numerous comprehensive reviews (e.g., Obrycki et al., 2004; Lundgren et al., 2009a; Hilbeck and Otto, 2015). Two major categories for how GM crops influence biological control, proposed by Lundgren et al. (2009a), are discussed below: (1) toxicity-based pathways, including natural enemy consumption of toxic plant or prey foods; and (2) crop-induced changes to the environment, including unintended alterations to the crop plant and a decrease in prey quality and/or density that alter functional and numerical responses as well as the community ecology of natural enemies.

Many natural enemies consume plant-provided non-prey foods (see Plant Nutrient-Mediated Interactions) and when these plant-provided resources are GM crops, they are likely to contain Bt toxins. The expression of transgenic proteins is influenced by many biotic and abiotic factors, including environment, geography, crop phenology and genetics, and the specific transgenic event and protein expressed (Fearing et al., 1997; Duan et al., 2002; Grossi-de-Sa et al., 2006; Obrist et al., 2006a; Lundgren et al., 2009a). Most Bt crops employ a constitutive promoter that expresses Bt proteins throughout the life of the plant in nearly all tissues. Natural enemies that engage in facultative phytophagy of these plants are therefore likely to be exposed to the Bt toxins. Despite this exposure, laboratory feeding assays and field studies do not report negative impacts (Pilcher et al., 1997; Armer et al., 2000; Lundgren and Wiedenmann, 2002; Geng et al., 2006; Ludy and Lang, 2006; Obrist et al., 2006b; Torres et al., 2006; Li et al., 2008), most likely due to the high specificity of Bt proteins against target pests and the lack of necessary physiological conditions in non-target arthropods. It is therefore unlikely this pathway has a significant impact on biological control in transgenic crops.

Natural enemies may be exposed to Bt toxins by consuming or parasitizing preyhosts that have fed on GM crops, a pathway similar to plant toxin-mediated interactions (see Plant Toxin-Mediated Interactions). One factor mitigating the exposure of natural enemies is that for crop pests that are highly susceptible to Bt toxins, ingestion of a very small amount of toxin elicits lethal effects. Exposure to natural enemies can be greater if the herbivore consuming a GM crop plant is only partially susceptible to the toxin and therefore consumes a greater quantity of plant tissue. Many herbivores do contain transgenic toxins

(e.g., Harwood et al., 2005; Meissle et al., 2005; Obrist et al., 2005, 2006b; Peterson et al., 2016), but accumulation in higher trophic levels is uncommon (Dutton et al., 2002; Obrist et al., 2006a; Paula and Andow, 2016). While tritrophic transfer of Bt proteins has been documented, it is at low levels (e.g., Harwood et al., 2005, 2007; Meissle et al., 2005; Zwahlen and Andow, 2005; Obrist et al., 2006a; Wei et al., 2008; Chen et al., 2009; Meissle and Romeis, 2009; Peterson et al., 2009, 2016; Tian et al., 2010; Han et al., 2015). Early studies reported that some predators had negative sub-lethal effects from exposure to Bt-containing prey (Hilbeck et al., 1998a,b; Ponsard et al., 2002) but it was subsequently revealed that this was the result of reduced prey quality rather than direct exposure to Bt toxins (Romeis et al., 2004; Torres and Ruberson, 2006).

The most likely action by which GM crops could influence natural enemy fitness and fecundity is through a reduction in prey quality and/or prey density. Numerous studies have shown that consumption of Bt-containing plant tissue negatively affects the growth and development of herbivorous species, thereby impacting their natural enemies (e.g., Lövei and Arpaia, 2005; Hilbeck and Schmidt, 2006; Romeis et al., 2006; Lawo et al., 2010; Garcia et al., 2012; Tian et al., 2014; Han et al., 2015). For example, Hilbeck et al. (1998a) reported that the generalist predator *C. carnea* experienced reduced larval survival and longer development time when fed a diet of European corn borer (ECB), *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae), that had consumed Bt corn. However, generalist predators are capable of preferential feeding on healthy prey (Ferry et al., 2006) and are able to shift their dietary preferences to consume the mixture of nutrients required for optimal fitness (Mayntz et al., 2005; Raubenheimer et al., 2007; Marques et al., 2015). Therefore, generalist predators may be able to compensate for reduced quality of select prey due to Bt toxin consumption, having a negligible impact on biological control. For entomopathogens, species that are specialists of Bt-targeted pests are likely to see population reductions, whereas generalists will continue to persist in Bt crop fields (Obrycki et al., 2004). Parasitoids often do not have the flexibility to select hosts unaffected by Bt toxins and are therefore more likely to be adversely affected (Bernal et al., 2004; Marvier et al., 2007; Wolfenbarger et al., 2008; Bernal, 2010). Specialist parasitoid populations are reduced due to a lack of suitable hosts and may also suffer direct mortality if they are developing inside of a host that suffers mortality due to ingestion of Bt toxins (Agrawal, 2000a). For hosts that are only partially susceptible to Bt toxins, reduced host quality can result in sublethal effects on parasitoids (e.g., Bernal et al., 2002; Baur and Boethel, 2003; Vojtech et al., 2005; Ramirez-Romero et al., 2007; Walker et al., 2007), but host-mediated impacts of Bt crops on parasitoids are not universal and vary depending on the plant, host, and parasitoid. For example, the soybean looper *Chrysodeixis includens* (Walker) (Lepidoptera: Noctuidae) is moderately susceptible to the Bt toxins expressed in transgenic cotton and exhibits slower development time and lower prepupal weight (Baur and Boethel, 2003). Parasitism by *Cotesia marginiventris* (Cresson) (Hymenoptera: Braconidae) on these hosts results in longer larval development time, reduced adult longevity, and reduced egg production. However, when

C. floridanum parasitizes loopers that have fed on Bt cotton, wasp pupal development time and adult longevity are unaffected, but fewer adults are produced per host (Baur and Boethel, 2003), revealing the difference in effects between species. In addition to development time, natural enemy size can be reduced if feeding on lower quality prey or hosts; smaller size in insects can result in reduced fecundity and dispersal capacity (Honék, 1993; Kazmer and Luck, 1995), further delaying natural enemy population growth (Lundgren et al., 2009a).

The majority of interactions discussed above operate at the scale of a single crop field or smaller. However, some effects of the proliferation of GM crops are observed at the landscape or community scale. For example, Bt maize has been associated with area wide suppression of ECB in the midwestern United States (Hutchison et al., 2010). Despite reduced ECB populations that confer economic benefits to growers planting non-Bt maize, management of this pest is still critical for seed corn, popcorn, and other crops not protected by Bt toxins. Therefore, suppression of ECB due to biological control by natural enemies such as the specialist parasitoid *Macrocentrus grandii* (Goidanich) (Hymenoptera: Braconidae) and the entomopathogenic microsporidian *Nosema pyrausta* (Paillot) (Microsporidia: Nosematidae) is a valuable service. Despite the large reduction in ECB populations, infection dynamics of *N. pyrausta* have not significantly changed (Lewis et al., 2009), although parasitism rates by *M. grandii* were lowest when ECB hosts were found in small aggregations (White and Andow, 2005). Therefore, the area wide suppression of Bt-targeted prey or hosts does not always affect the interactions of pests with their natural enemies.

In addition to transgenic Bt crops, other herbicide-resistant and insecticidal GM crops are commercially available or under review by governmental agencies. The adoption of herbicide-tolerant crops that confer resistance to herbicides such as glyphosate, glufosinate, and 2,4-Dichlorophenoxyacetic acid (2,4-D) has been rapid. In the United States, 89% of corn and upland cotton and 94% of soybeans planted in 2015 had GM herbicide-tolerance traits (United States Department of Agriculture National Agricultural Statistics Service, 2015). Furthermore, herbicide-tolerant canola, alfalfa, and sugar beets are currently being grown in the United States, albeit in reduced frequency. This adoption has led to changes in the agricultural landscape, including reduced within-field plant diversity (Heard et al., 2005; Culpepper, 2006; Pleasants and Oberhauser, 2013), potentially affecting natural enemies and conservation biological control. The potential consequence of GM herbicide-tolerant crops on biological control is addressed in detail by Lundgren et al. (2009a). Transgenic insecticidal traits other than Bt have been studied; for example, potatoes, rice, maize, sugarcane, wheat, and other crops have been engineered to express snowdrop lectin GNA, a protein produced by the common snowdrop plant *Galanthus nivalis* (Asparagales: Amaryllidaceae) that expresses anti-hemipteran properties (Gatehouse et al., 1996; Sudhakar et al., 1998; Wang et al., 2005; Zhangsun et al., 2007; Duan et al., 2015). However, negative impacts of snowdrop lectin on natural enemies have been reported (Birch et al., 1999; Sétamou et al., 2002a,b,c; Horger vorst et al., 2006; Li and Romeis, 2009). The

next generation of transgenic insecticidal crops in the commercial pipeline utilizes RNA interference (RNAi), where small double stranded RNA molecules expressed in the plant selectively silence targeted genes in herbivores that feed on the plant (Siomi and Siomi, 2009). For the western corn rootworm (WCR), silencing the *DvSnf7* gene using genetically modified RNAi maize induces mortality of this pest (Baum et al., 2007; Bolognesi et al., 2012) but the interactions between RNAi crops and biological control are not fully understood. While the reported spectrum of insecticidal activity of *DvSnf7* RNAi is limited to a subset of species related to the WCR (Bachman et al., 2013), further risk-assessment is clearly required. The potential hazards of GM RNAi crops to natural enemies include off-target gene silencing, silencing of the targeted gene in non-target organisms, immune stimulation, and saturation of the RNAi machinery; however, these interactions may be highly complex and difficult to predict (see reviews by Lundgren and Duan, 2013; Casacuberta et al., 2015; Roberts et al., 2015). Consequently, understanding the potential effect that GM crops have on natural enemy-pest dynamics will allow for better integration of this technology with biological control services. Genetically engineered biotech crops undoubtedly afford significant levels of pest suppression; research on the compatibility of this approach with biological control is critical to address the long-term integration of both approaches.

DISCUSSION

Top-Down vs. Bottom-Up Control of Herbivorous Populations

As emphasized throughout this review, IPM ideally integrates a range of approaches to reduce damage caused by insect pests. Two of these approaches, HPR and biological control, are essentially forms of bottom-up and top-down control of herbivore populations. Whether breeding for increased plant resistance and the use of biological control are compatible and complementary approaches depends, in large part, on the mechanisms involved in HPR and the effects they have on biological control agents. Plant breeding for increased toxicity to herbivores will likely have negative effects on any biological control agents of these herbivores, whether due to direct ingestion of plant toxins or the effects of reduced host or prey size. In this respect, the array of interactions described in the Plant Toxin-Mediated Interactions and Plant Nutrient-Mediated Interactions sections are expected to apply here. An increasing number of studies have demonstrated that HPR has negative consequences for biological control agents through reduced body size or survivorship of individual natural enemies, raising the concern that such approaches are incompatible. Perhaps true in some circumstances, this is not always the case. Even if these control tactics negatively interact, the net effect in suppressing pest populations may be greater than use of either strategy alone. While rarely done, studies evaluating the joint effects of HPR and biological control efforts on pest population dynamics are essential to design effective and sustainable IPM strategies to minimize pest damage. Conversely, efforts to increase HPR by selecting for varieties that increase production of volatiles

attractive to biological control agents are clearly compatible with biological control approaches. These interactions have been discussed in the Semiochemically Mediated Interactions and Case study: Maize Volatiles, Western Corn Rootworm, and Entomopathogenic Nematodes sections. Too often, however, little is known about the mechanisms underlying plant resistance to herbivory.

In turn, parasitoids can reduce herbivore pressure allowing for increased plant yields. Parasitoids, especially solitary species, can reduce damage done by herbivores, resulting in direct yield benefits to the plant; even gregarious parasitoids, which often induce increased feeding by individual herbivores, can reduce long-term population sizes of herbivores. Indeed, the widespread success of many insect biological control programs speaks to the ability of parasitoids (and predators) to have positive effects on plant production and yield. An underappreciated facet of this interaction between parasitoids and plant fitness/yield is the potential for parasitoids to reduce the likelihood of evolution of herbivore resistance to plant resistance traits. This is discussed further in section “Biological Control Can Reduce the Likelihood of Resistance Evolution.”

Considerations for the Use of Volatiles to Recruit Biological Control Agents

Most studies involving HIPVs are undertaken in laboratory and greenhouse settings, with fewer studies conducted on the efficacy of HIPVs as host-plant resistance mechanisms in cropping systems at the field scale (Orre et al., 2010; Simpson et al., 2011a,b). Our understanding of arthropod responses to chemical compounds is still evolving, but efforts in developing HIPV strategies for crops are already in place via baiting/lures (Kaplan, 2012) or via bioengineering (Degenhardt et al., 2003, 2009). However, efforts to increase natural enemy efficacy by increasing plant attractiveness via HIPVs cannot ignore potential side effects. Extensive reviews of the challenges and the future of HIPV use in pest management have been published (Dicke, 2009, 2015; Alba et al., 2012; Kaplan, 2012; Heil, 2014) and there are many unknown factors and risks associated with the use of HIPV-based pest management tactics. Cropping systems are often considered low-diversity environments because of monocultural practices but in reality there are a multitude of organisms in any given field emitting and receiving chemical cues. We know that HIPVs targeted to attract natural enemies also attract herbivores, plant parasites, and members of the fourth trophic level. Releasing HIPV technology without examining the ecological factors present may render the technology ineffective. Several studies have shown that application of synthetic elicitors such as methyl jasmonate (MeJA) to induce elevated plant volatile production can also attract herbivores (Ballhorn et al., 2013) as well as hyperparasitoids (Kaplan, 2012; Heil, 2014), both outcomes that would be counterproductive to the potential for increased rates of parasitism by primary parasitoids. Additional spatio-temporal considerations must be understood to apply this technology in a large field setting. Moreover, it is unclear how the intentional use of HIPV technology impacts the net-efficiency of the HIPV-emitting crop. For example, the use of synthetic green

leaf volatiles and MeJA to induce increased HIPV production in field grown maize did not result in increased parasitism rates by parasitoids of *S. frugiperda* (von Mérey et al., 2011, 2012). An essential question that needs additional exploration is whether an increase in biological control due to HIPV-emission will equate to increased crop yields.

Biological Control Can Reduce the Likelihood of Resistance Evolution

Pesticide resistance is listed as the third most serious threat to global agriculture (behind soil erosion and water pollution) (Pimentel, 2005). Resistance is a pest population's decreased response to a pesticide or control agent (including plant defense traits) as a result of previous exposure (McKenzie, 1996) and over 540 arthropod species have developed resistance to at least one pesticide (Arthropod Pesticide Resistance Database, 2016). The evolution of resistance to GM crops is of particular concern. For example, the WCR developed resistance to Cry3Bb1 Bt proteins with cross-resistance to mCry3A within 8 years of commercial release in the U.S. (Gassmann et al., 2011; Wangila et al., 2015). The impacts of resistance are often severe and far-reaching: they can lead to economic losses and increased pesticide usage. Delaying or preventing adaptation to pesticides, insecticidal GM crops and host plant defense traits can be achieved through the adoption of an integrated resistance management plan, and biological control can play a large role in these efforts. The impact of biological control on the rate of evolution of pest resistance is dependent upon whether natural enemies disproportionately attack resistant preyhosts (thereby slowing resistance evolution) or susceptible preyhosts (thereby accelerating resistance evolution) (Gould et al., 1991). In a high-dose/refuge strategy, such as that used for Bt crops, susceptible pests developing in refuges are frequently found at higher densities than resistant pests feeding on high-dose plants. Therefore, if natural enemies preferentially attack hosts found at higher densities (positive density-dependent mortality), the rate of resistance evolution will be faster than if natural enemies prefer less dense hosts (inverse density-dependent mortality) or are unaffected by host density (density-independent mortality) (Heimpel et al., 2005). For example, *Coleomegilla maculata* De Geer (Coleoptera: Coccinellidae) exhibits inverse density-dependent predation on the egg masses of the Colorado potato beetle *Leptinotarsa decemlineata* Say (Coleoptera: Chrysomelidae), decreasing the rate at which this pest develops resistance to Bt potatoes (Arpaia et al., 1997). However, the introduction of alternative prey can alter feeding patterns of this generalist predator, thereby affecting its influence on resistance evolution (Mallampalli et al., 2005).

Natural enemies can enhance resistance management for plant defense traits by inflicting mortality on those pests that have developed resistance (Liu et al., 2014). In oilseed rape *Brassica napus* L. (Brassicaceae) expressing Bt toxins, for example, the parasitoid *Cotesia vestalis* (Halliday) (Hymenoptera: Braconidae) dies with their host if developing inside a Bt-susceptible *P. xylostella* larva, but does not suffer negative effects when parasitizing Bt-resistant caterpillars (Schuler et al., 1999).

Susceptible *P. xylostella* are killed within 5 days of feeding on Bt plants and consumption of Bt leaves is significantly reduced for susceptible larvae than resistant larvae. Consequently, the parasitoid *C. plutellae* is more attracted to Bt-resistant hosts, as plants with greater feeding damage release more HIPVs, which are attractive to the parasitoid (Schuler et al., 1999). Additionally, natural enemies can slow the evolution of resistance if they increase the fitness costs associated with resistance to crop traits (Raymond et al., 2007) but alternatively may amplify selection for resistance if they attack susceptible prey or hosts more frequently (Gould et al., 1991). For example, susceptible *H. virescens* feeding on Bt tobacco *Nicotiana tabacum* L. (Solanales: Solanaceae) took longer to develop, exposing them to greater parasitism by *Campoletis sonorensis*, and had higher movement rates, increasing risk of infection by the entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson (Johnson and Gould, 1992; Johnson et al., 1997a,b).

As described, biological control can influence the rate of resistance evolution via top-down influence. However, the manner in which host plant resistance traits are implemented can also have an effect on the evolution of resistance through bottom-up selection. The durability of plant resistance traits is affected by a multitude of factors that influence selection pressure on herbivorous pests, such as planting of a monoculture of resistant plants vs. mixtures or refuges of non-resistant plants, the mechanism and efficacy of the resistance traits, and the use of pyramiding multiple resistance traits (Stout, 2013). To achieve the greatest durability of plant defense traits, and therefore a more stable and sustainable pest management strategy, both top-down and bottom-up methods for delaying evolution of resistance by arthropod pests should be employed.

How Can We Integrate Host Plant Resistance and Biological Control?

Historically, developers of HPR and biological control programs have worked independently, seeking to find "single-solution approaches to pest problems" (Thomas and Waage, 1996). Communication between such disparate groups such as plant breeders and natural enemy ecologists may not be inherently high. In reality, there are at least four distinct groups that should come together to better integrate plant defense traits and biological control: (1) HPR researchers (including plant breeders), (2) biological control researchers, (3) ecologists studying community and tritrophic interactions, and (4) extension professionals who are implementing IPM programs and working directly with producers and their advisors (Thomas and Waage, 1996). How can these fields and groups be brought together? Currently, plant breeding for HPR includes the selection of plant traits with the goal of enhancing direct defenses against herbivorous pests, with little consideration for enhancing plant traits that could improve indirect defenses through the action of natural enemies against pests (Cortesero et al., 2000). Evaluating the impacts of plant resistance characteristics on common natural enemies in the assessment of plant varieties during breeding for HPR would aid in bringing these two methods together. Additionally, fundamental

ecological literature and applied host plant resistance literature have suffered from a lack of integration, an observation that has persisted for nearly 30 years (Kogan, 1986; Stout, 2013). An adherence by the host plant resistance community to the three traditional categories of resistance: antibiosis, antixenosis and tolerance (Painter, 1951) may also account for the lack of consideration of the third trophic level (Stout, 2013). Induced, indirect host plant resistance, such as what is seen when herbivore feeding or oviposition on plants triggers the attraction of natural enemies, does not fit into the three traditional categories proposed by Painter (1951). To further our understanding of the interactions between plant defense traits and biological control, experts that can conduct research using natural history, molecular and genetic tools, and field experimentation must be brought together (Agrawal, 2000a).

Practical Implementation of Host Plant Resistance and Biological Control in Integrated Pest Management

A successful IPM plan must account for the ecology and biology of the targeted pest(s), environmental factors, and agricultural management. It must be localized; a one size fits all approach will never be effective, yet area wide suppression programs encompassing large regions are sometimes necessary (Schellhorn et al., 2015). This is a significant challenge in making prescriptions. An HPR-biological control combination targeting the same pest may work in one region, but not another. Similarly, this combination may work for one type of pest, but not another, even within the same field. While HPR and biological control are two of the key pillars of IPM, other essential management tactics include cultural control and chemical control. Another key management tactic is the “stimulo-deterrant diversion” or “push-pull” strategy. Host plant resistance traits can contribute to the “push” component, while biological control by natural enemies may be enhanced by concentration of pests due to the “pull” component (Eigenbrode et al., 2016). Finding a compromise between the strategies of host plant resistance and biological control may prove to be advantageous for selecting management strategies that maximize pest suppression and minimize the likelihood of resistance by reducing selection pressure on pests. For example, glandular pubescence was bred into commercial potato clones for defense against aphids and leafhoppers (Tingey, 1982). In the absence of natural enemies, aphid populations are the lowest on plants with high trichome density; however, when natural enemies are present, biological control is greatest on plants with intermediate trichome density (Obrycki et al., 1983). Therefore, plants with intermediate trichome density were recommended for potato IPM due to their partial resistance to aphids, compatibility with natural enemies, and reduced risk for development of pest resistance (Obrycki et al., 1983). The concept of pairing a partially resistant crop plant with biological control was proposed by van Emden (1988) as two of the three components of a “pest management triad” for aphid control (the third being use of selective insecticides to cause mortality of pests but not natural enemies). Cortesero et al. (2000) identified leaf domatia, trichomes (in intermediate density), plant signaling via

volatiles, and extrafloral nectaries as the most promising plant defense traits for positive synergy with biological control.

Plants experience a wide range of biotic associations (both beneficial and antagonistic) above- and belowground that interact in complex ways (Bezemer and van Dam, 2005; van Dam and Heil, 2011). Herbivory and pathogen pressures experienced belowground can influence above ground interactions between plants, herbivores, and higher trophic levels (e.g., Soler et al., 2007, 2012). Approaches that use beneficial root associates such as arbuscular mycorrhizal fungi and rhizobacteria can not only increase root production and have benefits on yield and aboveground growth, they can stimulate aboveground defensive chemistry providing protection against aboveground herbivores (Gehring and Bennett, 2009; Orrell and Bennett, 2013).

Any recommendations that are given to maximize the compatibility of host plant resistance and biological control must also consider other important agronomic and practical factors, such as water availability and water use efficiency, fertilization and nutrient availability, weed management, and disease management. However, multiple goals can sometimes be achieved by the adoption of a single practice. For example, indirect host plant resistance, pathogen resistance, and biological control can be simultaneously supported in the case with leaf domatia on grape leaves: both predatory and fungivorous mites use these structures for protection and their presence can decrease incidence of arthropod pests and powdery mildew, a major disease of grapes (Agrawal, 2000a; Norton et al., 2000). For crop producers, agronomic traits other than insect resistance, and ultimately yield, will be the deciding factors for variety or hybrid selection. For crops where the seed market is dominated by transgenics, there may be less choice for the farmer; often only the highest yielding hybrids are chosen for transformation; in order to have the Bt or herbicide resistance traits desired, a smaller pool of varieties are available. Plant breeding often focuses on enhancing agronomic traits, such as drought tolerance, with higher yields as a major driving factor. Therefore, breeding for resistance to arthropod pests may not be the highest priority. Many plant defense traits have been inadvertently lost or weakened through domestication and selective breeding to enhance yields (Brattsen, 1991; Loughrin et al., 1995; Pickett et al., 1997; Rasmann et al., 2005; Chen et al., 2015a,b). Often, indirect defenses that rely upon the attraction or provisioning of natural enemies have also been lost, although efforts have been made to restore these plant traits, such as E β C-production due to an oregano transgene in maize to attract nematodes to attack rootworm larvae (see Case study: Maize Volatiles, Western Corn Rootworm, and Entomopathogenic Nematodes) or artificial domatia added to commercial cotton plants, which increased the abundance of certain predators (Agrawal et al., 2000). Wild relatives of cotton do have leaf domatia (Fryxell, 1978) and molecular mapping has been used to identify the genes that affect pubescence in cotton (Wright et al., 1999), allowing for the selective expression of pubescence at the leaf vein axils (domatia) that could positively affect natural enemies and biological control in cotton. Looking back to wild relatives of domesticated plant species could be informative for discovering plant defense traits capable of controlling pest species.

Host plant resistance and biological control are both well-suited for adoption in developing countries due to their low cost and lack of need for specialized equipment. The costs of HPR are often built into the price of seed (and may be a one-time expense if farmers can harvest and plant their own seeds subsequently). Biological control may be completely free, if natural control or conservation biological control is used. However, the use of entomopathogens may require application equipment. These biological control methods are in contrast to other types of management, such as chemical control, which may require the use of expensive equipment that is not accessible to farmers in developing countries. A review of these considerations can be found in Thomas and Waage (1996). Finally, HPR and biological control are compatible with the ecological intensification theory of agricultural production, which focuses on the conservation and promotion of biodiversity to support ecosystem services in cropland (Geertsema et al., 2016).

CONCLUSION

In one of the first reviews to address the interactions between host plant resistance and biological control for pest management, Bergman and Tingey (1979) stated that “interactions between plant resistance and arthropod predators and parasites remain poorly known.” Since that time, a large body of literature has

addressed this important question. However, we will need to continue to explore the dynamic interactions between host plant resistance and biological control as these tritrophic interactions are impacted by changing global conditions, such as climate. It is now clear that the mechanisms by which plant defense traits and natural enemies interact are complex and may be synergistic, disruptive, or anywhere on the continuum between. Each is clearly a powerful tool for suppressing herbivore populations and continued efforts to utilize these methods in IPM are essential for environmentally and economically sustainable global crop production. This review provided synthesis for the many facets of these interactions and encompassed the many critical implications these interactions have for agriculture today.

AUTHOR CONTRIBUTIONS

All authors listed, have made substantial, direct and intellectual contribution to the work, and approved it for publication.

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REFERENCES

- Agrawal, A., Karban, R., and Colfer, R. (2000). How leaf domatia and induced resistance affect herbivores, natural enemies and plant performance. *Oikos* 89, 70–80. doi: 10.1034/j.1600-0706.2000.890108.x
- Agrawal, A., Kobayashi, C., and Thaler, J. S. (1999). Influence of prey availability and induced host-plant resistance on omnivory by western flower thrips. *Ecology* 80, 518–523. doi: 10.1890/0012-9658(1999)080[0518:IOPAAI]2.0.CO;2
- Agrawal, A. A. (2000a). Mechanisms, ecological consequences and agricultural implications of tri-trophic interactions. *Curr. Opin. Plant Biol.* 3, 329–395. doi: 10.1016/S1369-5266(00)00089-3
- Agrawal, A. A. (2000b). Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. *Oikos* 89, 493–500. doi: 10.1034/j.1600-0706.2000.890308.x
- Agrawal, A. A. (2011). Current trends in the evolutionary ecology of plant defence. *Funct. Ecol.* 25, 420–432. doi: 10.1111/j.1365-2435.2010.01796.x
- Agrawal, A. A., and Karban, R. (1997). Domatia mediate plant-arthropod mutualism. *Nature* 387, 562–563. doi: 10.1038/42384
- Alba, J. M., Bleeker, P. M., Glas, J. J., Schimmel, B. C. J., Wijk, M., van Sabelis, M. W., et al. (2012). “The impact of induced plant volatiles on plant-arthropod interactions,” in *Arthropod-Plant Interactions*, eds G. Smagghe and I. Diaz (Dordrecht: Springer International), 15–73.
- Alborn, H. T., Turlings, T. C. J., Jones, T. H., Stenhamer, G., Loughrin, J. H., and Tumlinson, J. H. (1997). An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276, 945–949. doi: 10.1126/science.276.5314.945
- Ali, J. G., and Agrawal, A. A. (2012). Specialist versus generalist insect herbivores and plant defense. *Trends Plant Sci.* 17, 293–302. doi: 10.1016/j.tplants.2012.02.006
- Anbesse, S., and Ehlers, R.-U. (2013). *Heterorhabditis* sp. not attracted to synthetic (E)- β -caryophyllene, a volatile emitted by roots upon feeding by corn rootworm. *J. Appl. Entomol.* 137, 88–96. doi: 10.1111/j.1439-0418.2012.01753.x
- Armer, C. A., Berry, R. E., and Kogan, M. (2000). Longevity of phytophagous heteropterans predators feeding on transgenic Bt-potato plants. *Entomol. Exp. Appl.* 95, 329–333. doi: 10.1046/j.1570-7458.2000.00672.x
- Arpaia, S., Gould, F., and Kennedy, G. (1997). Potential impact of *Coleomegilla maculata* predation on adaptation of *Leptinotarsa decemlineata* to Bt-transgenic potatoes. *Entomol. Exp. Appl.* 82, 91–100. doi: 10.1046/j.1570-7458.1997.00117.x
- Arthropod Pesticide Resistance Database (2016)). *Arthropod Pesticide Resistance Database*. Available at: www.pesticideresistance.org/
- Athey, K. J., Dreyer, J., Kowles, K. A., Penn, H. J., Sitvarin, M. I., and Harwood, J. D. (2016). Spring forward: molecular detection of early season predation in agroecosystems. *Food Webs* doi: 10.1016/j.fooweb.2016.06.001
- Bachman, P. M., Bolognesi, R., Moar, W. J., Mueller, G. M., Paradise, M. S., Ramaseshadri, P., et al. (2013). Characterization of the spectrum of insecticidal activity of a double-stranded RNA with targeted activity against Western Corn Rootworm (*Diabrotica virgifera virgifera* LeConte). *Transgenic Res.* 22, 1207–1222. doi: 10.1007/s11248-013-9716-5
- Baker, E. A. (1974). The influence of environment on leaf wax development in *Brassica oleracea* var. gemmifera. *New Phytol.* 73, 955–966. doi: 10.1111/j.1469-8137.1974.tb01324.x
- Bakker, F. M., and Klein, M. E. (1992). “How cassava plants enhance the efficacy of their phytoseiid bodyguards,” in *Proceedings of the 8th International Symposium on Insect-Plant Relationships*, Dordrecht. 353–354.
- Ballhorn, D. J., Kautz, S., and Heil, M. (2013). Distance and sex determine host plant choice by herbivorous beetles. *PLoS ONE* 8:e55602. doi: 10.1371/journal.pone.0055602
- Barbosa, P., Gross, P., and Kemper, J. (1991). Influence of plant allelochemicals on the tobacco hornworm and its parasitoid, *Cotesia congregata*. *Ecology* 72, 1567–1575. doi: 10.2307/1940956
- Barbosa, P., Saunders, J. A., Kemper, J., Trumbule, R., Olechno, J., and Martinat, P. (1986). Plant allelochemicals and insect parasitoids: effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hypothesis annulipes* (Cresson) (Hymenoptera: Ichneumonidae). *J. Chem. Ecol.* 12, 1319–1328. doi: 10.1007/BF01012351
- Baum, J. A., Bogaert, T., Clinton, W., Heck, G. R., Feldmann, P., Ilagan, O., et al. (2007). Control of coleopteran insect pests through RNA interference. *Nat. Biotechnol.* 25, 1322–1326. doi: 10.1038/nbt1359

- Baur, M. E., and Boethel, D. J. (2003). Effect of Bt-cotton expressing Cry1A(c) on the survival and fecundity of two hymenopteran parasitoids (Braconidae, Encyrtidae) in the laboratory. *Biol. Control* 26, 325–332. doi: 10.1016/S1049-9644(02)00160-3
- Bellows, T. S., Fisher, T. W., Caltagirone, L. E., Dahlsten, D. L., Gordh, G., and Huffaker, C. B. (1999). *Handbook of Biological Control*. Amsterdam: Elsevier.
- Benrey, B., and Denno, R. F. (1997). The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly and its larval parasitoid. *Ecology* 78, 987–999. doi: 10.1890/0012-9658(1997)078[0987:TSGHMH]2.0.CO;2
- Bentley, B. L. (1977). Extrafloral nectaries and protection by pugnacious bodyguards. *Annu. Rev. Ecol. Evol. Syst.* 8, 407–427. doi: 10.1146/annurev.es.08.110177.002203
- Bentley, B. L. (1983). "Nectaries in agriculture, with an emphasis on the tropics," in *The Biology of Nectaries*, eds B. Bentley and T. Elias (New York, NY: Columbia University Press), 204–222.
- Berenbaum, M. R. (1983). Coumarins and caterpillars: a case for coevolution. *Evolution* 37, 163–179. doi: 10.2307/2408184
- Berenbaum, M. R. (1990). Evolution of specialization in insect-umbellifer associations. *Annu. Rev. Entomol.* 35, 319–343. doi: 10.1146/annurev.en.35.010190.001535
- Bergman, J. M., and Tingey, W. M. (1979). Aspects of interaction between plant genotypes and biological control. *Bull. Entomol. Soc. Am.* 25, 275–279. doi: 10.1093/besa/25.4.275
- Bernal, J. S. (2010). "Genetically modified crops and biological control with egg parasitoids," in *Egg Parasitoids in Agroecosystems with Emphasis on Trichogramma*, eds F. Consoli, J. R. Parra, and R. A. Zucchi (Dordrecht: Springer International), 443–465.
- Bernal, J. S., Griset, J. G., and Gillogly, P. O. (2002). Impacts of developing on Bt maize intoxicated hosts on fitness parameters of a stem borer parasitoid. *J. Entomol. Sci.* 37, 27–40.
- Bernal, J. S., Prasifka, J. R., Sétamou, M., and Heinz, K. M. (2004). "Transgenic insecticidal cultivars in integrated pest management: challenges and opportunities," in *Integrated Pest Management: Potential, Constraints and Challenges*, eds O. Koul, G. S. Dhaliwal, and G. W. Cuperus (Oxfordshire: CAB International), 123–145.
- Bernays, E. A., and Graham, M. (1988). On the evolution of host specificity in phytophagous arthropods. *Ecology* 69, 886–892. doi: 10.2307/1941237
- Bezemer, T. M., and van Dam, N. M. (2005). Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol.* 20, 617–624. doi: 10.1016/j.tree.2005.08.006
- Birch, A. N. E., Geoghegan, I. E., Majerus, M. E. N., McNicol, J. W., Hackett, C. A., Gatehouse, A. M. R., et al. (1999). Tri-trophic interactions involving pest aphids, predatory 2-spot ladybirds and transgenic potatoes expressing snowdrop lectin for aphid resistance. *Mol. Breed.* 5, 75–83. doi: 10.1023/A:1009659316170
- Bolognesi, R., Ramaseshadri, P., Anderson, J., Bachman, P., Clinton, W., Flanagan, R., et al. (2012). Characterizing the mechanism of action of double-stranded RNA activity against western corn rootworm (*Diabrotica virgifera virgifera* LeConte). *PLoS ONE* 7:e47534. doi: 10.1371/journal.pone.0047534
- Bottger, G. T., and Patana, R. (1966). Growth, development and survival of certain Lepidoptera fed gossypol in the diet. *J. Econ. Entomol.* 59, 1166–1169. doi: 10.1093/jee/59.5.1166
- Bottrell, D. G., Barbosa, P., and Gould, F. (1998). Manipulating natural enemies by plant variety selection and modification: a realistic strategy? *Annu. Rev. Entomol.* 43, 347–367. doi: 10.1146/annurev.ento.43.1.347
- Brattsen, L. B. (1991). Bioengineering of crop plants and resistant biotype evolution in insects: counteracting coevolution. *Arch. Insect. Biochem. Physiol.* 17, 253–267. doi: 10.1002/arch.940170408
- Bruinsma, M., Posthumus, M. A., Mumm, R., Mueller, M. J., van Loon, J. J. A., and Dicke, M. (2009). Jasmonic acid-induced volatiles of *Brassica oleracea* attract parasitoids: effects of time and dose, and comparison with induction by herbivores. *J. Exp. Bot.* 60, 2575–2587. doi: 10.1093/jxb/erp101
- Buitenhuis, R., Shipp, L., Scott-Dupree, C., Brommitt, A., and Lee, W. (2014). Host plant effects on the behavior and performance of *Amblyseius swirskii* (Acar: Phytoseiidae). *Exp. Appl. Acarol.* 62, 171–180. doi: 10.1007/s10493-013-9735-1
- Bush, G. L. (1974). "Sympatric speciation in phytophagous parasitic insects," in *Evolutionary Strategies of Parasitic Insects and Mites*, ed. P. W. Price (New York, NY: Plenum), 187–206.
- Cameron, J. W., and Anderson, L. D. (1966). Husk tightness, earworm egg numbers, and starchiness of kernels in relation to resistance of corn to the corn earworm. *J. Econ. Entomol.* 59, 556–558. doi: 10.1093/jee/59.3.556
- Campbell, B. C., and Duffey, S. S. (1979). Tomatine and parasitic wasps: potential incompatibility of plant antibiotics with biological control. *Science* 205, 700–702. doi: 10.1126/science.205.4407.700
- Carrière, Y., Ellers-Kirk, C., Sisterson, M., Antillam, L., Whitlow, M., Dennehy, T. J., et al. (2003). Long-term regional suppression of pink bollworm by *Bacillus thuringiensis* cotton. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1519–1523. doi: 10.1073/pnas.0436708100
- Casacuberta, J. M., Devos, Y., du Jardin, P., Ramon, M., Vaucheret, H., and Nogue, F. (2015). Biotechnological uses of RNAi in plants: risk assessment considerations. *Trends Biotechnol.* 33, 145–147. doi: 10.1016/j.tibtech.2014.12.003
- Chapman, R. F. (1977). The role of the leaf surface in food selection by acridids and other insects. *Colloq. Int. Centre Natl. Rech. Sci.* 265, 133–149.
- Chen, M., Ye, G. Y., Liu, Z. C., Fang, Q., Hu, C., Peng, Y. F., et al. (2009). Analysis of Cry1Ab toxin bioaccumulation in a food chain of Bt rice, an herbivore and a predator. *Ecotoxicology* 18, 230–238. doi: 10.1007/s10646-008-0276-z
- Chen, Y. H., Gols, R., and Benrey, B. (2015a). Crop domestication and its impact on naturally selected trophic interactions. *Annu. Rev. Entomol.* 60, 35–58. doi: 10.1146/annurev-ento-010814-020601
- Chen, Y. H., Gols, R., Stratton, C. A., Brevik, K. A., and Benrey, B. (2015b). Complex tritrophic interactions in response to crop domestication: predictions from the wild. *Entomol. Exp. Appl.* 157, 40–59. doi: 10.1111/eea.12344
- Ciosi, M., Toepfer, S., Li, H., Haye, T., Kuhlmann, U., Wang, H., et al. (2009). European populations of *Diabrotica virgifera virgifera* are resistant to aldrin, but not methyl-parathion. *J. Appl. Entomol.* 133, 307–314. doi: 10.1111/j.1439-0418.2008.01363.x
- Cook, O. F. (1904). An enemy of the cotton-boll-weevil. *Science* 19, 862–864. doi: 10.1126/science.19.492.862-a
- Cook, O. F. (1905). Habits of the Kelep or Guatemalan cotton boll weevil ant. *U. S. Dep. Agric. Bull. Bureau Entomol.* 49, 1–15.
- Cortesero, A. M., Stapel, J. O., and Lewis, W. J. (2000). Understanding and manipulating plant attributes to enhance biological control. *Biol. Control* 17, 35–49. doi: 10.1006/bcon.1999.0777
- Culpepper, A. S. (2006). Glyphosate induced weed shifts. *Weed Technol.* 20, 277–281. doi: 10.1073/pnas.1013154107
- De Clercq, P., Mohaghegh, J., and Tirry, L. (2000). Effect of host plant on the functional response of the predator *Podisus nigrispinus* (Heteroptera: Pentatomidae). *Biol. Control* 18, 65–70. doi: 10.1006/bcon.1999.0808
- De Geyter, N., Gholami, A., Goormachtig, S., and Goossens, A. (2012). Transcriptional machineries in jasmonate-elicited plant secondary metabolism. *Trends Plant Sci.* 17, 349–359. doi: 10.1016/j.tplants.2012.03.001
- De Moraes, C. M., Lewis, W. J., Paré, P. W., Alborn, H. T., and Tumlinson, J. H. (1998). Herbivore-infested plants selectively attract parasitoids. *Nature* 393, 570–573. doi: 10.1038/31219
- Degen, T., Dillmann, C., Marion-Poll, F., and Turlings, T. C. J. (2004). High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135, 1928–1938. doi: 10.1104/pp.104.039891
- Degenhardt, J., Gershenson, J., Baldwin, I. T., and Kessler, A. (2003). Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Curr. Opin. Biotechnol.* 14, 169–176. doi: 10.1016/S0958-1669(03)00025-9
- Degenhardt, J., Hiltbold, I., Köllner, T. G., Frey, M., Gierl, A., Gershenson, J., et al. (2009). Restoring a maize root signal that attracts insect-killing nematodes to control a major pest. *Proc. Natl. Acad. Sci. U.S.A.* 106, 13213–13218. doi: 10.1073/pnas.0906365106
- Dicke, M. (1999). Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomol. Exp. Appl.* 91, 131–142. doi: 10.1046/j.1570-7458.1999.00475.x
- Dicke, M. (2009). Behavioural and community ecology of plants that cry for help. *Plant Cell Environ.* 32, 654–665. doi: 10.1111/j.1365-3040.2008.01913.x
- Dicke, M. (2015). Herbivore-induced plant volatiles as a rich source of information for arthropod predators: fundamental and applied aspects. *J. Indian Inst. Sci.* 95, 35–42.
- Duan, J. J., Head, G., McKee, M. J., Nickson, T. E., Martin, J. H., and Sayegh, F. S. (2002). Evaluation of dietary effects of transgenic corn pollen expressing

- Cry3Bb1 protein on a non-target ladybird beetle, *Coleomegilla maculata*. *Entomol. Exp. Appl.* 104, 271–280. doi: 10.1046/j.1570-7458.2002.01013.x
- Duan, J. J., Lundgren, J. G., Naranjo, S. E., and Marvier, M. (2010). Extrapolating non-target risk of Bt crops from laboratory to field. *Biol. Lett.* 6, 74–77. doi: 10.1098/rsbl.2009.0612
- Duan, X. L., Hou, Q. L., and Liang, R. Q. (2015). Expression of two synthetic lectin genes sGNA and sNTL in transgenic wheat enhanced resistance to aphids. *Res. J. Biotechnol.* 10, 11–18.
- Dudareva, N., Klempien, A., Muhlemann, J. K., and Kaplan, I. (2013). Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytol.* 198, 16–32. doi: 10.1111/nph.12145
- Dutton, A., Klein, H., Romeis, J., and Bigler, F. (2002). Uptake of Bt-toxin by herbivores feeding on transgenic maize and consequences for the predator *Chrysoperla carnea*. *Ecol. Entomol.* 27, 441–447. doi: 10.1046/j.1365-2311.2002.00436.x
- Eigenbrode, S. D., Birch, A. N. E., Lindzey, S., Meadow, R., and Snyder, W. E. (2016). A mechanistic framework to improve understanding and application of push-pull systems in pest management. *J. Appl. Ecol.* 53, 202–212. doi: 10.1111/1365-2664.12556
- Eigenbrode, S. D., Castagnola, T., Roux, M.-B., and Steljes, L. (1996). Mobility of three generalist predators is greater on cabbage with glossy leaf wax than on cabbage with a wax bloom. *Entomol. Exp. Appl.* 81, 335–343. doi: 10.1046/j.1570-7458.1996.00104.x
- Eigenbrode, S. D., and Espelie, K. E. (1995). Effects of plant epicuticular lipids on insect herbivores. *Annu. Rev. Entomol.* 40, 171–194. doi: 10.1146/annurev.en.40.010195.001131
- Eigenbrode, S. D., Espelie, K. E., and Shelton, A. M. (1991a). Behavior of neonate diamondback moth larvae [*Plutella xylostella* (L.)] on leaves and on extracted leaf waxes of resistant and susceptible cabbages. *J. Chem. Ecol.* 17, 1691–1704. doi: 10.1007/BF00984697
- Eigenbrode, S. D., and Jetter, R. (2002). Attachment to plant surface waxes by an insect predator. *Integr. Comp. Biol.* 42, 1091–1099. doi: 10.1093/icb/42.6.1091
- Eigenbrode, S. D., Kabalo, N. N., and Stoner, K. A. (1999). Predation, behavior, and attachment by *Chrysoperla plorabunda* larvae on *Brassica oleracea* with different surface waxblooms. *Entomol. Exp. Appl.* 90, 225–235. doi: 10.1046/j.1570-7458.1999.00443.x
- Eigenbrode, S. D., Moodie, S., and Castagnola, T. (1995). Predators mediate host plant resistance to a phytophagous pest in cabbage with glossy leaf wax. *Entomol. Exp. Appl.* 77, 335–342. doi: 10.1111/j.1570-7458.1995.tb02331.x
- Eigenbrode, S. D., and Shelton, A. M. (1990). Behavior of neonate diamondback moth larvae (Lepidoptera: Plutellidae) on glossy-leaved resistant *Brassica oleracea* L. *Environ. Entomol.* 19, 1566–1571. doi: 10.1093/ee/19.5.1566
- Eigenbrode, S. D., Stoner, K. A., Shelton, A. M., and Kain, W. C. (1991b). Characteristics of glossy leaf waxes associated with resistance to diamondback moth (Lepidoptera: Plutellidae) in *Brassica oleracea*. *J. Econ. Entomol.* 84, 1609–1618. doi: 10.1093/jee/84.5.1609
- Eilenberg, J., Hajek, A., and Lomer, C. (2001). Suggestions for unifying the terminology in biological control. *Biocontrol* 46, 387–400. doi: 10.1023/A:1014193329979
- Eubanks, M. D., and Denno, R. F. (1999). The ecological consequences of variation in plants and prey for an omnivorous insect. *Ecology* 80, 1253–1266. doi: 10.1890/0012-9658(1999)080[1253:TECOVI]2.0.CO;2
- Fearing, P. L., Brown, D., Vlachos, D., Meghji, M., and Privalle, L. (1997). Quantitative analysis of CryIA(b) expression in Bt maize plants, tissues, and silage and stability of expression over successive generations. *Mol. Breed.* 3, 167–176. doi: 10.1023/A:1009611613475
- Felton, G. W., and Tumlinson, J. H. (2008). Plant-insect dialogs: complex interactions at the plant-insect interface. *Trends Plant Sci.* 17, 250–259. doi: 10.1016/j.tplants.2008.07.001
- Ferry, N., Mulligan, E. A., Stewart, C. N., Tabashnik, B. E., Port, G. R., and Gatehouse, A. M. R. (2006). Prey-mediated effects of transgenic canola on a beneficial, non-target, carabid beetle. *Transgenic Res.* 15, 501–514. doi: 10.1007/s11248-006-0021-4
- Fox, L. R., Letourneau, D. K., Eisenbach, J., and van Nouhuys, S. (1990). Parasitism rates and sex ratios of a parasitic wasp: effects of herbivore and plant quality. *Oecologia* 83, 414–419. doi: 10.1007/BF00317569
- Francis, F., Lognay, G., Wathelet, J.-P., and Haubrige, E. (2001). Effects of allelochemicals from first (Brassicaceae) and second (*Myzus persicae* and *Brevicoryne brassicae*) trophic levels on *Adalia bipunctata*. *J. Chem. Ecol.* 27, 243–256. doi: 10.1023/A:1005672220342
- Fryxell, P. A. (1978). *The Natural History of the Cotton Tribe*. College Station, TX: Texas A & M University Press.
- Garcia, M., Ortego, F., Castanera, P., and Farinos, G. P. (2012). Assessment of prey-mediated effects of the coleopteran-specific toxin Cry3Bb1 on the generalist predator *Atheta coraria* (Coleoptera: Staphylinidae). *Bull. Entomol. Res.* 102, 293–302. doi: 10.1017/S0007485311000666
- Gassmann, A. J., Petzold-Maxwell, J. L., Keweshan, R. S., and Dunbar, M. W. (2011). Field-evolved resistance to Bt maize by western corn rootworm. *PLoS ONE* 6:e22629. doi: 10.1371/journal.pone.0022629
- Gatehouse, A. M. R., Down, R. E., Powell, K. S., Sauvion, N., Rahb, Y., Newell, C. A., et al. (1996). Effects of GNA-expressing transgenic potato plants on peach-potato aphid, *Myzus persicae*. *Entomol. Exp. Appl.* 79, 295–307. doi: 10.1011/j.1570-7458.1996.tb00837.x
- Geertsema, W., Rossing, W. A. H., Landis, D. A., Bianchi, F. J. J. A., van Rijn, P. C. J., Schaminée, J. H. J., et al. (2016). Actionable knowledge for ecological intensification of agriculture. *Front. Ecol. Environ.* 14:209–216. doi: 10.1002/fee.1258
- Geervliet, J. B. F., Ariëns, S., Dicke, M., and Vet, L. E. M. (1998). Long-distance assessment of patch profitability through volatile infochemicals by the parasitoids *Cotesia glomerata* and *C. rubecula* (Hymenoptera: Braconidae). *Biol. Control* 11, 113–121. doi: 10.1006/bcon.1997.0585
- Gehring, C., and Bennett, A. (2009). Mycorrhizal fungal-plant-insect interactions: the importance of a community approach. *Environ. Entomol.* 38, 93–102. doi: 10.1603/022.038.0111
- Geng, J.-H., Shen, Z.-R., Song, K., and Zheng, L. (2006). Effect of pollen of regular cotton and transgenic Bt+CpTI cotton on the survival and reproduction of the parasitoid wasp *Trichogramma chilonis* (Hymenoptera: Trichogrammatidae) in the laboratory. *Environ. Entomol.* 35, 1661–1668. doi: 10.1093/ee/35.6.1661
- Ghising, K., Harmon, J. P., Beauzay, P. B., Prischmann-Voldseth, D. A., Helms, T. C., Ode, P. J., et al. (2012). Impact of Rag1 aphid resistant soybeans on *Binodoxys communis* (Hymenoptera: Braconidae), a parasitoid of soybean aphid (Hemiptera: Aphididae). *Environ. Entomol.* 41, 282–288. doi: 10.1603/EN11196
- Gols, R., Bukovinsky, T., van Dam, N. M., Dicke, M., Bullock, J. M., and Harvey, J. A. (2008a). Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations. *J. Chem. Ecol.* 34, 132–143. doi: 10.1007/s10886-008-9429-z
- Gols, R., Witjes, L. M. A., van Loon, J. J. A., Posthumus, M. A., Dicke, M., and Harvey, J. A. (2008b). The effect of direct and indirect defenses in two wild brassicaceous plant species on a specialist herbivore and its gregarious endoparasitoid. *Entomol. Exp. Appl.* 128, 99–108. doi: 10.1111/j.1570-7458.2008.00681.x
- Gould, F., Kennedy, G. G., and Johnson, M. T. (1991). Effects of natural enemies on the rate of herbivore adaptation to resistant host plants. *Entomol. Exp. Appl.* 58, 1–14. doi: 10.1011/j.1570-7458.1991.tb01445.x
- Gray, M. E., Sappington, T. W., Miller, N. J., Moeser, J., and Bohn, M. O. (2009). Adaptation and invasiveness of western corn rootworm: intensifying research on a worsening pest. *Annu. Rev. Entomol.* 54, 303–321. doi: 10.1146/annurev.ento.54.110807.090434
- Grazia-Tommasini, M. (1995). “*Frankliniella occidentalis* and other thrips harmful to vegetable and ornamental crops in Europe,” in *Biological Control of Thrips Pests*, eds A. J. M. Loomans, J. C. van Lenteren, M. G. Tommasini, S. Maini, and J. Riudavets (Wageningen: Wageningen Agricultural University Press), 1–42.
- Grossi-de-Sa, M. F., Lucena, W., Souza, M. L., Nepomuceno, A. L., Osir, E. O., Amugune, N., et al. (2006). “Transgene expression and locus structure of Bt cotton,” in *Environmental Risk Assessment of Genetically Modified Organisms-Methodologies for Assessing Bt Cotton in Brazil*, eds A. Hilbeck, D. A. Andow, and E. M. G. Fontes (Wallingford: CAB International), 93–107.
- Gunasena, G. H., Vinson, S. B., Williams, H. J., and Stipanovic, R. D. (1989). Development and survival of the endoparasitoid *Campoletis sonorensis* (Hymenoptera: Ichneumonidae) reared from gossypol exposed *Heliothis virescens* (F) (Lepidoptera: Noctuidae). *Environ. Entomol.* 18, 886–891. doi: 10.1093/ee/18.5.886
- Hagenbucher, S., Olson, D. M., Ruberson, J. R., Wäckers, F. L., and Romeis, J. (2013a). Resistance mechanisms against arthropod herbivores in cotton and

- their interactions with natural enemies. *Crit. Rev. Plant Sci.* 32, 458–482. doi: 10.1080/07352689.2013.809293
- Hagenbucher, S., Wäckers, F. L., and Romeis, J. (2014a). Aphid honeydew quality as a food source for parasitoids is maintained in Bt cotton. *PLoS ONE* 9:e107806. doi: 10.1371/journal.pone.0107806
- Hagenbucher, S., Wäckers, F. L., and Romeis, J. (2014b). Indirect multi-trophic interactions mediated by induced plant resistance: impact of caterpillar feeding on aphid parasitoids. *Biol. Lett.* 10:20130795. doi: 10.1098/rsbl.2013.0795
- Hagenbucher, S., Wäckers, F. L., Wettstein, F. E., Olson, D. M., Ruberson, J. R., and Romeis, J. (2013b). Pest trade-offs in technology: reduced damage by caterpillars in Bt cotton benefits aphids. *Proc. R. Soc. B* 280: 20130042. doi: 10.1098/rspb.2013.0042
- Hajek, A. E. (2004). *Natural Enemies*. New York, NY: Cambridge University Press.
- Halkier, B. A., and Gershenson, J. (2006). Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.* 57, 303–333. doi: 10.1146/annurev.arplant.57.032905.105228
- Hamm, J. J., and Wiseman, B. R. (1986). Plant resistance and nuclear polyhedrosis virus for suppression of fall armyworm (Lepidoptera: Noctuidae). *Fla. Entomol.* 69, 541–549. doi: 10.2307/3495388
- Han, Y., Chen, J., Wang, H., Zhao, J., He, Y., and Hua, H. (2015). Prey-mediated effects of transgenic cry2Aa rice on the spider *Hylaphantes graminicola*, a generalist predator of *Nilaparvata lugens*. *BioControl* 60, 251–261. doi: 10.1007/s10526-014-9629-0
- Harvey, J. A., and Gols, R. (2011). Population-related variation in plant defense more strongly affects survival of an herbivore than its solitary parasitoid wasp. *J. Chem. Ecol.* 37, 1081–1090. doi: 10.1007/s10886-011-0024-3
- Harwood, J. D., Samson, R. A., and Obrycki, J. J. (2007). Temporal detection of Cry1Ab-endotoxins by coccinellid predators in fields of *Bacillus thuringiensis* corn. *Bull. Entomol. Res.* 97, 643–648. doi: 10.1017/S000748530700524X
- Harwood, J. D., Wallin, H., and Obrycki, J. J. (2005). Uptake of Bt-endotoxins by non-target herbivores and higher order arthropod predators: molecular evidence from a transgenic corn agroecosystem. *Mol. Ecol.* 14, 2815–2823. doi: 10.1111/j.1365-294X.2005.02611.x
- Hawley, C. J., Peairs, F. B., and Randolph, T. L. (2003). Categories of resistance at different growth stages in halt, a winter wheat resistant to the Russian wheat aphid (Homoptera: Aphididae). *J. Econ. Entomol.* 96, 214–219. doi: 10.1093/jee/96.1.214
- Heard, M. S., Rothery, P., Perry, J. N., and Firbank, L. G. (2005). Predicting long-term changes in weed populations under GMHT crop management. *Weed Res.* 45, 331–338. doi: 10.1111/j.1365-3180.2005.00465.x
- Heil, M. (2008). Indirect defence via tritrophic interactions. *New Phytol.* 178, 41–61. doi: 10.1111/j.1469-8137.2007.02330.x
- Heil, M. (2014). Herbivore-induced plant volatiles: targets, perception and unanswered questions. *New Phytol.* 204, 297–306. doi: 10.1111/nph.12977
- Heil, M. (2015). Extrafloral nectar at the plant-insect interface: a spotlight on chemical ecology, phenotypic plasticity, and food webs. *Annu. Rev. Entomol.* 60, 213–232. doi: 10.1146/annurev-ento-010814-020753
- Heil, M., and Ton, J. (2008). Long-distance signaling in plant defence. *Trends Plant Sci.* 13, 264–272. doi: 10.1016/j.tplants.2008.03.005
- Heimpel, G. E., Neuhauser, C., and Andow, D. A. (2005). Natural enemies and the evolution of resistance to transgenic insecticidal crops by pest insects: the role of egg mortality. *Environ. Entomol.* 34, 512–526. doi: 10.1603/0046-225X-34.3.512
- Higley, L. G., and Peterson, R. K. D. (2009). “Economic decision rules for IPM,” in *Integrated Pest Management: Concepts, Tactics, Strategies and Case Studies*, eds E. B. Radcliffe, W. D. Hutchison, and R. E. Cancelado (New York, NY: Cambridge University Press), 25–32.
- Hilbeck, A., Baumgartner, M., Fried, P. M., and Bigler, F. (1998a). Effects of transgenic *Bacillus thuringiensis* corn-fed prey on mortality and development time of immature *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environ. Entomol.* 27, 480–487. doi: 10.1093/ee/27.5.1255
- Hilbeck, A., Moar, W. J., Puszta-Carey, M., Filippini, A., and Bigler, F. (1998b). Toxicity of *Bacillus thuringiensis* Cry1Ab toxin to the predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). *Environ. Entomol.* 27, 1255–1263. doi: 10.1093/ee/27.5.1255
- Hilbeck, A., and Otto, M. (2015). Specificity and combinatorial effects of *Bacillus thuringiensis* cry toxins in the context of GMO environmental risk assessment. *Front. Environ. Sci.* 3:71. doi: 10.3389/fenvs.2015.00071
- Hilbeck, A., and Schmidt, J. E. U. (2006). Another view on Bt proteins – how specific are they and what else might they do? *Biopestic. Int.* 2, 1–50.
- Hilfiker, O., Groux, R., Bruessow, F., Kiefer, K., Zeier, J., and Reymond, P. (2014). Insect eggs induce a systemic acquired resistance in *Arabidopsis*. *Plant J.* 80, 1085–1094. doi: 10.1111/tpj.12707
- Hilker, M., and Meiners, T. (2006). Early herbivore alert: insect eggs induce plant defense. *J. Chem. Ecol.* 32, 1379–1397. doi: 10.1007/s10886-006-9057-4
- Hiltbold, I., Baroni, M., Toepfer, S., Kuhlmann, U., and Turlings, T. C. J. (2010a). Selection of entomopathogenic nematodes for enhanced responsiveness to a volatile root signal helps to control a major root pest. *J. Exp. Biol.* 213, 2417–2423. doi: 10.1242/jeb.041301
- Hiltbold, I., Baroni, M., Toepfer, S., Kuhlmann, U., and Turlings, T. C. J. (2010b). Selective breeding of entomopathogenic nematodes for enhanced attraction to a root signal did not reduce their establishment or persistence after field release. *Plant Signal. Behav.* 5, 1450–1452. doi: 10.4161/psb.5.11.13363
- Hiltbold, I., Erb, M., Robert, C. A. M., and Turlings, T. C. J. (2011). Systemic root signalling in a belowground, volatile-mediated tritrophic interaction. *Plant Cell Environ.* 34, 1267–1275. doi: 10.1111/j.1365-3040.2011.02327.x
- Hiltbold, I., Hibbard, B. E., French, B. W., and Turlings, T. C. J. (2012). Capsules containing entomopathogenic nematodes as a Trojan horse approach to control the western corn rootworm. *Plant Soil* 358, 11–25. doi: 10.1007/s11104-012-1253-0
- Hiltbold, I., Toepfer, S., Kuhlmann, U., and Turlings, T. C. J. (2010c). How maize root volatiles affect the efficacy of entomopathogenic nematodes in controlling the western corn rootworm? *Chemoecology* 20, 155–162. doi: 10.1007/s00049-009-0034-6
- Holling, C. S. (1966). The functional response of invertebrate predators to prey density. *Mem. Can. Entomol.* 116, 1109–1121.
- Honěk, A. (1993). Intraspecific variation in body size and fecundity in insects: a general relationship. *Oikos* 66, 483–492. doi: 10.2307/3544943
- Horgervorst, P. A. M., Ferry, N., Gatehouse, M. R., Wäckers, F. L., and Romeis, J. (2006). Direct effects of snowdrop lectin (GNA) on larvae of three aphid predators and fate of GNA after ingestion. *J. Insect Physiol.* 52, 614–624. doi: 10.1016/j.jinsphys.2006.02.011
- Hulspas-Jordan, P. M., and van Lenteren, J. C. (1978). The relationship between host plant leaf structure and parasitisation efficiency of the parasitic wasp *Encarsia formosa* Gahan (Hymenoptera: Aphelinidae). *Meded. Faculteit Landbouww. Rijksuniv. Gent* 43, 431–439.
- Hutchison, W. D., Burkness, E. C., Mitchell, P. D., Moon, R. D., Leslie, T. W., Fleischer, S. J., et al. (2010). Areawide suppression of European corn borer with Bt maize reaps savings to non-Bt maize growers. *Science* 330, 222–225. doi: 10.1126/science.1190242
- Jackson, J. J. (1996). Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 89, 366–372. doi: 10.1093/jee/89.2.366
- Jalalizand, A., Karimy, A., Ashouri, A., Hosseini, M., and Golparvar, A. R. (2012). Effect of host plant morphological features on functional response of *Orius albipennis* (Hemiptera: Anthocoridae) to *Tetranychus urticae* (Acari: Tetranychidae). *Res. Crops* 13, 378–384.
- Jeffries, M. J., and Lawton, J. H. (1984). Enemy free space and the structure of ecological communities. *Biol. J. Linn. Soc.* 23, 269–286. doi: 10.1007/s11356-009-0138-0
- Jenkins, J. N., Maxwell, F. G., and Lafever, H. N. (1966). The comparative preference of insects for ginned and glandless cotton. *J. Econ. Entomol.* 59, 352–356. doi: 10.1093/jee/59.2.352
- Johnson, M. T., and Gould, F. (1992). Interaction of genetically engineered host plant resistance and natural enemies of *Heliothis virescens* (Lepidoptera: Noctuidae) in tobacco. *Environ. Entomol.* 21, 586–597. doi: 10.1093/ee/21.3.586
- Johnson, M. T., Gould, F., and Kennedy, G. G. (1997a). Effect of an entomopathogen on adaptation of *Heliothis virescens* populations to transgenic host plants. *Entomol. Exp. Appl.* 83, 121–135. doi: 10.1046/j.1570-7458.1997.00165.x
- Johnson, M. T., Gould, F., and Kennedy, G. G. (1997b). Effects of natural enemies on relative fitness of *Heliothis virescens* genotypes adapted and not adapted to resistant host types. *Entomol. Exp. Appl.* 82, 219–230. doi: 10.1046/j.1570-7458.1997.00133.x

- Kaplan, I. (2012). Attracting carnivorous arthropods with plant volatiles: the future of biocontrol or playing with fire? *Biol. Control* 60, 77–89. doi: 10.1016/j.biocontrol.2011.10.017
- Kazana, E., Pope, T. W., Tibbles, L., Bridges, M., Pickett, J. A., Bones, A. M., et al. (2007). The cabbage aphid: a walking mustard oil bomb. *Proc. R. Soc. B* 274, 2271–2277. doi: 10.1098/rspb.2007.0237
- Kazmer, D. J., and Luck, R. F. (1995). Field tests of the size-fitness hypothesis in the egg parasitoid *Trichogramma pretiosum*. *Ecology* 76, 412–425. doi: 10.2307/1941200
- Kessler, A. (2015). The information landscape of plant constitutive and induced secondary metabolite production. *Curr. Opin. Insect Sci.* 8, 47–53. doi: 10.1016/j.cois.2015.02.002
- Kessler, A., and Baldwin, I. T. (2002). Plant responses to insect herbivory: the emerging molecular analysis. *Annu. Rev. Plant Biol.* 53, 299–328. doi: 10.1146/annurev.arplant.53.100301.135207
- Kim, J., Tooker, J. F., Luthe, D. S., De Moraes, C. M., and Felton, G. W. (2012). Insect eggs can enhance wound response in plants: a study of tomato *Solanum lycopersicum* L. and *Helicoverpa zea* Boddie. *PLoS ONE* 7:e37420. doi: 10.1371/journal.pone.0037420
- Kirk, W. D. J., and Terry, L. I. (2003). The spread of the western flower thrips *Frankliniella occidentalis* (Pergande). *Agric. For. Entomol.* 5, 301–310. doi: 10.1046/j.1461-9563.2003.00192.x
- Kogan, M. (1986). "Plant defense strategies and host-plant resistance," in *Ecological Theory and Integrated Pest Management Practice*, ed. M. Kogan (New York, NY: John Wiley and Sons), 83–134.
- Kogan, M. (1998). Integrated pest management: historical perspectives and contemporary developments. *Annu. Rev. Entomol.* 43, 243–270. doi: 10.1146/annurev.ento.43.1.243
- Köllner, T. G., Held, M., Lenk, C., Hiltbold, I., Turlings, T. C. J., Gershenzon, J., et al. (2008). A maize (E)- β -caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *Plant Cell* 20, 482–494. doi: 10.1105/tpc.107.051672
- Konno, K. (2011). Plant latex and other exudates as plant defense systems: roles of various defense chemicals and proteins contained therein. *Phytochemistry* 72, 1510–1530. doi: 10.1016/j.phytochem.2011.02.016
- Koptur, S. (1992). "Extrafloral nectary-mediated interactions between insects and plants," in *Insect-Plant Interactions*, ed. E. Bernays (Boca Raton, FL: CRC Press), 81–129.
- Koveos, D. S., and Broufas, G. D. (2000). Functional response of *Euseius finlandicus* and *Amblyseius andersoni* to *Panonychus ulmi* on apple and peach leaves in the laboratory. *Exp. Appl. Acarol.* 24, 247–256. doi: 10.1023/A:1006431710313
- Krieger, R. I., Feeny, P. P., and Wilkinson, C. F. (1971). Detoxification enzymes in guts of caterpillars: an evolutionary answer to plant defenses. *Science* 172, 579–581. doi: 10.1126/science.172.3983.579
- Krips, O. E., Kleijn, P. W., Willems, P. E. L., Gols, G. J. Z., and Dicke, M. (1999). Leaf hairs influence searching efficiency and predation rate of the predatory mite *Phytoseiulus persimilis* (Acari: Phytoseiidae). *Exp. Appl. Acarol.* 23, 119–131. doi: 10.1023/A:1006098410165
- Kumar, A., Kumar, N., Siddiqui, A., and Tripathi, C. P. M. (1999). Prey-predator relationship between *Lipaphis erysimi* Kalt. (Hom., Aphididae) and *Coccinella septempunctata* L. (Col., Coccinellidae). II. Effect of host plants on the functional response of the predator. *J. Appl. Entomol.* 125, 591–601. doi: 10.1046/j.1439-0418.1999.00367.x
- Kuo, H.-L. (1986). "Resistance of oats to cereal aphids: effects on parasitism by *Aphelinus asychis* (Walker)," in *Interactions of Plant Resistance and Parasitoids and Predators of Insects*, eds D. J. Boethel and R. D. Eikenbary (West Sussex: Ellis Horwood Limited), 125–137.
- Kurtz, B., Hiltbold, I., Turlings, T. C. J., Kuhlmann, U., and Toepfer, S. (2009). Comparative susceptibility of larval instars and pupae of the western corn rootworm to infection by three entomopathogenic nematodes. *BioControl* 54, 255–262. doi: 10.1007/s10526-008-9156-y
- Lampert, E. C., Dyer, L. A., and Bowers, M. D. (2010). Caterpillar chemical defense and parasitoid success: *Cotesia congregata* parasitism of *Ceratomia catalpa*. *J. Chem. Ecol.* 36, 992–998. doi: 10.1007/s10886-010-9840-0
- Lampert, E. C., Dyer, L. A., and Bowers, M. D. (2011a). Chemical defense across three trophic levels: *Catalpa bignonioides*, the caterpillar *Ceratomia catalpa*, and its endoparasitoid *Cotesia congregata*. *J. Chem. Ecol.* 37, 1063–1070. doi: 10.1007/s10886-011-0018-1
- Lampert, E. C., Zangerl, A. R., Berenbaum, M. R., and Ode, P. J. (2011b). Generalist and specialist host-parasitoid associations respond differently to wild parsnip (*Pastinaca sativa*) defensive chemistry. *Ecol. Entomol.* 36, 52–61. doi: 10.1111/j.1365-2311.2010.01244.x
- Lampert, E. C., Zangerl, A. R., Berenbaum, M. R., and Ode, P. J. (2008). Tritrophic effects of xanthotoxin on the polyembryonic parasitoid *Copidosoma sosares* (Hymenoptera: Encyrtidae). *J. Chem. Ecol.* 34, 783–790. doi: 10.1007/s10886-008-9481-8
- Landis, D. A., Wratten, S. D., and Gurr, G. M. (2000). Habitat management to conserve natural enemies of arthropod pests in agriculture. *Annu. Rev. Entomol.* 45, 175–201. doi: 10.1146/annurev.ento.45.1.175
- Lawo, N. C., Wäckers, F. L., and Romeis, J. (2010). Characterizing indirect prey-quality mediated effects of a Bt crop on predatory larvae of the green lacewing, *Chrysoperla carnea*. *J. Insect Physiol.* 56, 1702–1710. doi: 10.1016/j.jinsphys.2010.06.012
- Lazník, Ž., and Trdán, S. (2013). An investigation on the chemotactic responses of different entomopathogenic nematode strains to mechanically damaged maize root volatile compounds. *Exp. Parasitol.* 134, 349–355. doi: 10.1016/j.exppara.2013.03.030
- Leather, S. R., and Walsh, P. J. (1993). Sub-lethal plant defenses: the paradox remains. *Oecologia* 93, 153–155. doi: 10.1007/BF00317663
- Lee, J. H., and Kang, T. J. (2004). Functional response of *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) to *Aphis gossypii* Glover (Homoptera: Aphididae) in the laboratory. *Biol. Control* 31, 306–310. doi: 10.1016/j.bioco.2004.04.011
- Levin, D. A. (1973). The role of trichomes in plant defense. *Q. Rev. Biol.* 48, 3–15. doi: 10.1086/407484
- Levine, E., Spencer, J. L., Isard, S. A., Onstad, D. W., and Gray, M. E. (2002). Adaptation of the western corn rootworm to crop rotation: evolution of a new strain in response to a management practice. *Am. Entomol.* 48, 94–107. doi: 10.1093/ae/48.2.94
- Lewis, L. C., Bruck, D. J., Prasifka, J. R., and Raun, E. S. (2009). *Nosema pyrausta*: its biology, history, and potential role in a landscape of transgenic insecticidal crops. *Biol. Control* 48, 223–231. doi: 10.1016/j.bioco.2008.10.009
- Lewis, W. J., and Takaishi, K. (1990). Use of learned odours by a parasitic wasp in accordance with host and food needs. *Nature* 348, 635–636. doi: 10.1038/348635a0
- Li, X., Baudry, J., Berenbaum, M. R., and Schuler, M. A. (2004). Structural and functional divergence of insect CYP6B proteins: from specialist to generalist P450. *Proc. Natl. Acad. Sci. U.S.A.* 101, 2939–2944. doi: 10.1073/pnas.0308691101
- Li, Y., Meissle, M., and Romeis, J. (2008). Consumption of Bt maize pollen expressing Cry1Ab or Cry3Bb1 does not harm adult green lacewings, *Chrysoperla carnea* (Neuroptera: Chrysopidae). *PLoS ONE* 3:e2909. doi: 10.1371/journal.pone.0002909
- Li, Y. H., and Romeis, J. (2009). Impact of snowdrop lectin (*Galanthus nivalis* agglutinin; GNA) on adults of the green lacewing, *Chrysoperla carnea*. *J. Insect Physiol.* 55, 135–142. doi: 10.1016/j.jinsphys.2008.10.015
- Lin, J., Eckenrode, C. J., and Dickson, M. H. (1983). Variation in *Brassica oleracea* resistance to diamondback moth (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 76, 1423–1427. doi: 10.1093/jee/76.6.1423
- Lindgren, P. D., and Lukefahr, M. J. (1977). Effects of nectariless cotton on caged populations of *Campoletis sonorensis*. *Environ. Entomol.* 6, 586–588. doi: 10.1093/ee/6.4.586
- Liu, X., Chen, M., Collins, H. L., Onstad, D. W., Roush, R. T., Zhang, Q., et al. (2014). Natural enemies delay insect resistance to Bt crops. *PLoS ONE* 9:e90366. doi: 10.1371/journal.pone.0090366
- Loader, C., and Damman, H. (1991). Nitrogen content of food plants and vulnerability of *Pieris rapae* to natural enemies. *Ecology* 72, 1586–1590. doi: 10.2307/1940958
- Loughrin, J. H., Manukian, A., Heath, R. R., and Tumlinson, J. H. (1995). Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol.* 21, 1217–1227. doi: 10.1007/BF02228321
- Louis, J., Peiffer, M., Ray, S., Luthe, D. S., and Felton, G. W. (2013). Host-specific salivary elicitor(s) of European corn borer induce defenses in tomato and maize. *New Phytol.* 199, 66–73. doi: 10.1111/nph.12308
- Lövei, G. L., and Arpaia, S. (2005). The impact of transgenic plants on natural enemies: a critical review of laboratory studies. *Entomol. Exp. Appl.* 114, 1–14. doi: 10.1007/s11248-009-9297-5

- Lu, Y., Wu, K., Jiang, Y., Guo, Y., and Desneaux, N. (2012). Widespread adoption of Bt cotton and insecticide decrease promotes biocontrol services. *Nature* 487, 362–365. doi: 10.1038/nature11153
- Lu, Y., Wu, K., Jiang, Y., Xia, B., Li, P., Feng, H., et al. (2010). Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of Bt in cotton. *Science* 328, 1151–1154. doi: 10.1126/science.1187881
- Ludy, C., and Lang, A. (2006). Bt maize pollen exposure and impact on the garden spider, *Araneus diadematus*. *Entomol. Exp. Appl.* 118, 145–156. doi: 10.1111/j.1570-7458.2006.00375.x
- Lukefahr, M. J., and Griffin, J. A. (1956). The effects of food on the longevity and fecundity of pink bollworm moths. *J. Econ. Entomol.* 49, 876–877. doi: 10.1093/jee/49.6.876
- Lukefahr, M. J., and Rhyne, C. (1960). Effects of nectarless cottons on populations of three lepidopterous insects. *J. Econ. Entomol.* 53, 242–244. doi: 10.1093/jee/53.2.242
- Lundgren, J. G. (2009). *Relationships of Natural Enemies and Non-prey Foods*. Dordrecht: Springer International.
- Lundgren, J. G., and Duan, J. J. (2013). RNAi-based insecticidal crops: potential effects on nontarget species. *BioScience* 63, 657–665. doi: 10.1525/bio.2013.63.8.8
- Lundgren, J. G., Gassmann, A. J., Bernal, J., Duan, J. J., and Ruberson, J. (2009a). Ecological compatibility of GM crops and biological control. *Crop Prot.* 28, 1017–1030. doi: 10.1016/j.cropro.2009.06.001
- Lundgren, J. G., Hesler, L. S., Tilmon, K., Dashiell, K., and Scott, R. (2009b). Direct effects of soybean varietal selection and *Aphis glycines*-resistant soybeans on natural enemies. *Arthropod Plant Interact.* 3, 9–16. doi: 10.1007/s11829-008-9053-4
- Lundgren, J. G., and Wiedenmann, R. N. (2002). Coleopteran-specific Cry3Bb toxin from transgenic corn pollen does not affect the fitness of a non-target species, *Coleomegilla maculata* DeGeer (Coleoptera: Coccinellidae). *Environ. Entomol.* 31, 1213–1218. doi: 10.1603/0046-225X-31.6.1213
- Lutz, W., and Samir, K. C. (2010). Dimensions of global population projections: what do we know about future population trends and structures? *Philos. Trans. R. Soc. B* 365, 2779–2791. doi: 10.1098/rstb.2010.0133
- Macey, M. J. K., and Barber, H. N. (1970). Chemical genetics of wax formation on leaves of *Brassica oleracea*. *Phytochemistry* 9, 13–23. doi: 10.1016/S0031-9422(00)86608-X
- Madadi, H., Enkegaard, A., Brodsgaard, H. F., Kharrazi-Pakdel, A., Mohaghegh, J., and Ashouri, A. (2007). Host plant effects on the functional response of *Neoseiulus cucumeris* to onion thrips larvae. *J. Appl. Entomol.* 131, 728–733. doi: 10.1111/j.1439-0418.2007.01206.x
- Mallampalli, N., Gould, F., and Barbosa, P. (2005). Predation of Colorado potato beetle eggs by a polyphagous ladybeetle in the presence of alternate prey: potential impact on resistance evolution. *Entomol. Exp. Appl.* 114, 47–54. doi: 10.1111/j.0013-8703.2005.00232.x
- Mao, W., Rupasinghe, S., Zangerl, A. R., Schuler, M. A., and Berenbaum, M. R. (2006). Remarkable substrate-specificity of CYP6AB3 in *Depressaria pastinacella*, a highly specialized caterpillar. *Insect Mol. Biol.* 15, 169–179. doi: 10.1111/j.1365-2583.2006.00623.x
- Marques, R. V., Sarmento, R. A., Lemos, F., Pedro-Neto, M., Sabelis, M. W., Venzon, M., et al. (2015). Active prey mixing as an explanation for polyphagy in predatory arthropods: synergistic dietary effects on egg production despite a behavioural cost. *Funct. Ecol.* 29, 1317–1324. doi: 10.1111/1365-2435.12439
- Marvier, M., McCreedy, C., Regetz, J., and Kareiva, P. (2007). A meta-analysis of effects of Bt cotton and maize on nontarget invertebrates. *Science* 316, 1475–1477. doi: 10.1126/science.1139208
- Matthews, G. A., and Tunstall, J. P. (1994). *Insect Pests of Cotton*. Wallingford: CAB International.
- Mayntz, D., Raubenheimer, D., Salomon, M., Toft, S., and Simpson, S. J. (2005). Nutrient-specific foraging in invertebrate predators. *Science* 307, 111–113. doi: 10.1126/science.1105493
- McGovern, J. L., Zangerl, A. R., Ode, P. J., and Berenbaum, M. R. (2006). Furanoconuimins and their detoxification in a tri-trophic interaction. *Chemoecology* 16, 45–50. doi: 10.1007/s00049-005-0327-3
- McKenzie, J. A. (1996). *Ecological and Evolutionary Aspects of Insecticide Resistance*. Austin, TX: Academic Press.
- Meinke, L. J., Siegfried, B. D., Wright, R. J., and Chandler, L. D. (1998). Adult susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to selected insecticides. *J. Econ. Entomol.* 91, 594–600. doi: 10.1093/jee/91.3.594
- Meissle, M., and Romeis, J. (2009). The web-building spider *Theridion impressum* (Araneae: Theridiidae) is not adversely affected by Bt maize resistant to corn rootworms. *Plant Biotechnol. J.* 7, 645–656. doi: 10.1111/j.1467-7652.2009.00431.x
- Meissle, M., Vojtech, E., and Poppy, G. M. (2005). Effects of Bt maize-fed prey on the generalist predator *Poecilus cupreus* L. (Coleoptera: Carabidae). *Transgenic Res.* 14, 123–132. doi: 10.1007/s11248-004-6458-4
- Memelink, J., Verpoorte, R., and Kijne, J. W. (2001). ORCAnization of jasmonate-responsive gene expression in alkaloid metabolism. *Trends Plant Sci.* 6, 212–219. doi: 10.1016/S1360-1385(01)01924-0
- Milne, M., and Walter, G. H. (1997). The significance of prey in the diet of the phytophagous thrips, *Frankliniella schultzei*. *Ecol. Entomol.* 22, 74–81. doi: 10.1046/j.1365-2311.1997.00034.x
- Mithöfer, A., and Boland, W. (2008). Recognition of herbivory-associated molecular patterns. *Plant Physiol.* 146, 825–831. doi: 10.1104/pp.107.113118
- Moran, N., and Hamilton, W. D. (1980). Low nutritive quality as defense against herbivores. *J. Theor. Biol.* 86, 247–254. doi: 10.1016/0022-5193(80)90004-1
- Müller, C. (2008). “Resistance at the plant cuticle,” in *Induced Plant Resistance to Herbivory*, ed. A. Schaller (Dordrecht: Springer-Verlag), 107–129.
- Müller, C., Boevé, J.-L., and Brakefield, P. M. (2002). Host plant derived feeding deterrence towards ants in the turnip sawfly *Athalia rosae*. *Entomol. Exp. Appl.* 104, 153–157. doi: 10.1046/j.1570-7458.2002.01002.x
- Müller, R., de Vos, M., Sun, J. Y., Sonderby, I. E., Halkier, B. A., Wittstock, U., et al. (2010). Differential effects of indole and aliphatic glucosinolates on lepidopteran herbivores. *J. Chem. Ecol.* 36, 905–913. doi: 10.1007/s10886-010-9825-z
- Musser, R. O., Hum-Musser, S. M., Eichenseer, H., Peiffer, M., Ervin, G., Murphy, J. B., et al. (2002). Caterpillar saliva beats plant defences. *Nature* 416, 599–600. doi: 10.1038/416599a
- Naranjo, S. E. (2009). *Risk Assessment: Bt Crops and Invertebrate Non-target Effects-Revisited*. ISB News Report: Agricultural and Environmental Biotechnology. Available at: <http://www.isb.vt.edu/news/2009/Dec/BtCropsarticle.pdf>
- Naranjo, S. E. (2011). Impacts of Bt transgenic cotton on integrated pest management. *J. Agric. Food Chem.* 59, 5842–5851. doi: 10.1021/jf102939c
- Naranjo, S. E., Ellsworth, P. C., and Frisvold, G. B. (2015). Economic value of biological control in integrated pest management of managed plant systems. *Annu. Rev. Entomol.* 60, 621–645. doi: 10.1146/annurev-ento-010814-021005
- Netting, A. G., Macey, M. J. K., and Barber, H. N. (1972). Chemical genetics of a sub-glaucous mutant of *Brassica oleracea*. *Phytochemistry* 11, 579–585. doi: 10.1016/0031-9422(72)80015-3
- Nishida, R. (2002). Sequestration of defensive substances from plants by Lepidoptera. *Annu. Rev. Entomol.* 47, 57–92. doi: 10.1146/annurev.ento.47.09201.145121
- Norton, A. P., English-Loeb, G., Gadoury, D. G., and Seem, R. C. (2000). Mycophagous mites and foliar pathogens: leaf domatia mediate tritrophic interactions in grapes. *Ecology* 81, 490–499. doi: 10.1890/0012-9658(2000)081[0490:MMAFPL]2.0.CO;2
- Obrist, L. B., Dutton, A., Albajes, R., and Bigler, F. (2006a). Exposure of arthropod predators to Cry1Ab toxin in Bt maize fields. *Ecol. Entomol.* 31, 143–154. doi: 10.1111/j.0307-6946.2006.00762.x
- Obrist, L. B., Dutton, A., Romeis, J., and Bigler, F. (2006b). Biological activity of Cry1Ab toxin expressed by Bt maize following ingestion by herbivorous arthropods and exposure of the predator *Chrysoperla carnea*. *BioControl* 51, 31–48. doi: 10.1007/s10526-005-2936-8
- Obrist, L. B., Klein, H., Dutton, A., and Bigler, F. (2005). Effects of Bt maize on *Frankliniella tenuicornis* and exposure of thrips predators to prey-mediated Bt toxin. *Entomol. Exp. Appl.* 115, 409–416. doi: 10.1111/j.1570-7458.2005.00298.x
- Obrycki, J. J., Ruberson, J. R., and Losey, J. E. (2004). “Interactions between natural enemies and transgenic insecticidal crops,” in *Genetics, Evolution and Biological Control*, eds L. E. Ehler, R. Sforza, and T. Mateille (Wallingford: CAB International), 183–206.
- Obrycki, J. J., and Tauber, M. J. (1984). Natural enemy activity on glandular pubescent potato plants in the greenhouse: an unreliable predictor of effects in the field. *Environ. Entomol.* 13, 679–683. doi: 10.1093/ee/13.3.679

- Obrycki, J. J., Tauber, M. J., and Tingey, W. M. (1983). Predator and parasitoid interaction with aphid-resistant potatoes to reduce aphid densities: a two-year field study. *J. Econ. Entomol.* 76, 456–462. doi: 10.1093/jee/76.3.456
- Ode, P. J. (2006). Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. *Annu. Rev. Entomol.* 51, 163–185. doi: 10.1146/annurev.ento.51.110414.151110
- Ode, P. J. (2013). "Plant defences and parasitoid chemical ecology" in *Chemical Ecology of Insect Parasitoids*, eds E. Wajnberg and S. Colazza (Oxford: Wiley-Blackwell), 11–36.
- Ode, P. J., and Crompton, D. S. (2013). Compatibility of aphid resistance in soybean and biological control by the parasitoid *Aphidius colemani* (Hymenoptera: Braconidae). *Biol. Control* 64, 255–262. doi: 10.1016/j.biocontrol.2012.12.001
- Ode, P. J., Harvey, J. A., Reichelt, M., Gershenson, J., and Gols, R. (2016). Differential induction of plant chemical defenses by parasitized and unparasitized herbivores: consequences for reciprocal, multitrophic interactions. *Oikos* 125, 1398–1407. doi: 10.1111/oik.03076
- O'Dowd, D. J., and Willson, M. F. (1991). Associations between mites and leaf domatia. *Trend Ecol. Evol.* 6, 179–820.
- Oerke, E.-C., and Dehne, H.-W. (2004). Safeguarding production- losses in major crops and the role of crop protection. *Crop Prot.* 23, 275–285. doi: 10.1016/j.cropro.2003.10.001
- Orre, G. U. S., Wratten, S. D., Jonsson, M., and Hale, R. J. (2010). Effects of an herbivore-induced plant volatile on arthropods from three trophic levels in brassicas. *Biol. Control* 53, 62–67. doi: 10.1016/j.biocontrol.2009.10.010
- Orrell, P., and Bennett, A. E. (2013). How can we exploit above-ground interactions to assist in addressing the challenges of food security? *Front. Plant Sci.* 4:432. doi: 10.3389/fpls.2013.00432
- Painter, R. H. (1951). *Insect Resistance in Crop Plants*. Lawrence, KS: University of Kansas Press.
- Panda, N., and Khush, G. S. (1995). *Host Plant Resistance to Insects*. Wallingford: CABI.
- Páre, P. W., and Tumlinson, J. H. (1999). Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121, 325–331. doi: 10.1104/pp.121.2.325
- Patt, J. M., and Pfannenstiel, R. S. (2008). Odor-based recognition of nectar in cursorial spiders. *Entomol. Exp. Appl.* 127, 64–71. doi: 10.1111/j.1570-7458.2008.00669.x
- Patt, J. M., and Pfannenstiel, R. S. (2009). Characterization of restricted area searching behavior following consumption of prey and non-prey food in a cursorial spider, *Hibana futilis*. *Entomol. Exp. Appl.* 132, 13–20. doi: 10.1111/j.1570-7458.2009.00865.x
- Paula, D. P., and Andow, D. A. (2016). Uptake and bioaccumulation of Cry toxins by an aphidophagous predator. *Environ. Pollut.* 209, 164–168. doi: 10.1016/j.envpol.2015.11.036
- Pedigo, L. P., and Rice, M. E. (2014). *Entomology and Pest Management*, 6th Edn. Long Grove, IL: Waveland Press, Inc.
- Pereira, A. E., Wang, H. C., Zukoff, S. N., Meinke, L. J., French, B. W., and Siegfried, B. D. (2015). Evidence of field-evolved resistance to bifenthrin in western corn rootworm (*Diabrotica virgifera virgifera* LeConte) populations in western Nebraska and Kansas. *PLoS ONE* 10:e0142299. doi: 10.1371/journal.pone.0142299
- Peterson, J. A., Lundgren, J. G., and Harwood, J. D. (2011). Interactions of transgenic *Bacillus thuringiensis* insecticidal crops with spiders (Araneae). *J. Arachnol.* 39, 1–21. doi: 10.1636/M10-98.1
- Peterson, J. A., Obrycki, J. J., and Harwood, J. D. (2009). Quantification of Bt-endotoxin exposure pathways in carabid food webs across multiple transgenic events. *Biocontrol Sci. Technol.* 19, 613–625. doi: 10.1080/09583150902968972
- Peterson, J. A., Obrycki, J. J., and Harwood, J. D. (2016). Spiders from multiple functional guilds are exposed to Bt-endotoxins in transgenic corn fields via prey and pollen consumption. *Biocontrol Sci. Technol.* 26, 1–42. doi: 10.1080/09583157.2016.1193591
- Pfannenstiel, R. S. (2008). Spider predators of lepidopteran eggs in south Texas field crops. *Biol. Control* 46, 202–208. doi: 10.1016/j.biocontrol.2008.03.011
- Pfannenstiel, R. S., and Patt, J. M. (2012). Feeding on nectar and honeydew sugars improves survivorship of two nocturnal cursorial spiders. *Biol. Control* 63, 231–236. doi: 10.1016/j.biocontrol.2012.07.013
- Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. (1997). Developing sustainable pest control from chemical ecology. *Agric. Ecosyst. Environ.* 64, 149–156. doi: 10.1016/S0167-8809(97)00033-9
- Pilcher, C. D., Obrycki, J. J., Rice, M. E., and Lewis, L. C. (1997). Preimaginal development, survival, and field abundance of insect predators on transgenic *Bacillus thuringiensis* corn. *Environ. Entomol.* 26, 446–454. doi: 10.1093/ee/26.2.446
- Pilz, C., Keller, S., Kuhlmann, U., and Toepfer, S. (2009). Comparative efficacy assessment of fungi, nematodes and insecticides to control western corn rootworm larvae in maize. *BioControl* 54, 671–684. doi: 10.1007/s10526-009-9209-x
- Pimentel, D. (1961). An evaluation of insect resistance in broccoli, Brussels sprouts, cabbage, collards and kale. *J. Econ. Entomol.* 54, 156–158. doi: 10.1093/jee/54.1.156
- Pimentel, D. (2005). Environmental and economic costs of the application of pesticides primarily in the United States. *Environ. Dev. Sustain.* 7, 229–252. doi: 10.1007/s10668-005-7314-2
- Pleasants, J. M., and Oberhauser, K. S. (2013). Milkweed loss in agricultural fields because of herbicide use: effect on the monarch butterfly population. *Insect Conserv. Divers.* 6, 135–144. doi: 10.1111/j.1752-4598.2012.00196.x
- Poelman, E. H., Broekgaarden, C., van Loon, J. J. A., and Dicke, M. (2008). Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Mol. Ecol.* 17, 3352–3365. doi: 10.1111/j.1365-294X.2008.03838.x
- Poelman, E. H., Bruinsma, M., Zhu, F., Weldegergis, B. T., Boursault, A. E., Jongema, Y., et al. (2012). Hyperparasitoids use herbivore-induced plant volatiles to locate their parasitoid host. *PLoS Biol.* 10:e1001435. doi: 10.1371/journal.pbio.1001435
- Poelman, E. H., Zheng, S.-J., Zhang, Z., Heemskerk, N. M., and Cortesero, A.-M. (2011). Parasitoid-specific induction of plant responses to parasitized herbivores affects colonization by subsequent herbivores. *Proc. Natl. Acad. Sci. U.S.A.* 108, 19647–19652. doi: 10.1073/pnas.1110748108
- Ponsard, S., Gutierrez, A. P., and Mills, N. J. (2002). Effect of Bt-toxin (Cry1Ac) in transgenic cotton on the adult longevity of four heteropteran predators. *Environ. Entomol.* 31, 1197–1205. doi: 10.1603/0046-225X-31.6.1197
- Poppy, G. M., and Sutherland, J. P. (2004). Can biological control benefit from genetically-modified crops? Tritrophic interactions on insect-resistant transgenic plants. *Physiol. Entomol.* 29, 257–268. doi: 10.1111/j.0307-6962.2004.00382.x
- Price, P. W. (1986). "Ecological aspects of host plant resistance and biological control: interactions among three trophic levels," in *Interactions of Plant Resistance and Parasitoids and Predators of Insects*, eds D. J. Boethel and R. D. Eikenbary (West Sussex: Ellis Horwood Limited), 11–30.
- Price, P. W., Bouton, C. E., Gross, P., McPheron, B. A., Thompson, J. N., and Weis, A. E. (1980). Interactions among three trophic levels: influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Evol. Syst.* 11, 41–65. doi: 10.1111/j.1469-8137.2008.02545.x
- Rabb, R. L., and Bradley, J. R. (1968). The influence of host plants on parasitism of eggs of the tobacco hornworm. *J. Econ. Entomol.* 61, 1249–1252. doi: 10.1093/jee/61.5.1249
- Radcliffe, E. B., Hutchison, W. D., and Cancelado, R. E. (2009). *Integrated Pest Management: Concepts, Tactics, Strategies and Case Studies*. New York, NY: Cambridge University Press.
- Ramirez-Romero, R., Bernal, J. S., Chaufaux, J., and Kaiser, L. (2007). Impact assessment of Bt-maize on a moth parasitoid, *Cotesia marginiventris* (Hymenoptera: Braconidae), via host exposure to purified Cry1Ab protein or Bt-plants. *Crop Prot.* 26, 953–962. doi: 10.1016/j.cropro.2006.09.001
- Rasmann, S., Köllner, T. G., Degenhardt, J., Hiltpold, I., Toepfer, S., Kuhlmann, U., et al. (2005). Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434, 732–737. doi: 10.1038/nature03451
- Raubenheimer, D., Mayntz, D., Simpson, S. J., and Toft, S. (2007). Nutrient-specific compensation following diapause in a predator: implications for intraguild predation. *Ecology* 88, 2598–2608. doi: 10.1890/07-0012.1
- Ray, S., Gaffor, I., Acevedo, F. E., Helms, A., Chuang, W.-P., Tooker, J., et al. (2015). Maize plants recognize herbivore-associated cues from caterpillar frass. *J. Chem. Ecol.* 41, 781–792. doi: 10.1007/s10886-015-0619-1
- Raymond, B., Sayyed, A. H., Hails, R. S., and Wright, D. J. (2007). Exploiting pathogens and their impact on fitness costs to manage the evolution of

- resistance to *Bacillus thuringiensis*. *J. Appl. Ecol.* 44, 768–780. doi: 10.1111/j.1365-2664.2007.01285.x
- Reina-Pinto, J. J., and Yephremov, A. (2009). Surface lipids and plant defenses. *Plant Physiol. Biochem.* 47, 540–549. doi: 10.1016/j.plaphy.2009.01.004
- Reymond, P., Weber, H., Damond, M., and Farmer, E. E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* 12, 707–719. doi: 10.1105/tpc.12.5.707
- Riddick, E. W., and Simmons, A. M. (2014a). Do plant trichomes cause more harm than good to predatory insects? *Pest Manag. Sci.* 70, 1655–1665. doi: 10.1002/ps.3772
- Riddick, E. W., and Simmons, A. M. (2014b). Plant trichomes have mixed impacts on predatory insects. *Pest Manag. Sci.* 70, 1668–1668. doi: 10.1002/ps.3811
- Riederer, M., and Müller, C. (2006). *Annual Plant Reviews, Biology of the Plant Cuticle*. New York, NY: John Wiley & Sons.
- Roberts, A. F., Devos, Y., Lemgo, G. N. Y., and Zhou, X. G. (2015). Biosafety research for non-target organism risk assessment of RNAi-based GE plants. *Front. Plant Sci.* 6:958. doi: 10.3389/fpls.2015.00958
- Rogers, C. E. (1985). Extrafloral nectar: entomological implications. *Bull. Entomol. Soc. Am.* 31, 15–20. doi: 10.1093/besa/31.3.15
- Romeis, J., Dutton, A., and Bigler, F. (2004). *Bacillus thuringiensis* toxin (Cry1Ab) has no direct effect on larvae of the green lacewing *Chrysoperla carnea* (Stephens) (Neuroptera: Chrysopidae). *J. Insect Physiol.* 50, 175–183. doi: 10.1016/j.jinsphys.2003.11.004
- Romeis, J., Meissle, M., and Bigler, F. (2006). Transgenic crops expressing *Bacillus thuringiensis* toxins and biological control. *Nat. Biotechnol.* 24, 63–71. doi: 10.1038/nbt1180
- Rossiter, J. T., Jones, A. M., and Bones, A. M. (2003). A novel myrosinase-glucosinolate defense system in cruciferous specialist aphids. *Recent Adv. Phytochem.* 37, 127–142.
- Roth, S. K., and Lindroth, R. L. (1995). Elevated atmospheric CO₂: Effects on phytochemistry, insect performance and insect-parasitoid interactions. *Glob. Chang. Biol.* 1, 173–182. doi: 10.1111/j.1365-2486.1995.tb00019.x
- Ruhren, S., and Handel, S. (1999). Jumping spiders (Salticidae) enhance the seed production of a plant with extrafloral nectaries. *Oecologia* 119, 227–230. doi: 10.1007/s004420050780
- Rutledge, C. E., and O'Neil, R. J. (2005). *Orius insidiosus* (Say) as a predator of the soybean aphid, *Aphis glycines* Matsumura. *Biol. Control* 33, 56–64. doi: 10.1016/j.biocontrol.2005.01.001
- Schäfer, M., Fischer, C., Meldau, S., Seebald, E., Oelmüller, R., and Baldwin, I. T. (2011). Lipase activity in insect oral secretions mediates defense responses in *Arabidopsis*. *Plant Physiol.* 156, 1520–1534. doi: 10.1104/pp.111.173567
- Schellhorn, N. A., Parry, H. R., Macfadyen, S., Wang, Y., and Zalucki, M. P. (2015). Connecting scales: achieving in-field pest control from areawide and landscape ecology studies. *Insect Sci.* 22, 35–51. doi: 10.1111/1744-7917.12161
- Schmidt, R. A. (2014). Leaf structures affect predatory mites (Acari: Phytoseiidae) and biological control: a review. *Exp. Appl. Acarol.* 62, 1–17. doi: 10.1007/s10493-013-9730-6
- Schoonhoven, L. M., van Loon, J. J. A., and Dicke, M. (2005). *Insect-Plant Biology*, 2nd Edn. Oxford: Oxford University Press.
- Schuler, T. H., Potting, R. P., Denholm, I., and Poppy, G. M. (1999). Parasitoid behaviour and Bt plants. *Nature* 400, 825–826. doi: 10.1038/23605
- Schuster, M. F., and Calderon, M. (1986). "Interactions of host plant resistant genotypes and beneficial insects in cotton ecosystems," in *Interactions of Plant Resistance and Parasitoids and Predators of Insects*, eds D. J. Boethel and R. D. Eikenbary (New York, NY: Wiley), 84–97.
- Schuster, M. F., Calvin, P. D., and Langston, W. C. (1983). "Interaction of high tannin with bollworm control by Pydrin and Dipel," in *Proceedings of the 1983 Beltwide Cotton Production Research Conference*, ed. J. M. Brown (Memphis, TN: National Cotton Council of America), 72–73.
- Schuster, M. F., Lukefahr, M. J., and Maxwell, F. G. (1976). Impact of nectariless cotton on plant bugs and natural enemies. *J. Econ. Entomol.* 69, 400–402. doi: 10.1093/jee/69.3.400
- Sétamou, M., Bernal, J. S., Legaspi, J. C., and Mirkov, T. E. (2002a). Effects of snowdrop lectin (*Galanthus nivalis* agglutinin) expressed in transgenic sugarcane on fitness of *Cotesia flavipes* (Hymenoptera: Braconidae), a parasitoid of the nontarget pest *Diatraea saccharalis* (Lepidoptera: Crambidae). *Ann. Entomol. Soc. Am.* 95, 75–83. doi: 10.1603/0013-8746(2002)095[0075:EOSLGN]2.0.CO;2
- Sétamou, M., Bernal, J. S., Legaspi, J. C., and Mirkov, T. E. (2002b). Parasitism and location of sugarcane borer (Lepidoptera: Pyralidae) by *Cotesia flavipes* (Hymenoptera: Braconidae) on transgenic and conventional sugarcane. *Environ. Entomol.* 31, 1219–1225. doi: 10.1603/0046-225X-31.6.1219
- Sétamou, M., Bernal, J. S., Legaspi, J. C., Mirkov, T. E., and Legaspi, B. C. Jr. (2002c). Evaluation of lectin-expressing transgenic sugarcane against stalkborers (Lepidoptera: Pyralidae): effects on life history parameters. *J. Econ. Entomol.* 95, 469–477. doi: 10.1603/0022-0493-95.2.469
- Settle, W. H., Ariawan, H., Astuti, E. T., Cahyana, W., Hakim, A. L., Hindayana, D., et al. (1996). Managing tropical rice pests through conservation of generalist natural enemies and alternative prey. *Ecology* 77, 1975–1988. doi: 10.2307/2265694
- Simpson, M., Gurr, G. M., Simmons, A. T., Wratten, S. D., James, D. G., Leeson, G., et al. (2011a). Field evaluation of the 'attract and reward' biological control approach in vineyards. *Ann. Appl. Biol.* 159, 69–78. doi: 10.1111/j.1744-7348.2011.00477.x
- Simpson, M., Gurr, G. M., Simmons, A. T., Wratten, S. D., James, D. G., Leeson, G., et al. (2011b). Attract and reward: combining chemical ecology and habitat manipulation to enhance biological control in field crops. *J. Appl. Ecol.* 48, 580–590. doi: 10.1111/j.1365-2664.2010.01946.x
- Siomi, H., and Siomi, M. C. (2009). On the road to reading the RNA-interference code. *Nature* 457, 396–404. doi: 10.1038/nature07754
- Smiley, J. (1986). Ant constancy at *Passiflora* extrafloral nectaries: effects on caterpillar survival. *Ecology* 67, 516–521. doi: 10.2307/1938594
- Smith, C. M. (2005). *Plant Resistance to Arthropods: Molecular and Conventional Approaches*. Dordrecht: Springer-Verlag.
- Soler, R., Harvey, J. A., Kamp, A. F. D., Vet, L. E. M., van der Putten, W. H., van Dam, N. M., et al. (2007). Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant volatile signals. *Oikos* 116, 367–376. doi: 10.1111/j.0030-1299.2007.15501.x
- Soler, R., van der Putten, W. H., Harvey, J. A., Vet, L. E. M., Dicke, M., and Bezemer, T. M. (2012). Root herbivore effects on aboveground multitrophic interactions: patterns, processes and mechanisms. *J. Chem. Ecol.* 38, 755–767. doi: 10.1007/s10886-012-0104-z
- Southwood, R. (1986). "Plant surfaces and insects- an overview," in *Insects and the Plant Surface*, eds B. Juniper and R. Southwood (London: Edward Arnold), 1–22.
- Stadler, B., and Mackauer, M. (1996). Influence of plant quality on interactions between the aphid parasitoid *Ephedrus californicus* (Baker) (Hymenoptera: Aphidiidae) and its host, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphidiidae). *Can. Entomol.* 128, 27–39. doi: 10.4039/Ent12827-1
- Stapel, J. O., Cortesero, A. M., De Moraes, C. M., Tumlinson, J. H., and Lewis, W. J. (1997). Effects of extrafloral nectar, honeydew and sucrose on searching behavior and efficiency of *Microplitis croceipes* (Hymenoptera: Braconidae) in cotton. *Environ. Entomol.* 26, 617–623. doi: 10.1093/ee/26.3.617
- Stavrinides, M. C., and Skirvin, D. J. (2003). The effect of chrysanthemum leaf trichome density and prey spatial distribution on predation of *Tetranychus urticae* (Acari: Tetranychidae) by *Phytoseiulus persimilis* (Acari: Phytoseiidae). *Bull. Entomol. Res.* 93, 343–350. doi: 10.1079/BER2003243
- Stephenson, A. G. (1982). The role of extrafloral nectaries of *Catalpa speciosa* in limiting herbivory and increasing fruit production. *Ecology* 63, 663–669. doi: 10.2307/1936786
- Stern, V. M., Smith, R. F., van den Bosch, R., and Hagen, K. S. (1959). The integrated control concept. *Hilgardia* 29, 81–101. doi: 10.3733/hilg.v29n0_2p081
- Stout, M. J. (2013). Reevaluating the conceptual framework for applied research on host-plant resistance. *Insect Sci.* 20, 263–272. doi: 10.1111/1744-7917.12011
- Stout, M. J., Workman, K. V., Bostock, R. M., and Duffey, S. S. (1998). Specificity of induced resistance in the tomato, *Lycopersicon esculentum*. *Oecologia* 113, 74–81. doi: 10.1007/s004420050355
- Sudhakar, D., Fu, X. D., Stoger, E., Williams, S., Spence, J., Brown, D. P., et al. (1998). Expression and immunolocalisation of the snowdrop lectin, GNA in transgenic rice plants. *Transgenic Res.* 7, 371–378. doi: 10.1023/A:1008856703464
- Sun, Y.-C., Li, F., Gao, F., and Ge, F. (2011). Effects of elevated CO₂ and plant genotype on interactions among cotton, aphids and parasitoids. *Insect Sci.* 18, 451–461. doi: 10.1111/j.1744-7917.2010.01328.x

- Sunderland, K. D., Fraser, A. M., and Dixon, A. F. G. (1986). Field and laboratory studies on money spiders (Linyphiidae) as predators of cereal aphids. *J. Appl. Ecol.* 24, 907–933. doi: 10.2307/2403989
- Taylor, R. M., and Pfannenstiel, R. S. (2008). Nectar feeding by wandering spiders on cotton plants. *Environ. Entomol.* 37, 996–1002. doi: 10.1093/ee/37.4.996
- Taylor, R. M., and Pfannenstiel, R. S. (2009). How dietary plant nectar affects the survival, growth, and fecundity of a cursorial spider *Cheiracanthium inclusum* (Araneae: Miturgidae). *Environ. Entomol.* 38, 1379–1386. doi: 10.1603/022.038.0505
- Thomas, M., and Waage, J. (1996). *Integration of Biological Control and Host Plant Resistance Breeding: A Scientific and Literature Review*. Wageningen: Technical Centre for Agricultural and Rural Cooperation (CTA).
- Thorbek, P., Sunderland, K. D., and Topping, C. J. (2004). Reproductive biology of agrobiont linyphiid spiders in relation to habitat, season and biocontrol potential. *Biol. Control* 30, 193–202. doi: 10.1016/j.biocontrol.2003.10.004
- Tian, D., Peiffer, M., Shoemaker, E., Tooker, J. F., Haubruege, E., Francis, F., et al. (2012). Salivary glucose oxidase from caterpillars mediates the induction of rapid and delayed-induced defenses in the tomato plant. *PLoS ONE* 7:e41947. doi: 10.1371/journal.pone.0036168
- Tian, J. C., Liu, Z. C., Chen, M., Chen, Y., Chen, X. X., Peng, Y. F., et al. (2010). Laboratory and field assessments of prey-mediated effects of transgenic Bt rice on *Ummelias insincticeps* (Araneida: Linyphiidae). *Environ. Entomol.* 39, 1369–1377. doi: 10.1603/EN10003
- Tian, J. C., Wang, X. P., Long, L. P., Romeis, J., Naranjo, S. E., Hellmich, R. L., et al. (2014). Eliminating host-mediated effects demonstrates Bt maize producing Cry1F has no adverse effects on the parasitoid *Cotesia marginiventris*. *Transgenic Res.* 23, 257–264. doi: 10.1007/s11248-013-9748-x
- Tinge, W. M. (1982). “Potential for plant resistance in management of arthropod pests,” in *Advances in Potato Pest Management*, eds J. H. Lashomb and R. A. Casagrande (Stroudsburg, PA: Hutchinson Ross), 268–288.
- Toepfer, S., Gueldenzoph, C., Ehlers, R.-U., and Kuhlmann, U. (2005). Screening of entomopathogenic nematodes for virulence against the invasive western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) in Europe. *Bull. Entomol. Res.* 95, 473–482. doi: 10.1079/BER2005379
- Toepfer, S., Peters, A., Ehlers, R.-U., and Kuhlmann, U. (2008). Comparative assessment of the efficacy of entomopathogenic nematode species at reducing western corn rootworm larvae and root damage in maize. *J. Appl. Entomol.* 132, 337–348. doi: 10.1111/j.1439-0418.2008.01274.x
- Tooker, J. F., Peiffer, M., Luthe, D. S., and Felton, G. W. (2010). Trichomes as sensors: detecting activity on the leaf surface. *Plant Signal. Behav.* 5, 73–75. doi: 10.4161/psb.5.1.10234
- Torres, J. A., and Ruberson, J. R. (2006). Interactions of Bt-cotton and the omnivorous bigeyed bug *Geocoris punctipes* (Say), a key predator in cotton fields. *Biol. Control* 39, 47–57. doi: 10.1016/j.biocontrol.2006.03.006
- Torres, J. B., Ruberson, J. R., and Adang, M. J. (2006). Expression of *Bacillus thuringiensis* Cry1Ac protein in cotton plants, acquisition by pests and predators: a tritrophic analysis. *Agric. For. Entomol.* 8, 191–202. doi: 10.1111/j.1461-9563.2006.00298.x
- Treacy, M. F., Benedict, J. H., Walmsley, M. H., Lopez, J. D., and Morrison, R. K. (1987). Parasitism of bollworm (Lepidoptera: Noctuidae) eggs on nectaried and nectarless cotton. *Environ. Entomol.* 16, 420–423. doi: 10.1093/ee/16.2.420
- Trichilo, P. J., and Leigh, T. F. (1986). Predation on spider mite eggs by the western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), an opportunist in a cotton agroecosystem. *Environ. Entomol.* 15, 821–825. doi: 10.1007/s10493-013-9711-9
- Turlings, T. C. J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G., and Dorn, S. (1998). The induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. *Biol. Control* 11, 122–129. doi: 10.1006/bcon.1997.0591
- Turlings, T. C. J., and Wäckers, F. L. (2004). “Recruitment of predators and parasitoids by herbivore-injured plants,” in *Advances in Insect Chemical Ecology*, eds R. T. Cardé and J. Millar (Cambridge: Cambridge University Press), 21–75.
- United Nations (2004). *World Population to 2300*. Available at: <http://www.un.org/esa/population/publications/longrange2/WorldPop2300final.pdf>
- United States Department of Agriculture National Agricultural Statistics Service (2015). *Acreage report*. Available at: <http://www.usda.gov/nass/PUBS/TODAYRPT/acrg0615.pdf>
- van Dam, N. M., and Heil, M. (2011). Multitrophic interactions above and below ground: en route to the next level. *J. Ecol.* 99, 77–88. doi: 10.1111/j.1365-2745.2010.01761.x
- van Emden, H. F. (1988). The potential for managing indigenous natural enemies of aphids on field crops. *Philos. Trans. R. Soc. Lond. B* 318, 183–201. doi: 10.1098/rstb.1988.0004
- van Emden, H. F. (1991). The role of host plant resistance in insect pest mismanagement. *Bull. Entomol. Res.* 81, 123–126. doi: 10.1017/S0007485300051166
- van Lenteren, J. C. (2012). *IOBC Internet Book of Biological Control, version 6*. International Organization for Biological Control. Available at: http://www.iobc-global.org/download/IOBC_InternetBookBiCoVersion6Spring2012.pdf
- Van Zandt, P. A., and Agrawal, A. A. (2004). Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. *Oikos* 104, 401–409. doi: 10.1111/j.0030-1299.2004.12964.x
- Verkerk, R. H. J., and Wright, D. J. (1996). Common cabbage resistance mechanisms against the diamondback moth: still an open book? *Ann. Appl. Biol.* 128, 571–577. doi: 10.1111/j.1744-7348.1996.tb07116.x
- Vet, L. E. M., and Dicke, M. (1992). Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37, 141–172. doi: 10.1146/annurev.en.37.010192.001041
- Vojtech, E., Meissle, M., and Poppy, G. M. (2005). Effects of Bt maize on the herbivore *Spodoptera littoralis* (Lepidoptera: Noctuidae) and the parasitoid *Cotesia marginiventris* (Hymenoptera: Braconidae). *Transgenic Res.* 14, 133–144. doi: 10.1007/s11248-005-2736-z
- von Berg, K., Traugott, M., Symondson, W. O. C., and Scheu, S. (2008). Impact of abiotic factors on predator-prey interactions: DNA-based gut content analysis in a microcosm experiment. *Bull. Entomol. Res.* 98, 257–261. doi: 10.1017/S0007485308006007
- von Mérey, G. E., Veyrat, N., de Lange, E., Degen, T., Mahuku, G., Valdez, R. L., et al. (2012). Minor effects of two elicitors of insect and pathogen resistance on volatile emissions and parasitism of *Spodoptera frugiperda* in Mexican maize fields. *Biol. Control* 60, 7–15. doi: 10.1016/j.biocontrol.2011.09.010
- von Mérey, G. E., Veyrat, N., Mahuku, G., Valdez, R. L., Turlings, T. C. J., and D’Alessandro, M. (2011). Dispensing synthetic green leaf volatiles in maize fields increases the release of sesquiterpenes by the plants, but has little effect on the attraction of pest and beneficial insects. *Phytochemistry* 72, 1838–1847. doi: 10.1016/j.phytochem.2011.04.022
- Wäckers, F. L., and Bonifay, C. (2004). How to be sweet? Extrafloral nectar allocation by *Gossypium hirsutum* fits optimal defense theory predictions. *Ecology* 85, 1512–1518. doi: 10.1890/03-0422
- Wäckers, F. L., Zuber, D., Wunderlin, R., and Keller, F. (2001). The effect of herbivory on temporal and spatial dynamics of extrafloral nectar production in cotton and castor. *Ann. Bot.* 87, 365–370. doi: 10.1006/anbo.2000.1342
- Walde, S. (1995). How quality of host plant affects a predator-prey interaction in biological control. *Ecology* 76, 1206–1219. doi: 10.2307/1940927
- Walker, G. P., Cameron, P. J., MacDonald, F. M., Madhusudhan, V. V., and Wallace, A. R. (2007). Impacts of *Bacillus thuringiensis* toxins on parasitoids (Hymenoptera: Braconidae) of *Spodoptera litura* and *Helicoverpa armigera* (Lepidoptera: Noctuidae). *Biol. Control* 40, 142–151. doi: 10.1016/j.biocontrol.2006.09.008
- Walling, L. L. (2000). The myriad of plant responses to herbivores. *J. Plant Growth Regul.* 19, 195–216.
- Walter, D. E. (1996). Living on leaves: mites, tomentia, and leaf domatia. *Annu. Rev. Entomol.* 41, 101–114. doi: 10.1146/annurev.en.41.010196.000533
- Wang, Z. Y., Zhang, K. W., Sun, X. F., Tang, K. X., and Zhang, J. R. (2005). Enhancement of resistance to aphids by introducing the snowdrop lectin gene GNA into maize plants. *J. Biosci.* 30, 627–638. doi: 10.1007/BF02703563
- Wangila, D. S., Gassmann, A. J., Petzold-Maxwell, J. L., French, B. W., and Meinke, L. J. (2015). Susceptibility of Nebraska western corn rootworm (Coleoptera: Chrysomelidae) populations to Bt corn events. *J. Econ. Entomol.* 108, 742–751. doi: 10.1093/jee/tou063
- Wäschke, N., Meiners, T., and Rostás, M. (2013). “Foraging strategies of parasitoids in complex chemical environments,” in *Chemical Ecology of Insect Parasitoids*, eds É Wajnberg and S. Colazza (Oxford: John Wiley & Sons), 37–63.
- Wei, W., Schuler, T. H., Clark, S. J., Stewart, C. N., and Poppy, G. M. (2008). Movement of transgenic plant-expressed Bt Cry1Ac proteins through high

- trophic levels. *J. Appl. Entomol.* 132, 1–11. doi: 10.1111/j.1439-0418.2007.01242.x
- White, J. A., and Andow, D. A. (2005). Host-parasitoid interactions in a transgenic landscape: spatial proximity effects of host density. *Environ. Entomol.* 34, 1493–1500. doi: 10.1603/0046-225X-34.6.1493
- Wilson, L. J., Bauer, L. R., and Walter, G. H. (1996). ‘Phytophagous’ thrips are facultative predators of two-spotted spider mites (Acaria: Tetranychidae) on cotton in Australia. *Bull. Entomol. Res.* 86, 297–305. doi: 10.1017/S0007485300052597
- Winder, L. (1990). Predation of the cereal aphid *Sitobion avenae* by polyphagous predators on the ground. *Ecol. Entomol.* 15, 105–110. doi: 10.1111/j.1365-2311.1990.tb00789.x
- Winder, L., Hirst, D. J., Carter, N., Wratten, S. D., and Sopp, P. I. (1994). Estimating predation on the grain aphid *Sitobion avenae* by polyphagous predators. *J. Appl. Ecol.* 31, 1–12. doi: 10.2307/2404594
- Wiseman, B. R., and Davis, F. M. (1990). Plant-resistance to insects attacking corn and grain-sorghum. *Fla. Entomol.* 73, 446–458. doi: 10.2307/3495461
- Wittstock, U., Agerbirk, N., Stauber, E. J., Olsen, C. E., Hippler, M., Mitchell-Olds, T., et al. (2004). Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. U.S.A.* 101, 4859–4864. doi: 10.1073/pnas.0308007101
- Wolfenbarger, L. L., Naranjo, S. E., Lundgren, J. G., Bitzer, R. J., and Watrud, L. S. (2008). Bt crop effects on functional guilds of non-target arthropods: a meta-analysis. *PLoS ONE* 3:e2118. doi: 10.1371/journal.pone.0002118
- Wright, R. J., Thaxton, P. M., El-Zik, K. M., and Paterson, A. H. (1999). Molecular mapping of genes affecting pubescence of cotton. *J. Heredity* 90, 215–219. doi: 10.1093/jhered/90.1.215
- Wright, R. J., Witkowski, J. F., Echtenkamp, G., and Georgis, R. (1993). Efficacy and persistence of *Steinernema carpocapsae* (Rhabditida: Steinernematidae) applied through a center-pivot irrigation system against larval corn rootworms (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 86, 1348–1354. doi: 10.1093/jee/86.5.1348
- Wu, K.-M., Lu, Y.-H., Feng, H.-Q., Jiang, Y.-Y., and Zhao, J.-Z. (2008). Suppression of cotton bollworm in multiple crops in China in areas for Bt toxin-containing cotton. *Science* 321, 1676–1678. doi: 10.1126/science.1160550
- Zhangsun, D., Luo, S., Chen, R., and Tang, K. (2007). Improved *Agrobacterium*-mediated genetic transformation of GNA transgenic sugarcane. *Biologia* 62, 386–393. doi: 10.2478/s11756-007-0096-2
- Zwahlen, C., and Andow, D. A. (2005). Field evidence for the exposure of ground beetles to Cry1Ab from transgenic corn. *Environ. Biosafety Res.* 4, 113–117. doi: 10.1051/ebr:2005014

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Plant Defense against Herbivorous Pests: Exploiting Resistance and Tolerance Traits for Sustainable Crop Protection

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Interactions between plants and insect herbivores are important determinants of plant productivity in managed and natural vegetation. In response to attack, plants have evolved a range of defenses to reduce the threat of injury and loss of productivity. Crop losses from damage caused by arthropod pests can exceed 15% annually. Crop domestication and selection for improved yield and quality can alter the defensive capability of the crop, increasing reliance on artificial crop protection. Sustainable agriculture, however, depends on reduced chemical inputs. There is an urgent need, therefore, to identify plant defensive traits for crop improvement. Plant defense can be divided into resistance and tolerance strategies. Plant traits that confer herbivore resistance typically prevent or reduce herbivore damage through expression of traits that deter pests from settling, attaching to surfaces, feeding and reproducing, or that reduce palatability. Plant tolerance of herbivory involves expression of traits that limit the negative impact of herbivore damage on productivity and yield. Identifying the defensive traits expressed by plants to deter herbivores or limit herbivore damage, and understanding the underlying defense mechanisms, is crucial for crop scientists to exploit plant defensive traits in crop breeding. In this review, we assess the traits and mechanisms underpinning herbivore resistance and tolerance, and conclude that physical defense traits, plant vigor and herbivore-induced plant volatiles show considerable utility in pest control, along with mixed species crops. We highlight emerging approaches for accelerating the identification of plant defensive traits and facilitating their deployment to improve the future sustainability of crop protection.

Keywords: agro-ecosystem, arthropod, crop improvement, insect, natural enemy, trophic interactions

INTRODUCTION

Domestication of agricultural crops, estimated at 2500 species globally (Meyer et al., 2012), has involved artificial selection of desirable traits that enhance yield and quality of the harvested product. While breeding for agronomic targets in high input environments has successfully increased global crop productivity (Lynch, 2007), it has tended to produce modern crop varieties with relatively low levels of diversity (Khush, 2001). This reduced genetic diversity could limit the availability of varieties adapted for crop production under non-optimal conditions. Plant defensive

traits can be lacking or expressed weakly in domesticated plants as a consequence of selection for other desirable traits (Chen et al., 2015). This poses a particular challenge for improving the sustainability of crop production as it suggests that modern varieties would perform poorly in low input systems with restricted pesticide use. While crop productivity has increased over the past century, combined global crop losses due to weeds, pests and diseases can be up to 40% (Oerke and Dehne, 2004). Across all vegetation systems, foliage, sap and root feeding herbivores remove >20% of net plant productivity (Agrawal, 2011). These losses occur despite increased pesticide use over recent decades (Oerke and Dehne, 2004), highlighting the need to develop sustainable approaches for pest control with less reliance on chemical inputs. To address concerns regarding human health, environmental safety and pesticide resistance, plant defensive traits could be exploited more widely in crop protection strategies.

Focusing on arthropod herbivores as pests, this review seeks, first, to summarize the plant defense strategies that have been documented in agricultural crops, second, to consider the potential utility of different types of crop defense, and, third, to highlight opportunities and technologies for improving the identification and deployment of plant defensive traits, particularly to achieve sustainable pest management under a changing environment.

PLANT DEFENSE STRATEGIES TOWARD ARTHROPOD PESTS

Plants have been successful in colonizing most environments and their success is due in part to their ability to resist or tolerate herbivore attack (Hanley et al., 2007). In a crop protection context, the system developed by Stout (2013) is particularly useful in differentiating between two plant defense strategies and the underpinning traits: resistance and tolerance. Resistance occurs when plant structural or chemical traits deter herbivore feeding and thus minimize the amount of herbivore damage experienced by the plant. Tolerance occurs when plant traits reduce the negative effects of herbivore damage on crop yield. This differentiation can allow defensive traits to be matched to the risk posed by the target pest: i.e., a high risk pest that should be reduced to low densities or eliminated vs. a low risk pest that can be tolerated within certain abundance thresholds. To identify suitable plant traits for crop protection against specific pests, we need a basic understanding of the mechanisms underpinning defensive traits, and how environmental conditions affect trait expression.

An important consideration is the extent to which defensive traits will provide durable pest control. Since plant resistance traits typically deter herbivore feeding, they are likely to impose a strong selection pressure on the herbivore to overcome plant resistance (Janzen, 1980). In contrast, plant tolerance traits are often assumed to have no effect on herbivore fitness, and therefore unlikely to impose selection on the herbivore (Strauss and Agrawal, 1999; Stowe et al., 2000). Stinchcombe (2002) challenges this assumption, suggesting that

in some circumstances tolerance traits could influence herbivore performance, but few studies have investigated this possibility, particularly in a crop protection context. Either way, resistance traits are likely to impose a stronger selection pressure due to more severe impacts on pest fitness, suggesting that tolerance traits will be more stable (Weis and Franks, 2006) with greater chance of providing durable pest control.

RESISTANCE TRAITS AND MECHANISMS

The mechanism by which specific plant resistance traits deter herbivore feeding is likely to vary with the stage of insect establishment that they influence. Here, we summarize traits that are known to promote crop resistance to herbivores by (1) deterring pest landing, (2) preventing attachment and feeding, and (3) reducing plant palatability (Table 1).

Chemical Deterrence of Pest Settling and Feeding

Herbivore feeding and oviposition can induce plant defense, including emission of herbivore induced plant volatiles (HIPVs), which have been proposed as a new focus for crop pest resistance and biocontrol (Stenberg et al., 2015). Production of HIPVs signals herbivore presence that can attract natural enemies of the pest and even signal herbivore threat and induce defense responses in neighboring plants (e.g., Erb et al., 2015). A recent meta-analysis of HIPV studies (Rowen and Kaplan, 2016) concluded that domesticated plants tend to produce volatiles in larger quantities but of simpler composition compared to wild relatives (Chen et al., 2015; Rowen and Kaplan, 2016), suggesting that specific biosynthetic capabilities have been lost during crop breeding (Dicke, 2016). Wild relatives offer a genetic resource for reintroducing these traits into crops (Stenberg et al., 2015), and landraces can provide genetic variation in HIPV production and natural enemy attraction (e.g., parasitoids of maize stemborer: Tamiru et al., 2015). Engineering elevated volatile production into crop plants is feasible: for example, wheat plants modified to produce insect alarm pheromone both repelled aphids and attracted their natural enemies in controlled conditions, although this did not translate into improved aphid control in the field (Bruce et al., 2015).

'Priming' of plant defenses by cues that signal herbivore threat can allow rapid induction of plant defenses upon subsequent herbivore attack (Kim and Felton, 2013). Priming of inducible responses is an attractive proposition for crop breeding, allowing plant defense allocation to be balanced against the degree of herbivore pressure (Stenberg et al., 2015). The identity of plant elicitors and mechanisms of defense induction are emerging for several crop species (Huffaker et al., 2013; Huffaker, 2015), opening up opportunities for exploiting priming and defense induction traits in crop breeding (Stenberg et al., 2015).

Physical Barriers

Plant structural traits (e.g., trichomes, spinescence, waxy cuticles, sclerophyll) can act as a physical barrier to arthropod

TABLE 1 | Examples of traits and underpinning mechanisms conferring crop resistance or tolerance to target arthropod pests.

Defense strategy	Mechanism	Trait and mode of action	Target pest	Crop host	Reference
Resistance	(1) Chemical deterrence of pest settling and feeding	Engineered elevated production of repellent alarm pheromone	<i>Myzus persicae</i>	<i>Triticum aestivum</i>	Bruce et al., 2015
		HIPV-induced attraction of maize stemborer parasitoids	<i>Chilo partellus</i>	<i>Cotesia sesamiae</i>	Tamiru et al., 2015
		Plant elicitor peptides induce plant defenses that impair Beet armyworm growth and attract its parasitoids	<i>Spodoptera exigua</i>	<i>Zea mays</i>	Huffaker et al., 2013
	(2) Physical barriers to pest attachment, feeding and oviposition	Epicuticular waxes differentially affect herbivore attachment	<i>Sitona lineatus</i> , <i>Acyrtosiphum pisum</i>	<i>Pisum sativum</i>	White and Eigenbrode, 2000
		Leaf surface waxes contribute to reduced performance of diamondback moth on cabbage	<i>Plutella xylostella</i>	<i>Brassica</i> sp.	Hariprasad and van Emden, 2010
		Glandular trichomes reduce mite movement	<i>Tetranychus urticae</i>	<i>Fragaria x ananassa</i>	Figueiredo et al., 2013
		Glandular trichomes reduce growth of corn earworm	<i>Helicoverpa zea</i>	<i>Solanum lycopersicum</i>	Tian et al., 2012
		Non glandular trichomes impair Colorado potato beetle feeding and growth	<i>Leptinotarsa decemlineata</i>		
		High density of non glandular trichomes prevent mite oviposition on raspberry	<i>Tetranychus urticae</i>	<i>Rubus idaeus</i>	Graham et al., 2014; Karley et al., 2016
		Gramine alkaloid decreased aphid feeding, growth and survival	<i>Rhopalosiphum padi</i>	<i>Hordeum vulgare</i>	Zúñiga and Corcuera, 1986
	(3) Reduced plant palatability	Benzoxazinoid synthesis decreased aphid growth and survival	<i>Rhopalosiphum padi</i>	<i>Zea mays</i>	Ahmad et al., 2011
		Aliphatic and indole glucosinolates reduced larval consumption and growth and slowed development on mature plants	<i>Mamestra brassicae</i> <i>Pieris rapae</i>	<i>Brassica oleracea</i> var. <i>acephala</i>	Santolamazza-Carbone et al., 2016
		Diterpenoid kaurealexins deter feeding of corn borer larvae	<i>Ostrinia nubilalis</i>	<i>Zea mays</i>	Schmelz et al., 2011
Tolerance	(1) Photosynthesis and growth	Stimulate growth	<i>Amphorophora idaei</i>	<i>Rubus idaeus</i>	Johnson et al., 2012; Karley et al., 2016
		Increased root vigor	<i>Lepidiota stigma</i>	<i>Saccharum officinarum</i>	Allsop and Cox, 2002
	(2) Phenology	Delayed allocation to roots	<i>Diabrotica virgifera virgifera</i>	<i>Zea mays</i>	Robert et al., 2015

pest attachment, feeding and oviposition; the plant cuticle and trichome density are two traits of particular focus in crop protection. Epicuticular waxes form a slippery film or crystals that prevent pests from attaching to the plant surface (White and Eigenbrode, 2000), ovipositing or feeding (Hariprasad and van Emden, 2010). Trichomes can prevent pest attachment and limit pest movement on crops (e.g., Tian et al., 2012; Figueiredo et al., 2013). While the effect of glandular trichomes is likely to have a chemical basis (see Reduced Plant Palatability, below), non-glandular trichomes act as a physical deterrent: oviposition by the generalist phytophagous mite, *Tetranychus urticae*, was significantly reduced on raspberry genotypes with high leaf trichome densities (Karley et al., 2016), and with identification of underlying

genetic markers, this trait has potential utility in breeding for mite control (Graham et al., 2014). Trichomes can also have indirect negative (Michalska, 2003) and positive effects (Dai et al., 2010) on the target pest through their impact on the behavior of herbivore natural enemies. For example, abundance of the predatory mite *Typhlodromus pyri* on grape was associated positively with the presence of leaf trichomes, while its prey, the European red mite, favored grape varieties with low trichome density (Loughner et al., 2008). Trichomes tend to be more effective against insects that are small relative to trichome size; additionally, trichomes tend to deter sap feeding or leaf chewing insects to a greater extent than those feeding within plant tissues (Hanley et al., 2007).

Reduced Plant Palatability

Plant compounds that are toxic or impair gut function in arthropods, produced constitutively or induced by herbivore damage, can enhance crop resistance to pests; examples include alkaloids (Zúñiga and Corcuera, 1986), benzoxazinoids (Ahmad et al., 2011), glucosinolates (Santolamazza-Carbone et al., 2016), and terpenoids (Schmelz et al., 2011). Plant breeding has tended to select against high levels of defensive compounds (Chen et al., 2015) due to their detrimental effects on crop quality for consumption. Targeted expression of defensive compounds in non-harvested organs (e.g., gossypol in vegetative structures of cotton; Palle et al., 2013) might allow tissue-specific engineering of chemical resistance into crops, although indirect effects of plant quality on biocontrol by natural enemies should be tested (Ågren et al., 2012). Another intriguing avenue is through symbiosis between cereal grasses and *Epichloë* fungal endophytes, allowing crops to benefit from fungal production of insecticidal alkaloids (Simpson et al., 2014).

Many plants deposit granular minerals in tissues that deter insect attack and feeding. A well-known example is silica accumulation in grasses (up to 2–5% silica by mass: Massey et al., 2006), which is abrasive, damaging herbivore feeding structures, and reducing digestibility (Massey and Hartley, 2009). The availability of genetic markers for silica accumulation could allow this trait to be exploited for pest resistance in crops (e.g., in rice: Bryant et al., 2011).

TOLERANCE TRAITS AND MECHANISMS

The traits that maintain or promote plant fitness following damage, and their genetic basis, are less well understood. Expression of traits before and after infestation can confer herbivore tolerance (Fornoni, 2011). Plant tolerance traits (**Table 1**) are classically grouped into those that alter (i) physiological processes such as photosynthetic activity and growth, (ii) phenology, and (iii) use of stored nutrients (Strauss and Agrawal, 1999; Stowe et al., 2000; Tiffin, 2000). We focus on the first two categories as there are few examples of using stored nutrient reserves as a tolerance strategy, although storage organs are important for plant recovery from damage and offer an effective strategy against unpredictable herbivore attack if there is no tradeoff with plant productivity (Strauss and Agrawal, 1999).

Photosynthesis and Growth

In many plant species, partial defoliation leads to increased photosynthetic rate in the remaining plant tissues (Strauss and Agrawal, 1999; Retuerto et al., 2004), suggesting that compensatory photosynthesis is a common physiological response to leaf damage (Tiffin, 2000). However, increased photosynthetic activity is not a universal response to herbivory and does not always drive compensatory growth, possibly due to resource diversion into resistance traits (Tiffin, 2000). Herbivore identity can determine whether changes in photosynthetic rate and growth occur: for example, compensatory photosynthesis is induced by several insect herbivores of soybean and drybean, but

not by Mexican bean beetle (Peterson et al., 1998). By contrast, aphid feeding on the perennial crop red raspberry frequently stimulates plant growth and influences nitrogen physiology (Johnson et al., 2012), which could reflect tolerance to aphid herbivory through increased plant vigor (Karley et al., 2016). Similarly in sugarcane, clonal variation in tolerance to root-feeding whitegrub correlated with increased plant vigor (Allsop and Cox, 2002). Plant vigor can provide tolerance to herbivory in a range of plant species (Price, 1991); higher abundance and fitness of many insect herbivore groups on vigorous host plants (Cornelissen et al., 2008) could reflect increased ability of vigorous plants to tolerate attack. Although plant vigor is likely to be controlled by multiple loci, quantitative trait loci (QTL) studies have identified genetic markers for vigor (e.g., root and shoot vigor in raspberry: Graham et al., 2011, 2014) that could be deployed in crop breeding.

Activation of dormant buds after removal or damage to flowering or vegetative meristems is a further type of compensatory growth mechanism that allows plants to recover from herbivore attack that could be exploited in crop species with multiple meristems (Tiffin, 2000). In some circumstances, growth overcompensation is observed, which might be an attractive trait for improving crop tolerance in fertile agricultural conditions (Pilson, 2000), although any impact on the quality of the harvested product would need to be assessed.

Phenology

Delayed growth, flower and fruit production following herbivore damage could promote herbivore tolerance by postponing plant development until the threat of attack has passed (Tiffin, 2000). For example, delayed resource allocation to roots is thought to underpin tolerance of western corn rootworm in herbivore-tolerant maize (Robert et al., 2015). The utility of these traits will depend on whether delayed development has a negative impact on yield and quality if the delay leads to crop flowering, pollination or ripening during non-optimal conditions.

SELECTING TRAITS TO OPTIMIZE PLANT DEFENSE: OPPORTUNITIES AND CHALLENGES

Matching defensive traits to herbivore types to optimize pest control will depend on the nature of damage inflicted by the pest, whether direct feeding damage, removal of resources, visual spoiling or vectoring plant disease (**Figure 1**). Resistance traits are more desirable for maintaining disease vectors below threshold infestation densities. Tolerance traits are likely to be useful against non-vector pests that typically cause damage by removing resources and reducing plant growth (**Figure 1A**), although this has to be balanced against the possibility of pest spillover to neighboring crops or between cropping cycles. An important consideration is whether the target defensive trait has a negative impact on populations of beneficial organisms, particularly natural enemies of the pest. For example, while high trichome densities can reduce abundance of insect pests on cotton, trichomes can also impair the searching efficiency

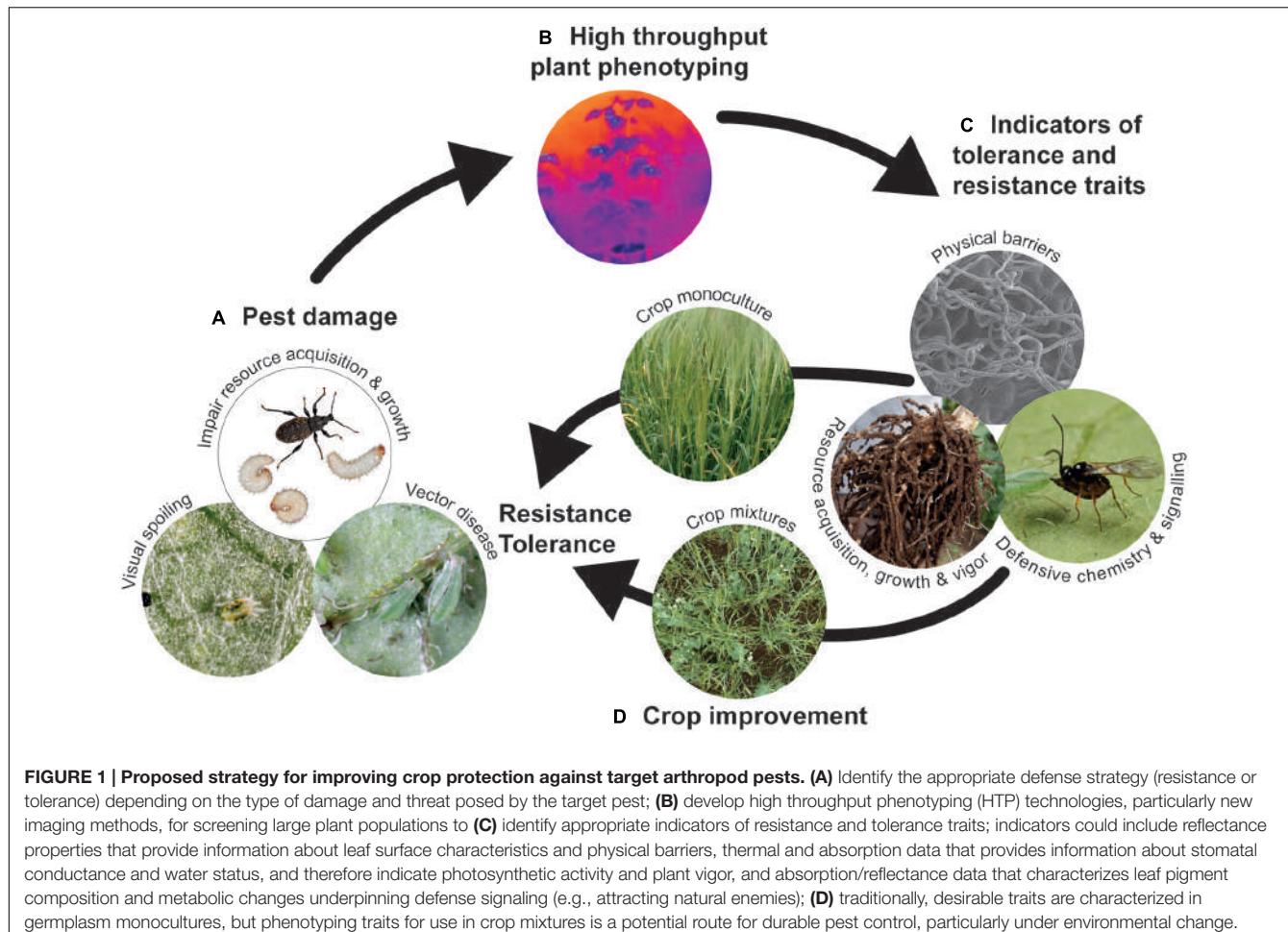


FIGURE 1 | Proposed strategy for improving crop protection against target arthropod pests. **(A)** Identify the appropriate defense strategy (resistance or tolerance) depending on the type of damage and threat posed by the target pest; **(B)** develop high throughput phenotyping (HTP) technologies, particularly new imaging methods, for screening large plant populations to **(C)** identify appropriate indicators of resistance and tolerance traits; indicators could include reflectance properties that provide information about leaf surface characteristics and physical barriers, thermal and absorption data that provides information about stomatal conductance and water status, and therefore indicate photosynthetic activity and plant vigor, and absorption/reflectance data that characterizes leaf pigment composition and metabolic changes underpinning defense signaling (e.g., attracting natural enemies); **(D)** traditionally, desirable traits are characterized in germplasm monocultures, but phenotyping traits for use in crop mixtures is a potential route for durable pest control, particularly under environmental change.

of herbivore natural enemies (Hagenbucher et al., 2013); by contrast, leafminers on tomato and their parasitoids are deterred by leaf trichomes, but trichomes and HIPVs have antagonistic effects on insect behavior (Wei et al., 2013). In some situations, incorporating plant traits that enhance natural enemy searching behavior might be more beneficial than enhancing pest resistance traits (Schmidt, 2014; Stenberg et al., 2015).

Technological advances in large-scale plant genotyping can accelerate selection of germplasm with desirable traits (Anderson and Mitchell-Olds, 2011), including herbivore defense. The rate-limiting step now resides in the ability to conduct high throughput phenotyping (HTP) to characterize desirable traits in large plant populations (Figure 1B). Imaging methodologies offer exciting opportunities for large-scale visualization of plant populations in controlled and field conditions, allowing semi-automated collection of light signals from the plant surface across a wide spectrum of wavelengths ranging between visible and infra-red (Fahlgren et al., 2015). Image-extracted traits provide information on canopy temperature, pigment composition and water status that can be linked to targeted measures of plant performance (Fahlgren et al., 2015). HTP approaches using imaging are already providing genetic markers for crop performance under

abiotic stress (e.g., Prashar et al., 2013), and there is significant potential for applying imaging techniques to phenotype plant responses to insect pests (Goggin et al., 2015). For example, imaging methods could provide non-destructive indicators of physiological processes, such as stomatal conductance and water status, leaf pigment composition or photosynthetic activity, or plant vigor (Figure 1C) that indicate genotypic differences in ability to tolerate or resist insect pest attack above and belowground.

While studies of plant defensive traits frequently focus on a single trait and target pest, the underlying genetic control and expression of traits is likely to involve a suite of traits (Agrawal, 2011) expressed to defend against multiple pests above- and below-ground. Depending on the dominant crop pests, it might be feasible to focus on a single defensive trait, such as silica accumulation, which is effective against a range of herbivore types (Reynolds et al., 2009; Guntzer et al., 2012). Although there is surprisingly little evidence for trade-offs in plant investment between multiple defenses (Koricheva et al., 2004), understanding the genetic control of multiple traits remains a significant challenge for crop breeders. An alternative approach is to take advantage of defensive traits associated with different crop types grown as cultivar- or species-mixtures (Figure 1D). Plant

diversification in crop systems often enhances natural enemy populations, suppresses arthropod pest populations and reduces crop damage (Letourneau et al., 2011) by providing a more complex habitat and heterogeneous resource for natural enemies, decreasing the density of preferred host plants, and interfering with host plant location and/or quality for herbivores (Jonsson et al., 2008; Letourneau et al., 2011). A good example of the latter effect is the negative impact of onions co-cropped with potato on attraction of potato aphids (Ninkovic et al., 2013). Increasing plant diversity in crop systems can confer additional benefits of yield stability and resource-use efficiency (Brooker et al., 2015). While there are many examples of the benefits of cultivating crop mixtures, particularly the ‘push–pull’ systems developed in sub-Saharan Africa for pest biocontrol (Pickett et al., 2014), there is significant opportunity for breeding crops with traits that optimize performance in mixtures (Ren et al., 2014).

CONCLUSION AND FUTURE PERSPECTIVES

Crop domestication over recent decades has focused on plant traits that improve yield, enhance quality for human consumption and make the crop more amenable to existing cropping methods (Chen et al., 2015). Now, however, there is increasing focus on improving the sustainability of agriculture by reducing reliance on pesticides and other chemical inputs (War et al., 2012). From the studies highlighted here, there is considerable potential to exploit HIPVs, physical defenses and plant vigor to protect crops (and crop mixtures) against focal pests and to promote activity of natural enemies. A major uncertainty, however, is the durability of crop protection under a changing climate, which is anticipated to increase pest pressures on crops. Elevated temperatures are likely to accelerate insect development and increase the number of insect generations each season (DeLucia et al., 2012), elevated CO₂ could decrease herbivore abundance but increase consumption

(Stiling and Cornelissen, 2007), while intermittent water stress can enhance performance in certain herbivore guilds (Huberty and Denno, 2004). The effect of climate factors, individually or in concert, on expression of plant defense traits is uncertain. Elevated temperature and CO₂ promote plant growth and volatile production, and can modulate defense signaling (DeLucia et al., 2012), which might strengthen expression of these tolerance/resistance traits. Conversely, these climate factors tend to reduce plant nutritional quality and decrease allocation to defensive compounds and physical structures, thus promoting plant consumption by herbivores (Stiling and Cornelissen, 2007; DeLucia et al., 2012), which suggests that crop protection from these physical and chemical resistance traits might be compromised under a changing climate. Applying imaging methods for HTP of target traits under conditions that mimic future climates (e.g., Rasmann et al., 2014), in parallel with optimizing crop defensive traits in mixtures, should assist crop scientists in identifying traits and trait combinations that are resilient to a changing environment, and that can be deployed as part of an integrated approach for sustainable crop protection.

AUTHOR CONTRIBUTIONS

The article was conceived by all authors, researched by CM and written by CM and AK, with corrections contributed by JG and RB.

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REFERENCES

- Agrawal, A. A. (2011). Current trends in the evolutionary ecology of plant defence. *Funct. Ecol.* 25, 420–432. doi: 10.1111/j.1365-2435.2010.01796.x
- Ågren, G. I., Stenberg, J. A., and Björkman, C. (2012). Omnivores as plant bodyguards - A model of the importance of plant quality. *Basic Appl. Ecol.* 13, 441–448. doi: 10.1016/j.baae.2012.07.005
- Ahmad, S., Veyrat, N., Gordon-Weeks, R., Zhang, Y., Martin, J., Smart, L., et al. (2011). Benzoxazinoid metabolites regulate innate immunity against aphids and fungi in maize. *Plant Physiol.* 157, 317–327. doi: 10.1104/pp.111.180224
- Allsop, P. G., and Cox, M. C. (2002). Sugarcane clones vary in their resistance to sugarcane whitegrubs. *Aust. J. Agric. Res.* 53, 1111–1136. doi: 10.1071/AR02035
- Anderson, J. T., and Mitchell-Olds, T. (2011). Ecological genetics and genomics of plant defences: evidence and approaches. *Funct. Ecol.* 25, 312–324. doi: 10.1111/j.1365-2435.2010.01785.x
- Brooker, R. W., Bennett, A. E., Cong, W.-F., Daniell, T. J., George, T. S., Hallett, P. D., et al. (2015). Improving intercropping: a synthesis of research in agronomy, plant physiology and ecology. *New Phytol.* 206, 107–117. doi: 10.1111/nph.13132
- Bruce, T. J. A., Aradottir, G. I., Smart, L. E., Martin, J. L., Caulfield, J. C., and Doherty, A. (2015). The first crop plant genetically engineered to release an insect pheromone for defence. *Nature* 511183. doi: 10.1038/srep11183
- Bryant, R., Proctor, A., Hawkridge, M., Jackson, A., Yeater, K., Counce, P., et al. (2011). Genetic variation and association mapping of silica concentration in rice hulls using a germplasm collection. *Genetica* 139, 1383–1398. doi: 10.1007/s10709-012-9637-x
- Chen, Y. H., Gols, R., and Benrey, B. (2015). Crop domestication and its impact on naturally selected trophic interactions. *Annu. Rev. Entomol.* 60, 35–58. doi: 10.1146/annurev-ento-010814-020601
- Cornelissen, T., Fernandes, G. W., and Vasconcellos-Neto, J. (2008). Size does matter: variation in herbivory between and within plants and the plant vigor hypothesis. *Oikos* 117, 1121–1130. doi: 10.1111/j.2008.0030-1299.16588.x
- Dai, H., Wang, Y., Du, Y., and Ding, J. (2010). Effects of plant trichomes on herbivores and predators on soybeans. *Insect Sci.* 17, 406–413. doi: 10.1111/j.1744-7917.2009.01305.x
- DeLucia, E. H., Nabity, P. D., Zavala, J. A., and Berenbaum, M. R. (2012). Climate change: resetting plant-insect interactions. *Plant Physiol.* 160, 1677–1685. doi: 10.1104/pp.112.204750
- Dicke, M. (2016). Induced plant volatiles: plant body odours structuring ecological networks. *New Phytol.* 210, 10–12. doi: 10.1111/nph.13896

- Erb, M., Veyrat, N., Robert, C. A. M., Xu, H., Frey, M., Ton, J., et al. (2015). Indole is an essential herbivore-induced volatile priming signal in maize. *Nat. Commun.* 6:6273. doi: 10.1038/ncomms7273
- Fahlgren, N., Gehan, M. A., and Baxter, I. (2015). Lights, camera, action: high-throughput plant phenotyping is ready for a close-up. *Curr. Opin. Plant Biol.* 24, 93–99. doi: 10.1016/j.pbi.2015.02.006
- Figueiredo, A. S. T., Resende, J. T. V., Morales, R. G. F., Gonçalves, A. P. S., and Da Silva, P. R. (2013). The role of glandular and non-glandular trichomes in the negative interactions between strawberry cultivars and spider mite. *Arthropod Plant Interact.* 7, 53–58. doi: 10.1007/s11829-012-9218-z
- Fornoni, J. (2011). Ecological and evolutionary implications of plant tolerance to herbivory. *Funct. Ecol.* 25, 399–407. doi: 10.1111/j.1365-2435.2010.01805.x
- Goggin, F. L., Lorence, A., and Topp, C. N. (2015). Applying high-throughput phenotyping to plant-insect interactions: picturing more resistant crops. *Curr. Opin. Insect Sci.* 9, 69–76. doi: 10.1016/j.cois.2015.03.002
- Graham, J., Hackett, C. A., Smith, K., Karley, A. J., Mitchell, C., Roberts, H., et al. (2014). Genetic and environmental regulation of plant architectural traits and opportunities for pest control in raspberry. *Ann. Appl. Biol.* 165, 318–328. doi: 10.1111/aab.12134
- Graham, J., Hackett, C. A., Smith, K., Woodhead, M., MacKenzie, K., Tierney, I., et al. (2011). Towards an understanding of the nature of resistance to *Phytophthora* root rot in red raspberry. *Theor. Appl. Genet.* 123, 585–601. doi: 10.1007/s00122-011-1609-5
- Guntzer, F., Keller, C., and Meunier, J. D. (2012). Benefits of plant silicon for crops: a review. *Agron. Sustain. Dev.* 32, 201–213. doi: 10.1007/s13593-011-0039-8
- Hagenbucher, S., Olson, D. M., Ruberson, J. R., Wäckers, F. L., and Romeis, J. (2013). Resistance mechanisms against arthropod herbivores in cotton and their interactions with natural enemies. *Crit. Rev. Plant Sci.* 32, 458–482. doi: 10.1080/07352689.2013.809293
- Hanley, M. E., Lamont, B. B., Fairbanks, M. M., and Rafferty, C. M. (2007). Plant structural traits and their role in anti-herbivore defence. *Perspect. Plant Ecol. Evol. Syst.* 8, 157–178. doi: 10.1016/j.ppees.2007.01.001
- Hariprasad, K. V., and van Emden, H. F. (2010). Mechanisms of partial plant resistance to diamondback moth (*Plutella xylostella*) in brassicas. *Int. J. Pest Manag.* 56, 15–22. doi: 10.1080/09670870902980834
- Huberty, A. F., and Denno, R. F. (2004). Plant water stress and its consequences for herbivorous insects: a new synthesis. *Ecology* 85, 1383–1398. doi: 10.1890/03-0352
- Huffaker, A. (2015). Plant elicitor peptides in induced defense against insects. *Curr. Opin. Insect Sci.* 9, 44–50. doi: 10.1016/10.1016/j.cois.2015.06.003
- Huffaker, A., Pearce, G., Veyrat, N., Erb, M., Turlings, T. C. J., Sartor, R., et al. (2013). Plant elicitor peptides are conserved signals regulating direct and indirect antiherbivore defense. *Proc. Natl. Acad. Sci. U.S.A.* 110, 5707–5712. doi: 10.1073/pnas.1214668110
- Janzen, D. H. (1980). When is it coevolution? *Evolution* 34, 611–612. doi: 10.2307/2408229
- Johnson, S. N., Young, M. W., and Karley, A. J. (2012). Protected raspberry production alters aphid-plant interactions but not aphid population size. *Agric. For. Entomol.* 14, 217–224. doi: 10.1111/j.1461-9563.2011.00561.x
- Jonsson, M., Wratten, S. D., Landis, D. A., and Gurr, G. M. (2008). Recent advances in conservation biological control of arthropods by arthropods. *Biol. Control* 45, 172–175. doi: 10.1016/j.bioccontrol.2008.01.006
- Karley, A. J., Mitchell, C., Brookes, C., McNicol, J., O'Neill, T., Roberts, H., et al. (2016). Exploiting physical defence traits for crop protection: leaf trichomes of *Rubus idaeus* have deterrent effects on spider mites but not aphids. *Ann. Appl. Biol.* 168, 159–172. doi: 10.1111/aab.12252
- Khush, G. S. (2001). Green revolution: the way forward. *Nat. Rev. Genet.* 2, 815–822. doi: 10.1038/35093585
- Kim, J., and Felton, G. W. (2013). Priming of antiherbivore defensive responses in plants. *Insect Sci.* 20, 273–285. doi: 10.1111/j.1744-7917.2012.01584.x
- Koricheva, J., Nykanen, H., and Gianoli, E. (2004). Meta-analysis of trade-offs among plant antiherbivore defenses: are plants jacks-of-all-trades, masters of all? *Am. Nat.* 163, E64–E75. doi: 10.1086/382601
- Letourneau, D. K., Armbrecht, I., Rivera, B. S., Lerma, J. M., Carmona, E. J., Daza, M. C., et al. (2011). Does plant diversity benefit agroecosystems? A synthetic review. *Ecol. Appl.* 21, 9–21. doi: 10.1890/09-2026.1
- Loughner, R., Goldman, K., Loeb, G., and Nyrop, J. (2008). Influence of leaf trichomes on predatory mite (*Typhlodromus pyri*) abundance in grape varieties. *Exp. Appl. Acarol.* 45, 111–122. doi: 10.1007/s10493-008-9183-5
- Lynch, J. P. (2007). Roots of the second green revolution. *Aust. J. Bot.* 55, 493–512. doi: 10.1071/bt06118
- Massey, F. P., Ennos, A. R., and Hartley, S. E. (2006). Silica in grasses as a defence against insect herbivores: contrasting effects on folivores and a phloem feeder. *J. Anim. Ecol.* 75, 595–603. doi: 10.1111/j.1365-2656.2006.01082.x
- Massey, F. P., and Hartley, S. E. (2009). Physical defences wear you down: progressive and irreversible impacts of silica on insect herbivores. *J. Anim. Ecol.* 78, 281–291. doi: 10.1111/j.1365-2656.2008.01472.x
- Meyer, R. S., DuVal, A. E., and Jensen, H. R. (2012). Patterns and processes in crop domestication: an historical review and quantitative analysis of 203 global food crops. *New Phytol.* 196, 29–48. doi: 10.1111/j.1469-8137.2012.04253.x
- Michalska, K. (2003). Climbing of leaf trichomes by eriophyid mites impedes their location by predators. *J. Insect Behav.* 16, 833–844. doi: 10.1023/b:joir.0000018323.55232.31
- Ninkovic, V., Dahlin, I., Vucetic, A., Petrovic-Obradovic, O., Glinwood, R., and Webster, B. (2013). Volatile exchange between undamaged plants - a new mechanism affecting insect orientation in intercropping. *PLoS ONE* 8:e69431. doi: 10.1371/journal.pone.0069431
- Oerke, E. C., and Dehne, H. W. (2004). Safeguarding production - losses in major crops and the role of crop protection. *Crop Prot.* 23, 275–285. doi: 10.1016/j.crop.2003.10.001
- Palle, S. R., Campbell, L. M., Pandeyal, D., Puckhaber, L., Tollack, L. K., Marcel, S., et al. (2013). RNAi-mediated Ultra-low gossypol cottonseed trait: performance of transgenic lines under field conditions. *Plant Biotechnol. J.* 11, 296–304. doi: 10.1111/pbi.12013
- Peterson, R. K. D., Higley, L. G., Haile, F. J., and Barrigossi, J. A. F. (1998). Mexican bean beetle (Coleoptera: Coccinellidae) injury affects photosynthesis of *Glycine max* and *Phaseolus vulgaris*. *Environ. Entomol.* 27, 373–381. doi: 10.1093/ee/27.2.373
- Pickett, J. A., Woodcock, C. M., Midgley, C. A. O., and Khan, Z. R. (2014). Push-pull farming systems. *Curr. Opin. Biotechnol.* 26, 125–132. doi: 10.1016/j.copbio.2013.12.006
- Pilson, D. (2000). The evolution of plant response to herbivory: simultaneously considering resistance and tolerance in *Brassica rapa*. *Evol. Ecol.* 14, 457–489. doi: 10.1023/A:1010953714344
- Prashar, A., Yildiz, J., McNicol, J. W., Bryan, G. J., and Jones, H. G. (2013). Infra-red thermography for high throughput field phenotyping in *Solanum tuberosum*. *PLoS ONE* 8:e65816. doi: 10.1371/journal.pone.0065816
- Price, P. W. (1991). The plant vigor hypothesis and herbivore attack. *Oikos* 62, 244–251. doi: 10.2307/3545270
- Rasmann, S., Pellissier, L., Defossez, E., Jactel, H., and Kunstler, G. (2014). Climate-driven change in plant-insect interactions along elevation gradients. *Funct. Ecol.* 28, 46–54. doi: 10.1111/1365-2435.12135
- Ren, W., Hu, L., Zhang, J., Sun, C., Tang, J., Yuan, Y., et al. (2014). Can positive interactions between cultivated species help to sustain modern agriculture? *Front. Ecol. Environ.* 12, 507–514. doi: 10.1890/130162
- Retuerto, R., Fernandez-Lema, B., Rodriguez, R., and Obeso, J. R. (2004). Increased photosynthetic performance in holly trees infested by scale insects. *Funct. Ecol.* 18, 664–669. doi: 10.1111/j.0269-8463.2004.00889.x
- Reynolds, O. L., Keeping, M. G., and Meyer, J. H. (2009). Silicon-augmented resistance of plants to herbivorous insects: a review. *Ann. Appl. Biol.* 155, 171–186. doi: 10.1111/j.1744-7348.2009.00348.x
- Robert, C. A. M., Schirmer, S., Barry, J., Wade French, B., Hibbard, B. E., and Gershenson, J. (2015). Belowground herbivore tolerance involves delayed overcompensatory root regrowth in maize. *Entomol. Exp. Appl.* 157, 113–120. doi: 10.1111/eea.12346
- Rowen, E., and Kaplan, I. (2016). Eco-evolutionary factors drive induced plant volatiles: a meta-analysis. *New Phytol.* 210, 284–294. doi: 10.1111/nph.13804
- Santolamazza-Carbone, S., Sotelo, T., Velasco, P., and Cartea, M. E. (2016). Antibiotic properties of the glucosinolates of *Brassica oleracea* var. acephala similarly affect generalist and specialist larvae of two lepidopteran pests. *J. Pest Sci.* 89, 195–206. doi: 10.1007/s10340-015-0658-y
- Schmelz, E. A., Kaplan, F., Huffaker, A., Dafoe, N. J., Vaughan, M. M., Ni, X., et al. (2011). Identity, regulation, and activity of inducible diterpenoid

- phytoalexins in maize. *Proc. Natl. Acad. Sci. U.S.A.* 108, 5455–5460. doi: 10.1073/pnas.1014714108
- Schmidt, R. A. (2014). Leaf structures affect predatory mites (Acaria: Phytoseiidae) and biological control: a review. *Exp. Appl. Acarol.* 62, 1–17. doi: 10.1007/s10493-013-9730-6
- Simpson, W. R., Faville, M. J., Moraga, R. A., Williams, W. M., McManus, M. T., and Johnson, R. D. (2014). Epichloë fungal endophytes and the formation of synthetic symbioses in Hordeae (= Triticeae) grasses. *J. Syst. Evol.* 52, 794–806. doi: 10.1111/jse.12107
- Stenberg, J. A., Heil, M., Ahman, I., and Bjorkman, C. (2015). Optimizing crops for biocontrol of pests and disease. *Trends Plant Sci.* 20, 698–712. doi: 10.1016/j.tplants.2015.08.007
- Stiling, P., and Cornelissen, T. (2007). How does elevated carbon dioxide (CO₂) affect plant–herbivore interactions? A field experiment and meta-analysis of CO₂-mediated changes on plant chemistry and herbivore performance. *Glob. Change Biol.* 13, 1823–1842. doi: 10.1111/j.1365-2486.2007.01392.x
- Stinchcombe, J. (2002). Can tolerance traits impose selection on herbivores? *Evol. Ecol.* 16, 595–602. doi: 10.1023/A:1021617418037
- Stout, M. J. (2013). Re-evaluating the conceptual framework for applied research on host-plant resistance. *Insect Sci.* 20, 263–272. doi: 10.1111/1744-7917.12011
- Stowe, K. A., Marquis, R. J., Hochwender, C. G., and Simms, E. L. (2000). The evolutionary ecology of tolerance to consumer damage. *Annu. Rev. Ecol. Syst.* 31, 565–595. doi: 10.1146/annurev.ecolsys.31.1.565
- Strauss, S. Y., and Agrawal, A. A. (1999). The ecology and evolution of plant tolerance to herbivory. *Trends Ecol. Evol.* 14, 179–185. doi: 10.1016/s0169-5347(98)01576-6
- Tamiru, A., Khan, Z. R., and Bruce, T. J. A. (2015). New directions for improving crop resistance to insects by breeding for egg induced defence. *Insect Sci.* 9, 51–55. doi: 10.1016/j.cois.2015.02.011
- Tian, D., Tooker, J., Peiffer, M., Chung, S. H., and Felton, G. W. (2012). Role of trichomes in defense against herbivores: comparison of herbivore response to woolly and hairless trichome mutants in tomato (*Solanum lycopersicum*). *Planta* 236, 1053–1066. doi: 10.1007/s00425-012-1651-9
- Tiffin, P. (2000). Mechanisms of tolerance to herbivore damage: what do we know? *Evol. Ecol.* 14, 523–536. doi: 10.1023/A:1010881317261
- War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., et al. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signal. Behav.* 7, 1306–1320. doi: 10.4161/psb.21663
- Wei, J., Yan, L., Ren, Q. I. N., Li, C., Ge, F., and Kang, L. E. (2013). Antagonism between herbivore-induced plant volatiles and trichomes affects tritrophic interactions. *Plant Cell Environ.* 36, 315–327. doi: 10.1111/j.1365-3040.2012.02575.x
- Weis, A. E., and Franks, S. J. (2006). Herbivory tolerance and coevolution: an alternative to the arms race? *New Phytol.* 170, 423–425. doi: 10.1111/j.1469-8137.2006.01745.x
- White, C., and Eigenbrode, S. D. (2000). Effects of surface wax variation in *Pisum sativum* on herbivorous and entomophagous insects in the field. *Environ. Entomol.* 29, 773–780. doi: 10.1603/0046-225x-29.4.773
- Zúñiga, G. E., and Corcueras, L. J. (1986). Effect of gramine in the resistance of barley seedlings to the aphid *Rhopalosiphum padi*. *Entomol. Exp. Appl.* 40, 259–262. doi: 10.1111/j.1570-7458.1986.tb00509.x

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Enhancing Integrated Pest Management in GM Cotton Systems Using Host Plant Resistance

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Cotton has lost many ancestral defensive traits against key invertebrate pests. This is suggested by the levels of resistance to some pests found in wild cotton genotypes as well as in cultivated landraces and is a result of domestication and a long history of targeted breeding for yield and fiber quality, along with the capacity to control pests with pesticides. Genetic modification (GM) allowed integration of toxins from a bacteria into cotton to control key Lepidopteran pests. Since the mid-1990s, use of GM cotton cultivars has greatly reduced the amount of pesticides used in many cotton systems. However, pests not controlled by the GM traits have usually emerged as problems, especially the sucking bug complex. Control of this complex with pesticides often causes a reduction in beneficial invertebrate populations, allowing other secondary pests to increase rapidly and require control. Control of both sucking bug complex and secondary pests is problematic due to the cost of pesticides and/or high risk of selecting for pesticide resistance. Deployment of host plant resistance (HPR) provides an opportunity to manage these issues in GM cotton systems. Cotton cultivars resistant to the sucking bug complex and/or secondary pests would require fewer pesticide applications, reducing costs and risks to beneficial invertebrate populations and pesticide resistance. Incorporation of HPR traits into elite cotton cultivars with high yield and fiber quality offers the potential to further reduce pesticide use and increase the durability of pest management in GM cotton systems. We review the challenges that the identification and use of HPR against invertebrate pests brings to cotton breeding. We explore sources of resistance to the sucking bug complex and secondary pests, the mechanisms that control them and the approaches to incorporate these defense traits to commercial cultivars.

Keywords: *Gossypium*, genetic resistance, plant breeding, resistance traits, plant defense mechanisms, arthropod control

COTTON – VALUE AS A CROP

Cotton (*Gossypium* sp.) is a major crop in many countries around the world and its fiber is a major raw material for apparel, bed linen, and many other products (Lee and Fang, 2015). About 35 million ha of cotton are planted in the world each year, producing about 26 million tones of lint (ICAC, 2015). The word ‘cotton’ refers to four separate species in the genus *Gossypium* that are grown for the fibers covering the epidermis of their seeds: *G. arboreum*, *G. barbadense* (Pima

cotton), *G. herbaceum*, and *G. hirsutum* (Upland cotton) (Wendel and Cronn, 2001; Wendel and Grover, 2015). This review will focus on *G. hirsutum* cotton, as it comprises around 95% of global cotton production.

CHALLENGES TO PEST MANAGEMENT

Arthropod pests have likely affected cotton since it was domesticated at least 3,000 years ago (Lee and Fang, 2015). A large number of arthropod species have been described as cotton pests, but only less than 40 of them are considered key pests of the crop (Wilson et al., 2013; Luttrell et al., 2015). They directly decrease yield or reduce fiber quality, and their management is a key challenge for cotton growers worldwide. Potential losses up to 40% occur from invertebrate pests alone in cotton (James, 2001; Oerke, 2006). Significantly, even after implementation of control measures, it is estimated that losses of about 12% occur to invertebrate pests (Oerke, 2006). The economic implications of invertebrate pests encompass both crop losses and the costs of control, which mainly consists of insecticides and their application (James, 2001; Naranjo, 2011).

Domestication and Loss of Plant Resistance to Invertebrate Pests

Plant domestication has successfully increased agricultural productivity supply for humans, although this selection has usually focused on major and highly recognizable traits such as yield and quality, inadvertently losing some others such as adaptation to extreme weather or plant resistance to herbivores (Koricheva, 2002; Macfadyen and Bohan, 2010; Chen et al., 2015). This pattern can be found in the history of the domestication of cotton.

A brief review of the history of domestication in *G. hirsutum* reveals how and why plant resistance traits may have been lost. Although, each of the four domesticated *Gossypium* species has a unique history of domestication and utilization, they were all domesticated in parallel so that the short lint covering the seed was transformed to be a source of textile fiber (Brubaker et al., 1999; Wendel and Cronn, 2001). Following this initial domestication and geographical spread of cotton, some preferred traits were specifically selected, such as: compact and annual growing habits, early maturity, photoperiod neutrality, longer and stronger fiber, and higher yield (more abundant lint on the seed) (Brubaker et al., 1999; Applequist et al., 2001; Gross and Strasburg, 2010). Invertebrate pests probably benefited from selecting cotton plants for increased yield and fiber quality, as this most likely led to trade-offs with the traits controlling invertebrate resistance (Chen et al., 2015). Furthermore, modern high input systems lead to cultivars with higher nutritional value for invertebrates.

The domestication and selection for desirable production and agronomic traits in cotton has gone through phases that have resulted in limited genetic diversity within modern cotton cultivars. Firstly, intense selection during the initial domestication (Iqbal et al., 2001), secondly, industrialization and demand for higher yields of improved-quality cotton meant the

US became the focus of cotton germplasm improvement for *G. hirsutum* during the second half of the 19th century (Moore, 1956). Finally, the Mexican boll weevil (*Anthonomus grandis*) appeared in Texas in 1892 causing a significant reduction in cotton production in the southern US. Rapid selection for shorter season cultivars which avoided severe losses to the boll weevil (Smith et al., 1999; Allen, 2008) resulted in a further bottleneck for genetic diversity. There has been some reintroduction of diversity during the last century due importation of genetic stocks of wild *G. hirsutum* cotton imported from Mexico as part of the search for resistance to the cotton boll weevil. However, there are few reports of commercial cultivars with effective plant resistance to sucking bugs, spider mites, aphids, mealybugs or whitefly.

Reliance on Insecticides and the Genesis of Integrated Pest Management (IPM)

The development and commercialisation of synthetic pesticides (insecticides and acaricides) during the mid-20th century offered highly efficacious and cost effective control of many pests, leading to significant increases in productivity. They also reduced emphasis on selection for traits that may confer resistance to pests. Further, reliance on pesticides lead to selection of pesticide resistance in key pest species, the resurgence of secondary pest outbreaks (e.g., spider mites, aphids) induced by the destruction of natural enemies with pesticides applications (Wilson et al., 1998; Wu and Guo, 2003; Luttrell et al., 2015), elevated costs and environmental contamination (Naranjo, 2011; Wilson et al., 2013). These issues were the catalyst for the development of the IPM approach which considers all available pest control techniques and their combination to reduce both pest populations and reliance on pesticides (FAO, 2015). This can include a wide array of strategies and tactics, e.g., effective sampling, use of economic thresholds, conservation or augmentation of natural enemies and host plant resistance (HPR). Pesticides are an important tool in IPM systems but used primarily to manage pest populations that justify control. The use of pesticides is based on economic thresholds and with preference for use of more selective options that control the target pests but have less negative effect on natural enemies. However, the practical implementation of IPM approaches is often difficult due to the lack of compatibility between conservation of natural enemies and the availability of selective pesticides, as well as to the higher cost of more selective compounds (if available) compared with older broad-spectrum compounds.

GM Cotton

In many cotton systems the primary pests are lepidopterans such as *Helicoverpa* or *Heliothis* sp., *Earias* sp., and *Pectinophora* sp. Capacity to manage these pests without spraying insecticides would strongly support IPM approaches. Genetic modification (GM) of cotton containing genes to express protein(s) from the bacteria *Bacillus thuringiensis* (*Bt*), which are highly effective at killing the larvae of some lepidopterans (Naranjo, 2011; Wilson et al., 2013), was introduced in the mid-1990s and greatly reduced pesticide use. *Bt*-cotton is highly efficacious against target pests (Lu et al., 2012), at the same time having a negligible effect on

non-target insects (Whitehouse et al., 2005, 2014; Tian et al., 2015) and causing little or no harm to most other organisms, including people (Mendelsohn et al., 2003; Herman et al., 2009). Globally, 25 million hectares were planted in 2013 to *Bt*-cotton, representing 68% of all cotton grown in the world. Including other crops, 76 million hectares were planted to genetically engineered crops producing insecticidal proteins from *Bt* (James, 2014).

However, GM cotton is not a ‘perfect’ solution. Firstly, target pest species may become resistant, requiring the implementation of strategies to reduce this risk (Downes and Mahon, 2012). This risk is especially high for cultivars expressing a single *Bt* protein. Several of these genes therefore need to be stacked to delay the development of resistance in the target insect population (Downes and Mahon, 2012; Tabashnik et al., 2013). However, HPR traits may help support resistance management for the *Bt*-cottons as Carrière et al. (2004) and Williams et al. (2011) reported that the presence of the terpenoid gossypol, which provides resistance to a range of cotton pests, can contribute to delaying the development of insect resistance against Cry proteins. Secondly, *Bt*-cotton crops can sometimes provide a more favorable environment for other pests that are not susceptible to the *Bt* proteins. The sucking bug complex in particular was historically controlled co-incidentally by insecticides applied against lepidopteran pests (Naranjo, 2011; Wilson et al., 2013). Consequently, with dramatically reduced pesticide use against lepidopteran pests the sucking pest complex has increased in importance in most *Bt*-cotton systems. These ‘emergent’ pests may require targeted control, which creates further issues as control options are often limited and the less expensive options, such as pyrethroids or organophosphates, are disruptive of natural enemy populations. Use of these compounds against sucking pests ultimately leads to an increase in risks of secondary pests outbreaks, such as spider mites, aphids, or whitefly (Naranjo, 2011; Wilson et al., 2013). These secondary pests then require control, hence, selecting them for pesticide resistance. In Australia for example, spider mites have become resistant to both organophosphates (Herron et al., 1998) and pyrethroids (Herron et al., 2001). Although insecticide applications have greatly decreased with the adoption of *Bt*-cotton, even with the presence of some important outbreaks caused by secondary pests (Naranjo, 2011), some specific situations have been reported with increases in the number of applications required due to these outbreaks (Catarino et al., 2015).

Among the key pests that are challenges in *Bt*-cotton systems are the sucking bugs, spider mites, thrips, silverleaf whitefly, and aphids (Wilson et al., 2013; Luttrell et al., 2015). Sucking bugs are currently considered the primary pest in many of the *Bt*-cotton growing regions such as Australia (Wilson et al., 2013), China (Lu et al., 2010), India (Sharma et al., 2005), and the United States (Naranjo, 2011) and in most seasons will require targeted control. The sucking bug complex comprises primarily of *Adelphocoris* sp., *Lygus* sp., *Creontiades dilutus* and *C. pacificus*, mealybugs (*Phenacoccus solenopsis*, *Pseudococcus corymbatus*, *Pulvinaria maxima*, and *Saissetia nigra*) and the green vegetable bug (*Nezara viridula*). These species feed on young squares and

bolls, causing their abortion or damage to developing bolls. Spider mites (predominantly *Tetranychus urticae*) feed on the underside of leaves by sucking out the contents of the mesophyll cells, resulting in reduced yield and fiber quality (Wilson, 1993). Thrips (predominantly *Frankliniella* sp. and *Thrips* sp.) are able to damage cotton seedlings and therefore cause a delay in plant growth and maturity, sometimes reducing yield when the attack is severe (Sadras and Wilson, 1998; Cook et al., 2013). Conversely, later in the season thrips are also considered beneficial insects as they are key predators of spider mites (Trichilo and Leigh, 1986; Wilson et al., 1996; Milne and Walter, 1998). Silverleaf whitefly (*Bemisia tabaci*) secretes honeydew which contaminates lint, causing difficulties in the mill when the fiber is processed (Hequet and Abidi, 2002). The development of silverleaf whitefly populations resistant to a wide range of insecticides exacerbates the problem (Rao et al., 2012). Cotton aphids (*Aphis gossypii*) cause a similar damage to the lint as they excrete honeydew when they feed on the plants. They are vectors for viruses (Ellis et al., 2013) and their feeding distorts plant growth and causes a reduction in photosynthetic activity (Shannag et al., 1998).

AVAILABLE SOURCES AND TRAITS FOR HOST PLANT RESISTANCE

Controlling these ‘emergent’ sucking pests with pesticides poses a risk to successful IPM approaches, and at the same time undermines the value of GM technology, as *Bt*-cotton facilitates the control of non-target pests by their natural enemies (Tian et al., 2015). HPR could support sustainable IPM in GM cotton systems by reducing the need to apply insecticides against emergent pests or other secondary pests. Cultivars resistant to key emergent or secondary pests would require less pesticide applications, thus reducing costs, increasing the population of beneficial insects and helping the environment.

Sources of Resistance in *Gossypium* sp.

The first step to improve HPR to invertebrate pests is to identify the resistance traits that can be incorporated into elite cotton cultivars through breeding. These traits can be found in the cotton genetic pool or created through molecular techniques. Therefore, the availability of gene pools with enough variability to include some genotypes with high levels of HPR is essential. The genus *Gossypium* comprises about 50 species with a high genetic diversity between them. It appeared between 10 and 15 million years ago and diversified in three different centers of origin: Africa–Arabia, Australia, and Central America (Wendel and Grover, 2015). The genus can be divided into eight diploid genome groups ($2n = 26$ chromosomes), as well as five allotetraploid species ($2n = 52$). Of these, only four species are grown commercially (*G. arboreum*, *G. barbadense*, *G. herbaceum*, and *G. hirsutum*). The African *G. herbaceum* and the Indian *G. arboreum* are both diploids while the American *G. barbadense* and *G. hirsutum* are both allotetraploids (Wendel and Grover, 2015). The diversity within the cultivated species has declined due to domestication and breeding for increased

productivity, as described in Chapter 2. Despite this lack of diversity, especially in *G. hirsutum*, there has been research to identify HPR traits to key pests, summarized in **Table 1**. The bollworm complex has been excluded from the table as this review focuses on management of emergent or secondary pests in *Bt*-cotton systems.

In many of the cases, sources of resistance have been identified but not incorporated to commercial cultivars, probably because of the time and effort that is required. Only in situations where pest control costs have been very extreme or unaffordable (e.g., jassids in India/Africa), has there been a strong effort to breed for HPR (**Table 1**). Sometimes HPR has been identified in the target species, for example high leaf hair density in some *G. hirsutum* populations while in other cases higher HPR have been identified in other cultivated species, for instance *G. arboreum* and *G. barbadense* are more resistant than *G. hirsutum* to some pests such as spider mites and thrips (Miyazaki et al., 2012; Zhang et al., 2014b). Similarly, significant differences have been found in gossypol content between *Gossypium* species (Khan et al., 1999; Stipanovic et al., 2005; Hagenbucher et al., 2013a), and within cotton cultivars (Cai et al., 2010).

Less domesticated populations and wild *Gossypium* species can also be valuable sources of HPR traits. Resistance to various cotton pests have been reported in these diploid cottons (**Table 1**), though in many cases the cause of resistance is unknown. These include; *G. arboreum* against thrips and spider mites (Stanton et al., 1992; Miyazaki et al., 2012), *G. armourianum* and *G. raimondii* against jassids (Pushpam and Raveendran, 2006), *G. australe* and *G. lobatum* against spider mites (Schuster et al., 1972), *G. darwini* against thrips (Zhang et al., 2013), *G. tomentosum* against jassids and thrips (Knight, 1952; Zhang et al., 2013), *G. thurberi* against whitefly (Walker and Natwick, 2006) and *G. trilobum* against spider mites and silverleaf whitefly (Miyazaki et al., 2012, 2013a). However, introgression of resistance from wild species is a very long process and sometimes unsuccessful due to the difficulty of introducing HPR traits from a diploid into a tetraploid (Ganesh Ram et al., 2008), usually by creating a synthetic tetraploid, while improving or maintaining yield and fiber quality. Landraces and old cultivars may also offer valuable HPR traits, and as they are tetraploid the process of introgression is significantly shorter. The value of all of these underutilized *Gossypium* genetic resources will be reinforced with the development of new molecular techniques which will greatly enhance the introgression of the resistant traits into commercial cultivars.

Plant Defense Mechanisms

Host plant resistance against herbivorous invertebrate pests is generally defined as “the sum of genetically inherited qualities that results in a plant of one cultivar or species being less damaged by a pest arthropod than a susceptible plant lacking these qualities” (Panda and Khush, 1995; Smith, 2005). Among its benefits as a pest control measure, HPR is durable, easy to use, environmentally friendly and compatible with other management practices (Smith, 2005; Wilson et al., 2013). On the other hand, breeding for HPR is generally a slow and difficult

process that has mostly been overlooked in preference to use of chemical control of pests. In recent times, breeding for HPR is becoming a more feasible alternative due to several facts: the reduction in the impact of the Lepidopteran pests by *Bt*-cotton, increasing pest resistance to insecticides, enactment of strict environmental regulations on insecticides and their use, and advances in molecular technologies.

Plant defense mechanisms have been traditionally classified into three main categories (Painter, 1958; Panda and Khush, 1995; Smith and Clement, 2012): antixenosis or non-preference mechanisms, that prevent or deter the herbivore from feeding on the plant; antibiosis mechanisms, that affect the insects performance and survival by a physical or chemical trait; and tolerance, that represents the plant's ability to compensate for herbivore damage and yield productivity. Currently, tolerance is usually regarded as a plant defense strategy separate from resistance (Rosenthal and Kotanen, 1994; Núñez-Farfán et al., 2007). Resistance is to cover “those plant traits that reduce the extent of injury done to a plant by a herbivore” as in practice antixenosis and antibiosis are often difficult to separate (Stout, 2013). Resistance mechanisms or categories can also be direct (e.g., antibiosis, leaf morphology) and indirect (e.g., attraction of natural enemies of the herbivore), and they can be expressed constitutively (e.g., leaf morphology) or be induced following a cascade of processes after some damage is caused by the herbivory (e.g., induced chemical responses) (Schuman and Baldwin, 2016). All of these mechanisms are unusually controlled polygenetically (Stout and Davis, 2009; Smith and Clement, 2012), but a number of cases of single-gene resistance have also been reported (Kaloshian, 2004; Stuart, 2015).

HPR Traits Available in Cotton

Traits providing HPR in cotton can include one or several defense mechanisms functioning in a complex way. Some of the morphological traits provide a mechanical barrier to the pest, such as trichomes or hairs on leaves, while others influence the general growing habit and appearance of the plant, such as okra leaf or red coloration of the plant (Jenkins and Wilson, 1996; Wilson and Sadras, 1998) or even the microclimate conditions present on the leaf, such as in okra leaves (Wilson, 1994b). There is also a wide array of chemical compounds used by cotton plants to defend themselves from herbivores, such as flavonoids, tannins and particularly terpenoids such as gossypol (Wink, 1988; Sadras and Felton, 2010; Hagenbucher et al., 2013a). The latter is produced by plants of the genus *Gossypium* and has been shown to be toxic to many pests that affect cotton (Jenkins and Wilson, 1996; Cai et al., 2010; Hagenbucher et al., 2013a). The application of HPR traits is complex as different traits can operate at the same time to provide a given level of resistance. A number of reviews focused on HPR traits in cotton are available (Jenkins and Wilson, 1996; Wilson and Sadras, 1998; Sadras and Felton, 2010; Hagenbucher et al., 2013a). In the present review, HPR traits will be discussed from the point of view of the genetic source providing the resistance and the prospects for the incorporation of these traits in commercial cultivars.

TABLE 1 | Genetic sources of host plant resistance and identified traits employed in cotton against pests usually considered as secondary.

Pest	Source of resistance	Resistance trait(s)	Grown commercially (Y/N)	Reference
Sucking bug complex	<i>Gossypium hirsutum</i> cultivars and breeding lines	Nectarless plus probably antibiosis	Y	Benedict et al., 1981; Bourland and Myers, 2015
	<i>G. hirsutum</i> cultivars and breeding lines	Glandless	N	Leigh et al., 1985
	<i>G. hirsutum</i> cultivars and breeding lines	Antibiosis	Y	Tingey et al., 1973
	<i>G. hirsutum</i> breeding line	Reduced oviposition preference	N	Tingey et al., 1973
	<i>G. hirsutum</i> cultivars and breeding lines	High leaf hair density	Y	Meredith and Schuster, 1979
Spider mites	<i>G. hirsutum</i> okra-leaf cultivars	Okra leaf	Y	Wilson, 1994b
	<i>G. barbadense</i>	Antibiosis	Y	Schuster et al., 1972; Miyazaki et al., 2012; Zhang et al., 2013
Thrips	<i>G. arboreum</i> single genotype	Antibiosis	N	Miyazaki et al., 2012
	<i>G. hirsutum</i> landraces	Antibiosis	N	Schuster et al., 1972
	<i>G. australe</i>	Antibiosis	N	Schuster et al., 1972
	<i>G. lobatum</i>	Antibiosis	N	Schuster et al., 1972
	<i>G. barbadense</i>	Unknown, <i>G. barbadense</i> -related	N	Zhang et al., 2013
	<i>G. hirsutum</i> glandless Acala lines	Glandless	N	Zhang et al., 2014a
	<i>G. hirsutum</i> high leaf hair density lines	High leaf hair density	N	Rummel and Quisenberry, 1979
	<i>G. arboreum</i> single genotype	Unknown	N	Stanton et al., 1992
	<i>G. tomentosum</i>	Tomentum in leaves	N	Zhang et al., 2013
	<i>G. darwinii</i>	Not reported	N	Zhang et al., 2013
Silverleaf whitefly	<i>G. hirsutum</i> okra leaf genotypes	Reduced feeding preference	N	Chu et al., 2002; Miyazaki et al., 2013a
	<i>G. hirsutum</i> glabrous leaf genotypes	Reduced oviposition preference	N	Butler et al., 1991; Miyazaki et al., 2013a
	<i>G. thurberi</i>	Okra and glabrous leaves, plus probably antibiosis	N	Walker and Natwick, 2006
	<i>G. arboreum</i> single genotype	Antibiosis	N	Miyazaki et al., 2013a, 2014
	<i>G. armourianum</i>	Leave thickness, plus probably antixenosis	N	Pushpam and Raveendran, 2006
Jassids or Leafhoppers	<i>G. raimondii</i>	High leaf hair density	N	Pushpam and Raveendran, 2006
	<i>G. hirsutum</i> selections	High leaf hair density and length	Y	Muttuthamby et al., 1969
	<i>G. hirsutum</i> selections	High leaf hair density and length	N	McLoud et al., 2015
	<i>G. hirsutum</i> old accessions	Unknown	N	Knutson et al., 2014
	<i>G. tomentosum</i>	Tomentum in leaves	N	Knight, 1952

Traits for direct resistance mechanisms are frequently targeted in HPR breeding because they usually have major effects and they are also easier to identify and select for. On the other hand, traits for indirect HPR are not as simple to identify and are rarely targeted. Traits for both constitutive and induced HPR can play a major role controlling HPR, but constitutive mechanisms are more usually targeted as once they are identified, plants carrying them can be selected without having to perform a bioassay. For that reason, traits for constitutive morphological resistance, such as a high leaf hair density or thickness are often initially targeted in breeding programs. Other traits for constitutive HPR, such as constitutive chemical compounds, can also be relatively simple to target. However, the initial identification of the specific compounds involved in the resistance is often

more challenging than identifying morphological HPR traits. Antibiosis traits can have the biggest impact on HPR and are probably the most successfully used in cotton, both in breeding for secondary pests (Table 1) and in main pests (*Bt*-cotton). However, identifying antibiosis is not as straightforward as other HPR traits such as morphological traits, often requiring the use of bioassays.

Using HPR Traits against Emergent and Secondary Pests in Cotton

Although, not an emergent pest in *Bt*-cotton systems, the cotton boll weevil has historically been the catalyst for considerable effort toward selection of HPR genotypes (Bourland and Myers, 2015). In areas where it was a pest there was a shift in

the cultivated germplasm toward short-season early maturing cultivars to reduce the period of exposure to the pest (Smith et al., 1999). Cotton boll weevil has since been eradicated from most areas of the eastern USA and this has allowed a significant increase in cotton productivity in these areas (Allen, 2008). Unfortunately, cotton boll weevil is causing major challenges to cotton production in some parts of South America, especially in Brazil where it is currently considered the most important cotton pest (Lima et al., 2012).

Resistance to spider mites has been studied and reviewed by Wilson and Sadras (1998) and Miyazaki et al. (2012, 2013b). Okra leaf (Wilson, 1994b) has been related to an increased resistance to this pest. However, biochemical traits seem to offer more effective resistance, as reported for *G. arboreum* and *G. barbadense* genotypes (Miyazaki et al., 2013b) and some *G. hirsutum* landraces (Schuster et al., 1972; **Table 1**).

Gossypium barbadense cultivars possess a major gene conferring a higher level of resistance to thrips, according to the segregation of resistant plants reported by Zhang et al. (2013). Glandless cotton (no gossypol glands; Zhang et al., 2014a) and high leaf hair density genotypes (Rummel and Quisenberry, 1979) have also been reported to provide some level of HPR to thrips, but the exact mechanisms have not been studied. Tolerance or compensatory responses have also been reported in damaged cotton seedlings by thrips (Sadras and Wilson, 1998; Wilson et al., 2003).

Several morphological traits have been associated with partial resistance to silverleaf whitefly. Okra shaped leaves (Chu et al., 2002), and very smooth (glabrous) or very hairy leaves harbor less whiteflies than moderately hairy leaves (Butler et al., 1991; Miyazaki et al., 2013a). Very high level of resistance against SLW has been reported in the wild diploid species *G. thurberi* (Walker and Natwick, 2006), which has both okra and glabrous leaf traits. Whitefly resistance has also been associated with biochemical traits, and particularly with the amount of total sugars, tannins, flavonoids, phenols, and gossypol (Butler et al., 1990).

Regarding the sucking bug complex, compensatory or tolerant responses have also been reported in later stages of the plant for damage caused by *Lygus* sp. (Barman and Parajulee, 2013) and *Creontiades dilutus* (Duggan et al., 2007), although the effect of the genotype was not studied. Nectariless (absence of glands exuding nectar) cotton genotypes have been reported to harbor lower plant bug populations (Benedict et al., 1981; Bourland and Myers, 2015). High leaf hair densities have also been reported to provide a higher level of resistance (Meredith and Schuster, 1979). High leaf hair density has also been associated with resistance to the cotton jassid or leafhoppers (Muttuthamby et al., 1969; Bhat et al., 1982; McLoud et al., 2015), as it interferes with oviposition.

With the exception of the nectariless trait, indirect mechanisms of HPR have never been targeted in cotton, and rarely in other crops (Wäckers, 2005). However, there are some new promising achievements in this field, such as the selection of maize plants with a high emission of induced plant volatiles that attract natural enemies of the target pest (Tamiru et al., 2015). Further exploration of these mechanisms in cotton genotypes may be worthwhile within an IPM strategy.

BREEDING APPROACHES FOR RESISTANCE TO EMERGING AND SECONDARY PESTS

There is sufficient genetic diversity to warrant HPR breeding programs to a range of emerging pests within *G. hirsutum* and its primary and secondary gene pools. The success of HPR breeding, as for any other program, depends on the complexity of the inheritance of the trait and the ease and reproducibility of the phenotype. The major additional complication for breeding for HPR is that it is essential to understand the nature of the resistance, and the potential benefits and risks from that characteristic. Resistance mechanisms often mean a trade-off for the plant, either among these mechanisms and other plant traits (Strauss et al., 2002), or among different defense mechanisms working on the plant (Kariñho-Betancourt and Núñez-Farfán, 2015), which has also been demonstrated in cotton (Rudgers et al., 2004). For instance, resistance to one pest may result in increased susceptibility to other pests, such as a leaf hairness which provides resistance against jassids (Muttuthamby et al., 1969) but can make plants more susceptible to spider mites (Wilson and Sadras, 1998). Ecological interactions are also important as HPR traits can reduce a target pest but also negatively affect beneficial populations, such as the nectariless trait where leaves do not develop the extrafloral nectaries, making the cotton less attractive to plant bugs but also reducing abundance of beneficials species that use nectaries as supplementary food (Adjei-Maafo and Wilson, 1983). This result suggests that some HPR traits can lead to 'enemy-free space' and thereby inadvertently advantage a non-target herbivore species (Hagenbucher et al., 2013b). Interactions at multitrophic levels must also be considered as HPR traits may directly affect both beneficials and non-target herbivores. For instance, the presence of extrafloral nectaries can attract and increase the population of natural enemies by providing them food (Adjei-Maafo and Wilson, 1983; Wäckers, 2005) but can also enhance the fitness of some herbivores, such as plant bugs, or make the crop more attractive for oviposition of *Helicoverpa punctigera* moths that also use nectar as a supplementary food source (Benedict et al., 1981; Flint et al., 1992). Nevertheless, most commercial *G. hirsutum* varieties have extrafloral nectaries.

Interactions between HPR traits, GM traits and herbivores are also important. In most *Bt*-cotton systems the sucking bug complex has become more important, requiring targeted control with insecticides. The cause of this increased pest status may be partially due to 'insecticide release' as they are no longer being coincidentally controlled by insecticide applications targeting lepidopteran pests (Naranjo et al., 2008). However, it has also been suggested that competitive release of the plant bug complex from competition with lepidopteran pests is also a possible contributing factor to increases in abundance of sucking bugs in *Bt*-cotton systems (e.g., Whitehouse et al., 2007; Zeilinger et al., 2011) or because *Bt*-cotton plants have less induced production of terpenoids due to reduced feeding damage from lepidopteran larvae (Hagenbucher et al., 2013b). In any case this example highlights the potential complexity and hence capacity for unexpected changes that could occur when combining GM and HPR traits.

Some traits come at a high metabolic cost or altered phenology that lowers yield, such as use of short season cultivars to avoid pest attack, or result in an unwanted side effect, for instance high leaf hairiness is incompatible with mechanized picking (Anthony and Rayburn, 1989), and gossypol in the seed is toxic to animals that are fed with cottonseed (Berardi and Goldblatt, 1980). However, the presence of gossypol has been removed by breeding glandless cotton cultivars (Cai et al., 2010), though these are more susceptible to invertebrate (both the fruit and leaves; Jenkins et al., 1966) and vertebrate pests (mice attacking seeds). A more effective approach has been the development of ultra-low gossypol cottonseed GM varieties, where gossypol production is selectively inhibited in the seeds but not in the rest of the plant (Rathore et al., 2012). Due to these issues, breeding for HPR is usually regarded very cautiously and a cost/benefit analysis must be applied to determine what HPR traits are targets for introgression into elite cultivars.

Identifying New Sources of HPR

Identifying new sources of resistance by phenotyping involves exposing a range of cotton genotypes to the pest population, either in the field, greenhouse or laboratory and assessing some measure of pest fitness (developmental rate, survival, fecundity, life span) and/or plant damage – essentially a large scale bioassay. Selection of genotypes can be directed by previous published literature, however, these studies are limited and mechanisms involved in the HPR reaction are not always reported. If there is no useful resistance available amongst domesticated *G. hirsutum* genotypes, the range of material tested will need to be expanded to include race lines and other *Gossypium* species. Once material has been assembled, experiments need to be set up in the field or greenhouse to evaluate pest fitness and plant damage responses. This can be challenging as the pest may not reliably appear at densities sufficient to discriminate between cotton genotypes, and experiments may require significant amounts of land or greenhouse space to allow a realistic number of genotypes to be evaluated with sufficient replication for the results to be statistically reliable. Non-target pest species may invade the experiments and require selective management and beneficial species may reduce pest abundance.

Culturing pests and releasing them onto candidate genotypes, either in the field, greenhouse or laboratory is an approach that has been used to ensure sufficient pest density with some success (Wilson, 1994a; Parajulee et al., 2006). This ensures more reliable results, but cultures must be maintained, keeping them free of other pest contaminants (e.g., spider mites in aphid cultures), free of problems with beneficial invertebrates attacking the pests (e.g., aphid or whitefly parasitoids invading cultures or field experiments) and vigorous so that they accurately represent the likely behaviors of ‘wild’ populations. Research in greenhouse situations can be indicative of field performance but conditions may mask differences in microclimate (Wilson, 1994b) and plants may perform differently in the field and greenhouse, such as differences in expression of leaf hairiness between field and greenhouse grown plants (Miyazaki et al., 2013a).

In an ideal situation the performance of the candidate genotypes would be evaluated under protected (no pests) and unprotected (pests present) scenarios to assess the resistance of the genotypes to the pest by comparing pest abundance and relative yield between protected and unprotected treatments. This again creates challenges with logistics of sampling pest abundance, managing other pests, land, labor and costs. These issues are all manageable in the search for sources of resistance, however, once resistance has been identified and a breeding program initiated to introgress traits into more desirable genetic backgrounds there is a need to screen many genotypes at successive stages in the HPR trait introgression process. In this situation the screening of genotypes in bioassays to confirm resistance to pests can quickly become a limiting factor.

Plant phenotyping for HPR is therefore a key limiting factor and improving the speed and accuracy is crucial to develop genotypes with effective HPR. High-throughput phenotyping using automation, robotics and remote data collection is changing the way cultivars are developed (Goggin et al., 2015). These new techniques can speed up the process of collecting and analyzing data, but the use of bioassays, with all their issues identified above, is still necessary. Eliminating a large proportion of genotypes early in the breeding process without the need of bioassays is therefore still desirable and might be possible by genotyping. New molecular tools could help in fulfilling this need, thus speeding up the HPR conventional breeding process, however, the HPR traits still need to be identified and characterized prior to the use of molecular tools.

Molecular Tools to Complement Phenotyping of HPR Traits

Once potential HPR traits have been identified, modern molecular techniques, which are evolving at a rapid pace, provide the opportunity to dramatically expedite breeding by avoiding the need to constantly assess the presence of HPR traits in genotypes by bioassay. The difficulty of bio-assaying for some HPR traits makes the identification of molecular markers that are closely linked to HPR traits and can be used as substitutes for performing HPR bio-assays, essential for breeding. The completion of the draft genome sequence for *G. hirsutum* cultivar TM-1 (Li et al., 2015; Zhang T. Z. et al., 2015) marks a major milestone as it facilitates a number of molecular assisted breeding strategies that can speed the identification of molecular markers linked to HPR traits. Next generation sequencing technologies and high throughput genotyping technologies has expedited the creation of high density genetic maps in cotton that have resulted in the identification of the causal gene for okra leaf (Zhu et al., 2015). The genes for other genetically simple HPR related traits such as nectariless and frego bract will be soon identified, resulting in “perfect” molecular markers that can be used as a diagnostic for the traits in young plants or seeds. In other species, several genes have been already identified as conferring HPR, for instance HPR in rice to brown planthopper (*Nilaparvata lugens*) provided by genes *Bph14* (Du et al., 2009) and *Bph3* (Hogenhout and Zipfel, 2015).

As the desired HPR is often found in agronomically poor germplasm, additional molecular markers located either side of the causal gene allows breeders to select for plants that contain little or no linkage drag that has often masked the benefits of an introgressed trait. Large scale genotyping platforms such as the Illumina CottonSNP63K array can readily identify chromosomal segment substitutions. Therefore by repeated backcrossing of the trait into an elite cultivar, linked markers to the trait(s) can be found after only a few rounds of backcrossing. Confirmation that the donor regions are linked to resistance can be performed in a further cycle of backcrossing, selfing and selection for resistant lines. This strategy is especially useful when traits are obtained from the secondary gene pool via synthetic tetraploid bridges. High throughput genotyping also makes possible obtaining linked markers via genome wide association studies on a range of cultivars and their pedigrees containing different levels of HPR, which avoids the time and energy required in the creation of specialized genetic populations. However, a robust and reliable phenotyping will still be necessary as the level of resistance needs to be confirmed in bioassays with the target pest during the discovery and validation phases.

Challenges and Potential Opportunities with Complex Traits

Marker assisted selection has generally been found to work well for simple genetic traits, or regions that exert a major quantitative influence, but have proven ineffective for genetically complex traits comprising many loci of small effect (Desta and Ortiz, 2014). Although, few quantitative genetic HPR analyses have been performed in cotton, from other plant systems it is thought that many important HPR traits are genetically complex (Stout and Davis, 2009; Smith and Clement, 2012). Genomic selection, a form of marker-assisted selection (Heffner et al., 2009) that has only recently became feasible in cotton, can enable genetically complicated HPR traits to be incorporated into elite cultivars (Desta and Ortiz, 2014). Genomic selection requires large populations to be accurately phenotyped and genotyped, such that there are markers covering the whole genome so that all genes are in linkage with at least one marker. The aim of genomic selection is to computationally predict genomic estimated breeding values, first by analyzing a training population composed of plant lines covering all important germplasm (i.e., founders) in the breeding program, and then validating the models on subsequent breeding populations. The advantage of this methodology is that it takes into account many regions which have a small effect from the different backgrounds of the breeding populations targeted. Genomic selection therefore has the ability to optimize the HPR of cultivars using existing variation within the breeding population.

New Methodologies for Generating and for Introgressing HPR Traits

There is significant scope for improving HPR by marker assisted breeding but introgressing traits from distant germplasm such as from the secondary gene pool, still remains a challenge and requires generations of crossing and selection. It also precludes

acquiring HPR from the tertiary gene pool that consists of diploid *Gossypium* species with a completely different genome type that generally show poor or no recombination with *G. hirsutum*. To access HPR traits from these species will require identification of the causal gene. These genes can then be transferred into cultivated *G. hirsutum* cotton by GM or gene editing technology. GM traits are subject to complex and expensive regulatory systems, that cannot be grown in some countries (Tabashnik et al., 2013; James, 2014) and so the HPR trait must possess a significant economic value to compensate for the regulatory investment. The regulatory status of genome editing is currently unknown, but as simple genome edits are indistinguishable from natural or induced mutations there is the possibility that these plants may not be subject to the same strict regulations as GM cotton. Genome editing might prove to be the main avenue for acquiring HPR from diverse *Gossypium* species, especially as both the *A_t* and *D_t* genomes present in *G. hirsutum* should be able to be edited simultaneously (Wang et al., 2014).

Natural genetic diversity for HPR against a pest is not always available or easily accessible. In such cases, new diversity can be induced using chemical mutagens, ionizing radiation or transposable elements. Mutation breeding of *G. hirsutum* has resulted in 'naked and tufted' seeds, herbicide resistance and plants with longer fiber (Auld et al., 2007; Bechere et al., 2009a,b) and may provide a means of obtaining novel forms of HPR especially via developmental or secondary metabolism changes.

The history of breeding for HPR against Lepidopteran pests illustrates that for some pests adequate control can only be achieved by using GM technology to access resistance that have evolved in other biological systems. There are a number of promising GM avenues that may help control the rise of emergent and secondary pests in *Bt*-cotton. Sap-sucking insects (Hemipterans) are generally not susceptible to *Bt*, however, Chougule et al. (2013) added a short pea aphid (*Acyrthosiphon pisum*) gut binding peptide to Cry2Aa that resulted in enhanced toxicity to both pea aphid and green peach aphid. A thorough understanding of the binding and mode of action of the Cry toxins may enable modified toxins to specifically target other important pests. Secondary plant metabolites are also a source of potential resistance (Birkett and Pickett, 2014). Small lipophilic molecules are a promising group of secondary metabolites that can have similar physiochemical properties and toxicities to pesticides or insect pheromones. These metabolites pathways can be engineered into plants to help manage pests, although the metabolic pathways are complex and may be energy intensive leading to a trade-offs with yield (Birkett and Pickett, 2014).

The discovery that ingested double stranded RNA can trigger RNA interference (RNAi) in nematodes (*Caenorhabditis elegans*) has opened up the possibility of plants expressing targeted RNA species that could silence essential genes in pest species resulting in their death or reduced fecundity (Fire et al., 1998). Mao et al. (2011) found that cotton plants expressing a dsRNA that targets a *Helicoverpa armigera* P450 monooxygenase gene (*CYP6AE14*) associated with detoxification of gossypol, resulted in reduced growth of bollworms and less plant damage. Yue et al. (2016) found that cotton expressing dsRNA against a *H. armigera* gene

involved in feeding behavior, resulted in significantly reduced leaf damage and smaller larval body size. This technology has the potential to be selective as it is based on the sequence of its target sequence, thus no effect should be observed on non-target species. The difficulties associated with the technology involve the selection of target genes that are required for a vital process to the pest species, and delivering the dsRNA at levels that are effective (Miller et al., 2012) as these RNAi plants usually inhibit, but do not kill, their target host (Mao et al., 2011; Zha et al., 2011). Expression of dsRNA in chloroplasts has resulted in higher levels of these transcripts and better efficacy against target insects (Jin et al., 2015; Zhang J. et al., 2015). However, plastid transformation is only possible in a limited number of plant species and is not currently practical in cotton. Foliar application of dsRNA targeted to pest species is also currently being explored as a novel form of insecticide. It is possible that this method of delivery will become more prevalent than GM, as it avoids plant registration costs, is more flexible and appears relatively stable (San Miguel and Scott, 2015).

CONCLUSION

The history of cotton production is linked with the history of the emergence of new pests. In recent times, these emergence events have generally been related to the use of insecticides and/or the emergence of *Bt*-cottons (Luttrell et al., 2015). However, there are few examples of successful deployment of HPR traits to the emergent pests or linked secondary pests in cotton cultivars. Recent research indicates that there is significant scope to improve HPR in cotton especially against key secondary pests.

REFERENCES

- Adjei-Maafo, I. K., and Wilson, L. T. (1983). Factors affecting the relative abundance of arthropods on nectariferous and nectarless cotton. *Environ. Entomol.* 12, 349–352. doi: 10.1093/ee/12.2.349
- Allen, C. T. (2008). “Boll weevil eradication: an areawide pest management effort,” in *Areawide Pest Management: Theory and Implementation*, eds O. Koul, G. Cuperus, and N. Elliot (Wallingford: CABI), 467–559.
- Anthony, W. S., and Rayburn, S. T. (1989). Cleanability of smooth- and hairy-leaf cottons - quality effects. *Trans. Am. Soc. Agric. Eng.* 32, 1127–1130. doi: 10.13031/2013.31122
- Applequist, W. L., Cronn, R., and Wendel, J. F. (2001). Comparative development of fiber in wild and cultivated cotton. *Evol. Dev.* 3, 3–17. doi: 10.1046/j.1525-142x.2001.00079.x
- Auld, D. L., Bechere, E., Krifa, M., Kebede, H., Hequet, E., Wright, R., et al. (2007). Registration of ‘Raider 276’, a high-yielding, improved-quality upland mutant cotton cultivar. *J. Plant Regist.* 1, 115–116. doi: 10.3198/jpr2007.01.0059crc
- Barman, A. K., and Parajulee, M. N. (2013). Compensation of *Lygus hesperus* induced preflower fruit loss in cotton. *J. Econ. Entomol.* 106, 1209–1217. doi: 10.1603/EC12173
- Bechere, E., Auld, D. L., Dotray, P. A., Gilbert, L. V., and Kebede, H. (2009a). Imazamox tolerance in mutation-derived lines of upland cotton. *Crop Sci.* 49, 1586–1592. doi: 10.2135/cropsci2008.09.0528
- Bechere, E., Auld, D. L., and Hequet, E. (2009b). Development of ‘naked-tufted’ seed coat mutants for potential use in cotton production. *Euphytica* 167, 333–339. doi: 10.1007/s10681-009-9890-y
- Benedict, J. H., Leigh, T. F., Hyer, A. H., and Wynholds, P. F. (1981). Nectariless cotton: effect on growth, survival, and fecundity of *Lygus* bugs. *Crop Sci.* 21, 28–30. doi: 10.2135/cropsci1981.0011183X002100010008x
- Berardi, L. C., and Goldblatt, L. A. (1980). “Gossypol,” in *Toxic Constituents of Plant Foodstuffs*, 2nd Edn, ed. I. E. Liener (New York, NY: Academic Press), 183–237.
- Bhat, M. G., Joshi, A. B., and Singh, M. (1982). Hairiness in relation to resistance to jassid (*Amrasca devastans* Distant) and other insect pests and quality characters in cotton (*Gossypium* spp.) - a review. *Agric. Rev.* 3, 1–8.
- Birkett, M. A., and Pickett, J. A. (2014). Prospects of genetic engineering for robust insect resistance. *Curr. Opin. Plant Biol.* 19, 59–67. doi: 10.1016/j.pbi.2014.03.009
- Bourland, F., and Myers, G. O. (2015). “Conventional cotton breeding,” in *Cotton*, 2nd Edn, ed. R. G. P. D. Fang (Madison, WI: American Society of Agronomy, Inc.), 205–288.
- Brubaker, C. L., Bourland, F. M., and Wendel, J. F. (1999). “The origin and domestication of cotton,” in *Cotton: Origin, History, Technology and Production*, ed. W. C. Smith (New York: John Wiley and Sons), 3–31.
- Butler, G. D. J., Wilson, F. J., and Fishler, G. (1991). Cotton leaf trichomes and populations of *Empoasca* lybica and *Bemisia tabaci*. *Crop Prot.* 10, 461–464. doi: 10.1016/S0261-2194(91)80117-X
- Butter, N. S., Kaur, B. K. V. G., Singh, T. H., and Raheja, R. K. (1990). Biochemical basis of resistance to whitefly *Bemisia tabaci* Genn. (Aleyrodidae:Hemiptera) in cotton. *Tropical Agric.* 69, 119–122.
- Cai, Y., Xie, Y., and Liu, J. (2010). Glandless seed and glandless plant research in cotton. A review. *Agron. Sustain. Dev.* 30, 181–190. doi: 10.1051/agro/2008024
- Carrière, Y., Ellers-Kirk, C., Biggs, R., Higginson, D. M., Dennehy, T. J., and Tabashnik, B. E. (2004). Effects of gossypol on fitness costs associated with resistance to Bt cotton in pink bollworm. *J. Econ. Entomol.* 97, 1710–1718. doi: 10.1603/0022-0493-97.5.1710
- Catarino, R., Ceddia, G., Areal, F. J., and Park, J. (2015). The impact of secondary pests on *Bacillus thuringiensis* (Bt) crops. *Plant Biotechnol. J.* 13, 601–612. doi: 10.1111/pbi.12363

This review outlines sources of germplasm and the opportunities to improve HPR in cotton against invertebrate pests in GM cotton systems. Unfortunately, traits providing a high level of HPR sometimes have other undesirable effects. Therefore, it is necessary to use caution when introgressing these HPR traits into elite cultivars. Modern techniques can also help to identify and expedite the process of incorporating HPR traits into elite germplasm.

Some caution is also required, as there is a risk that the target population of herbivores can overcome the improved defense mechanisms of the plant, leading to an “arms race.” Lessons from the development of pesticide resistance in many insect and mite species suggest that any HPR mechanism which is based on a single toxin affecting pest fitness would impose strong selection for resistance in the target pest population. Issues with emerging resistance in *Bt*-cottons reinforce this fact and highlight the need of integration of HPR within IPM tactics.

Ultimately, the success of incorporating HPR will depend on the benefit it can provide compared with current strategies to manage the pest and any potential agronomic cost in terms of yield and fiber quality compared with elite cultivars. Nevertheless, HPR represents an opportunity to improve the value to cotton production systems that the current pest resistant *Bt*-cottons offer.

AUTHOR CONTRIBUTIONS

CT, WS, and LW conceived and designed the review. CT drafted the review. CT, IW, WS, and LW wrote the review.

- Chen, Y. H., Gols, R., and Benrey, B. (2015). Crop domestication and its impact on naturally selected trophic interactions. *Annu. Rev. Entomol.* 60, 35–58. doi: 10.1146/annurev-ento-010814-020601
- Chougule, N. P., Li, H. R., Liu, S. J., Linz, L. B., Narva, K. E., Meade, T., et al. (2013). Retargeting of the *Bacillus thuringiensis* toxin Cyt2Aa against hemipteran insect pests. *Proc. Natl. Acad. Sci. U.S.A.* 110, 8465–8470. doi: 10.1073/pnas.1222144110
- Chu, C.-C., Natwick, E. T., and Henneberry, T. J. (2002). *Bemisia tabaci* (Homoptera: Aleyrodidae) biotype B colonization on okra- and normal-leaf upland cotton strains and cultivars. *J. Econ. Entomol.* 95, 733–738. doi: 10.1603/0022-0493-95.4.733
- Cook, D. R., Rogers Leonard, B., Burris, E., and Gore, J. (2013). Impact of thrips infesting cotton seedlings on cotton yield distribution and maturity. *J. Cotton Sci.* 17, 23–33.
- Desta, Z. A., and Ortiz, R. (2014). Genomic selection: genome-wide prediction in plant improvement. *Trends Plant Sci.* 19, 592–601. doi: 10.1016/j.tplants.2014.05.006
- Downes, S., and Mahon, R. (2012). Evolution, ecology and management of resistance in *Helicoverpa* spp. to Bt cotton in Australia. *J. Invertebrate Pathol.* 110, 281–286. doi: 10.1016/j.jip.2012.04.005
- Du, B., Zhang, W., Liu, B., Hu, J., Wei, Z., Shi, Z., et al. (2009). Identification and characterization of Bph14, a gene conferring resistance to brown planthopper in rice. *Proc. Natl. Acad. Sci. U.S.A.* 106, 22163–22168. doi: 10.1073/pnas.0912139106
- Duggan, B. L., Lei, T. T., and Wilson, L. J. (2007). “The response of cotton to real and simulated mirid damage in Australia,” in *Proceedings of the World Cotton Research Conference-4*, Lubbock, TX.
- Ellis, M. H., Silva, T. F., Stiller, W. N., Wilson, L. J., Vaslin, M. F. S., Sharman, M., et al. (2013). Identification of a new *Poherovirus* (family Luteoviridae) associated with cotton bunt top disease in Australia. *Aust. Plant Pathol.* 42, 261–269. doi: 10.1007/s13313-012-0177-8
- FAO (2015). *The Food and Agriculture Organisation (FAO) of the United Nations*. Available at: <http://www.fao.org/agriculture/crops/core-themes/theme/pests/ipm/en/> [accessed November 28 2015].
- Fire, A., Xu, S. Q., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 391, 806–811. doi: 10.1038/35888
- Flint, H. M., Wilson, F. D., Parks, N. J., Reynoso, R. Y., Stapp, B. R., and Szaro, J. L. (1992). Suppression of pink bollworm and effect on beneficial insects of a nectarless okra-leaf cotton germplasm line. *Bull. Entomol. Res.* 82, 379–384.
- Ganesh Ram, S., Hari Ramakrishnan, S., Thiruvengadam, V., and Kannan Bapu, J. R. (2008). Prefertilization barriers to interspecific hybridization involving *Gossypium hirsutum* and four diploid wild species. *Plant Breed.* 127, 295–300. doi: 10.1111/j.1439-0523.2007.01453.x
- Goggin, F. L., Lorence, A., and Topp, C. N. (2015). Applying high-throughput phenotyping to plant-insect interactions: picturing more resistant crops. *Curr. Opin. Insect Sci.* 9, 69–76. doi: 10.1016/j.cois.2015.03.002
- Gross, B. L., and Strasburg, J. L. (2010). Cotton domestication: dramatic changes in a single cell. *BMC Biol.* 8:137. doi: 10.1186/1741-7007-8-137
- Hagenbucher, S., Olson, D. M., Ruberson, J. R., Wäckers, F. L., and Romeis, J. (2013a). Resistance mechanisms against arthropod herbivores in cotton and their interactions with natural enemies. *Crit. Rev. Plant Sci.* 32, 458–482. doi: 10.1080/07352689.2013.809293
- Hagenbucher, S., Wäckers, F. L., Wettstein, F. E., Olson, D. M., Ruberson, J. R., and Romeis, J. (2013b). Pest trade-offs in technology: reduced damage by caterpillars in Bt cotton benefits aphids. *Proc. R. Soc. B Biol. Sci.* 280:20130042. doi: 10.1098/rspb.2013.0042
- Heffner, E. L., Sorrells, M. E., and Jannink, J. L. (2009). Genomic selection for crop improvement. *Crop Sci.* 49, 1–12. doi: 10.2135/cropsci2008.08.0512
- Hequet, E., and Abidi, N. (2002). Processing sticky cotton: implication of trehalulose in residue build-up. *J. Cotton Sci.* 6, 77–90.
- Herman, R. A., Chassy, B. M., and Parrott, W. (2009). Compositional assessment of transgenic crops: an idea whose time has passed. *Trends Biotechnol.* 27, 555–557. doi: 10.1016/j.tibtech.2009.07.003
- Herron, G. A., Edge, V. E., Wilson, L. J., and Rophail, J. (1998). Organophosphate resistance in spider mites (Acari:Tetranychidae) from cotton in Australia. *Exp. Appl. Acarol.* 22, 17–30. doi: 10.1023/A:1006029307049
- Herron, G. A., Rophail, J., and Wilson, L. J. (2001). The development of bifenthrin resistance in two-spotted spider mite (Acari: Tetranychidae) from Australian cotton. *Exp. Appl. Acarol.* 25, 301–310. doi: 10.1023/A:1017967118609
- Hogenhout, S. A., and Zipfel, C. (2015). Engineering insect-free cereals. *Nat. Biotechnol.* 33, 262–263. doi: 10.1038/nbt.3162
- ICAC (2015). *International Cotton Advisory Committee*. Available at: <http://www.icac.org> [accessed November 28, 2015].
- Iqbal, M. J., Reddy, O. U. K., El-Zik, K. M., and Pepper, A. E. (2001). A genetic bottleneck in the ‘evolution under domestication’ of upland cotton *Gossypium hirsutum* L. examined using DNA fingerprinting. *Theor. Appl. Genet.* 103, 547–554. doi: 10.1007/PL00002908
- James, C. (2001). *Global Review of Commercialized Transgenic Crops: 2001*. Ithaca, NY: ISAAA.
- James, C. (2014). *Global Status of Commercialized Biotech/GM Crops: 2014*. Ithaca, NY: ISAAA.
- Jenkins, J. N., Maxwell, L. G., and Lafever, H. N. (1966). The comparative preference of insects for glanded and glandless cotton. *J. Econ. Entomol.* 59, 352–356. doi: 10.1093/jee/59.2.352
- Jenkins, J. N., and Wilson, F. D. (1996). “Host plant resistance,” in *Cotton Insects and Mites: Characterization and Management*, eds E. G. King, J. R. Phillips, and R. J. Coleman (Memphis, TN: The Cotton Foundation), 563–597.
- Jin, S., Singh, N. D., Li, L., Zhang, X., and Daniell, H. (2015). Engineered chloroplast dsRNA silences cytochrome p450 monooxygenase, V-ATPase and chitin synthase genes in the insect gut and disrupts *Helicoverpa armigera* larval development and pupation. *Plant Biotechnol. J.* 13, 435–446. doi: 10.1111/pbi.12355
- Kaloshian, I. (2004). Gene-for-gene disease resistance: bridging insect pest and pathogen defense. *J. Chem. Ecol.* 30, 2419–2438. doi: 10.1007/s10886-004-7943-1
- Kariñho-Betancourt, E., and Núñez-Farfán, J. (2015). Evolution of resistance and tolerance to herbivores: testing the trade-off hypothesis. *Peer J.* 3:e789. doi: 10.7717/peerj.789
- Khan, M. A., Stewart, J. M., and Murphy, J. B. (1999). Evaluation of the *Gossypium* gene pool for foliar terpenoid aldehydes. *Crop Sci.* 39, 253–258. doi: 10.2135/cropsci1999.0011183X003900010039x
- Knight, R. L. (1952). The genetics of jassid resistance in cotton - I. The genes H1 and H2. *J. Genet.* 51, 47–66. doi: 10.1371/journal.pone.0072542
- Knutson, A., Isaacs, S., Campos, C., Campos, M., and Smith, C. W. (2014). Resistance to cotton fleahopper feeding in primitive and converted race stocks of cotton, *Gossypium hirsutum*. *J. Cotton Sci.* 18, 385–392.
- Koricheva, J. (2002). Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. *Ecology* 83, 176–190. doi: 10.1890/0012-9658(2002)083[0176:MAOSOV]2.0.CO;2
- Lee, J. A., and Fang, D. D. (2015). “Cotton as a world crop: origin, history and current status,” in *Cotton*, 2n Edn, ed. R. G. P. D. D. Fang (Madison, WI: American Society of Agronomy, Inc.), 1–23.
- Leigh, T. F., Hyer, A. H., Benedict, J. H., and Wynholds, P. F. (1985). Observed population increase, nymphal weight gain and oviposition nonpreference as indicators of *Lygus hesperus* knight (Heteroptera:Miridae) resistance in glandless cotton. *J. Econ. Entomol.* 78, 1109–1113. doi: 10.1093/jee/78.5.1109
- Li, F. G., Fan, G. Y., Lu, C. R., Xiao, G. H., Zou, C. S., Kohel, R. J., et al. (2015). Genome sequence of cultivated Upland Cotton (*Gossypium hirsutum* TM-1) provides insights into genome evolution. *Nat. Biotechnol.* 33, 524–530. doi: 10.1038/nbt.3208
- Lima, I. S., Degrande, P. E., Miranda, J. E., and Santos, W. J. (2012). Evaluation of the Boll Weevil *Anthonomus grandis* bohemani (Coleoptera: Curculionidae) suppression program in the state of Goiás, Brazil. *Neotrop. Entomol.* 42, 82–88. doi: 10.1007/s13744-012-0083-3
- Lu, B., Downes, S., Wilson, L., Gregg, P., Knight, K., Kauter, G., et al. (2012). Yield, development, and quality response of dual-toxin Bt cotton to *Helicoverpa* spp. infestations in Australia. *Entomol. Exp. Appl.* 145, 72–81. doi: 10.10111/j.1570-7458.2012.01313.x
- Lu, Y., Wu, K., Jiang, Y., Xia, B., Li, P., Feng, H., et al. (2010). Mirid bug outbreaks in multiple crops correlated with wide-scale adoption of Bt cotton in China. *Science* 328, 1151–1154. doi: 10.1126/science.1187881

- Luttrell, R. G., Teague, T. G., and Brewer, M. J. (2015). "Cotton insect pest management," in *Cotton*, 2nd Edn, ed. R. G. P. D. D. Fang (Madison, WI: American Society of Agronomy, Inc), 509–546.
- Macfadyen, S., and Bohan, D. A. (2010). Crop domestication and the disruption of species interactions. *Basic Appl. Ecol.* 11, 116–125. doi: 10.1016/j.baae.2009.11.008
- Mao, Y. B., Tao, X. Y., Xue, X. Y., Wang, L. J., and Chen, X. Y. (2011). Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms. *Trans. Res.* 20, 665–673. doi: 10.1007/s11248-010-9450-1
- McLoud, L. A., Knutson, A., Campos-Figueroa, M., Smith, C. W., and Hague, S. (2015). Evaluating pilose, a cultigen of *Gossypium hirsutum*, as a source of resistance to Cotton Fleahopper (Hemiptera: Miridae). *J. Econ. Entomol.* 108, 2048–2054. doi: 10.1093/jee/tov128
- Mendelsohn, M., Kough, J., Vaituzis, Z., and Matthews, K. (2003). Are Bt crops safe? *Nat. Biotechnol.* 21, 1003–1009. doi: 10.1038/nbt0903-1003
- Meredith, W. R., and Schuster, M. F. (1979). Tolerance of glabrous and pubescent cottons to tarnished plant bug. *Crop Sci.* 19, 484–488. doi: 10.2135/cropsci1979.0011183X001900040014x
- Miller, S. C., Miyata, K., Brown, S. J., and Tomoyasu, Y. (2012). Dissecting systemic RNA interference in the Red Flour Beetle *Tribolium castaneum*: parameters affecting the efficiency of RNAi. *PLoS ONE* 7:e47431. doi: 10.1371/journal.pone.0047431
- Milne, M., and Walter, G. H. (1998). Significance of mite prey in the diet of the onion thrips *Thrips tabaci* Lindeman (Thysanoptera: Thripidae). *Aust. J. Entomol.* 37, 120–124. doi: 10.1111/j.1440-6055.1998.tb01558.x
- Miyazaki, J., Stiller, W. N., Truong, T. T., Xu, Q., Hocart, C. H., Wilson, L. J., et al. (2014). Jasmonic acid is associated with resistance to twospotted spider mites in diploid cotton (*Gossypium arboreum*). *Funct. Plant Biol.* 41, 748–757. doi: 10.1071/FP13333
- Miyazaki, J., Stiller, W. N., and Wilson, L. J. (2012). Novel cotton germplasm with host plant resistance to twospotted spider mite. *Field Crops Res.* 134, 114–121. doi: 10.1002/ps.3813
- Miyazaki, J., Stiller, W. N., and Wilson, L. J. (2013a). Identification of host plant resistance to silverleaf whitefly in cotton: implications for breeding. *Field Crops Res.* 154, 145–152. doi: 10.1016/j.fcr.2013.08.001
- Miyazaki, J., Wilson, L. J., and Stiller, W. N. (2013b). Fitness of twospotted spider mites is more affected by constitutive than induced resistance traits in cotton (*Gossypium spp.*). *Pest Manag. Sci.* 69, 1187–1197. doi: 10.1002/ps.3546
- Moore, J. H. (1956). Cotton breeding in the old south. *Agric. History* 30, 95–104.
- Muttuthamby, S., Aslam, M., and Khan, M. A. (1969). Inheritance of leaf hairiness in *Gossypium hirsutum* L. cotton and its relationship with jassid resistance. *Euphytica* 18, 435–439. doi: 10.1007/BF00397794
- Naranjo, S. E. (2011). Impacts of Bt transgenic cotton on integrated pest management. *J. Agric. Food Chem.* 59, 5842–5851. doi: 10.1021/jf102939c
- Naranjo, S. E., Ruberson, J. R., Sharma, H. C., Wilson, L., and Wu, K. (2008). "The present and future role of insect-resistant genetically modified cotton in IPM," in *Integration of Insect-Resistant Genetically Modified Crops Within IPM Programs*, eds J. Romeis, A. M. Shelton, and G. G. Kennedy (Berlin: Springer), 159–194.
- Núñez-Farfán, J., Fornoni, J., and Valverde, P. L. (2007). "The evolution of resistance and tolerance to herbivores," in: annual review of ecology. *Evol. Syst.* 38, 541–566. doi: 10.1146/annurev.ecolsys.38.091206.095822
- Oerke, E. C. (2006). Crop losses to pests. *J. Agric. Sci.* 144, 31–43. doi: 10.1017/S0021859605005708
- Painter, R. H. (1958). Resistance of plants to insects. *Annu. Rev. Entomol.* 3, 267–290. doi: 10.1146/annurev.en.03.010158.001411
- Panda, N., and Khush, G. S. (1995). *Host Plant Resistance to Insects*. Wallingford Oxon: CAB International.
- Parajulee, M., Shrestha, R., and Leser, J. (2006). Sampling methods, dispersion patterns, and fixed precision sequential sampling plans for Western flower thrips (Thysanoptera; Thripidae) and cotton fleahoppers (Hemiptera: Miridae) in cotton. *Entomol. Soc. Am.* 6, 568–577.
- Pushpam, R., and Raveendran, T. S. (2006). Production of interspecific hybrids between *Gossypium hirsutum* and Jassid resistant wild species *G. raimondii* and *G. armourianum*. *Cytologia* 71, 407–418. doi: 10.1508/cytologia.71.407
- Rao, Q., Xu, Y. H., Luo, C., Zhang, H. Y., Jones, C. M., Devine, G. J., et al. (2012). Characterisation of neonicotinoid and pymetrozine resistance in strains of *Bemisia tabaci* (Hemiptera: Aleyrodidae) from China. *J. Integr. Agric.* 11, 321–326. doi: 10.1016/S2095-3119(12)60016-1
- Rathore, K. S., Sundaram, S., Sunilkumar, G., Campbell, L. M., Puckhaber, L., Marcel, S., et al. (2012). Ultra-low gossypol cottonseed: generational stability of the seed-specific, RNAi-mediated phenotype and resumption of terpenoid profile following seed germination. *Plant Biotechnol. J.* 10, 174–183. doi: 10.1111/j.1467-7652.2011.00652.x
- Rosenthal, J. P., and Kotanen, P. M. (1994). Terrestrial plant tolerance to herbivory. *Trends Ecol. Evol.* 9, 145–148. doi: 10.1016/0169-5347(94)90180-5
- Rudgers, J. A., Strauss, S. Y., and Wendel, J. F. (2004). Trade-offs among anti-herbivore resistance traits: insights from Gossypieae (Malvaceae). *Am. J. Bot.* 91, 871–880. doi: 10.3732/ajb.91.6.871
- Rummel, D. R., and Quisenberry, J. E. (1979). Influence of thrips injury on leaf development and yield of various cotton genotypes. *J. Econ. Entomol.* 72, 706–709. doi: 10.1093/jee/72.5.706
- Sadras, V., and Felton, G. (2010). "Mechanisms of cotton resistance to arthropod herbivory," in *Physiology of Cotton*, eds J. Stewart, D. Oosterhuis, J. Heitholt, and J. Mauney (Berlin: Springer), 213–228.
- Sadras, V. O., and Wilson, L. J. (1998). Recovery of cotton crops after early season damage by thrips (Thysanoptera). *Crop Sci.* 38, 399–409. doi: 10.2135/cropsci1998.0011183X003800020022x
- San Miguel, K., and Scott, J. G. (2015). The next generation of insecticides: dsRNA is stable as a foliar-applied insecticide. *Pest. Manag. Sci.* 72, 801–809. doi: 10.1002/ps.4056
- Schuman, M. C., and Baldwin, I. T. (2016). The layers of plant responses to insect herbivores. *Annu. Rev. Entomol.* 61, 373–394. doi: 10.1146/annurev-ento-010715-023851
- Schuster, M. F., Maxwell, F. G., and Jenkins, J. N. (1972). Resistance to the two spotted spider mite in certain *Gossypium hirsutum* races, *Gossypium* species, and glanded-glandless counterpart cottons. *J. Econ. Entomol.* 65, 1108–1110. doi: 10.1093/jee/65.4.1108
- Shannag, H. K., Thorvilson, H., and El-Shatnawi, M. K. (1998). Changes in photosynthetic and transpiration rates of cotton leaves infested with the cotton aphid *Aphis gossypii*: unrestricted infestation. *Ann. Appl. Biol.* 132, 13–18. doi: 10.1111/j.1744-7348.1998.tb05181.x
- Sharma, O. P., Bambawale, O. M., Dhandapani, A., Tanwar, R. K., Bhosle, B. B., Lavekar, R. C., et al. (2005). Assessment of severity of important diseases of rainfed Bt transgenic cotton in southern Maharashtra. *Indian Phytopathol.* 58, 483–485.
- Smith, C. M. (2005). *Plant Resistance to Arthropods: Molecular and Conventional Approaches*. Berlin: Springer.
- Smith, C. M., and Clement, S. L. (2012). Molecular bases of plant resistance to arthropods. *Annu. Rev. Entomol.* 57, 309–328. doi: 10.1146/annurev-ento-120710-100642
- Smith, C. W., Cantrell, R. G., Moser, H. S., and Oakley, S. R. (1999). "History of cultivar development in the United States," in *Cotton: Origin, History, Technology and Production*, ed. W. C. Smith (New York, NY: John Wiley and Sons), 99–171.
- Stanton, M. A., Stewart, J. M., and Tugwell, N. P. (1992). Evaluation of *Gossypium arboreum* L. germplasm for resistance to thrips. *Genet. Resour. Crop Evol.* 39, 89–95.
- Stipanovic, R. D., Puckhaber, L. S., Bell, A. A., Percival, A. E., and Jacobs, J. (2005). Occurrence of (+)- and (-)-gossypol in wild species of cotton and in *Gossypium hirsutum* var. marie-galante (Watt) Hutchinson. *J. Agric. Food Chem.* 53, 6266–6271. doi: 10.1021/jf050702d
- Stout, M., and Davis, J. (2009). "Keys to the increased use of host plant resistance in integrated pest management," in *Integrated Pest Management: Integration Development Process*, eds R. Peshin and A. K. Dhawan (Berlin: Springer), 163–181.
- Stout, M. J. (2013). Reevaluating the conceptual framework for applied research on host-plant resistance. *Insect Sci.* 20, 263–272. doi: 10.1111/1744-7917.12011
- Strauss, S. Y., Rudgers, J. A., Lau, J. A., and Irwin, R. E. (2002). Direct and ecological costs of resistance to herbivory. *Trends Ecol. Evol.* 17, 278–285. doi: 10.1086/665654

- Stuart, J. (2015). Insect effectors and gene-for-gene interactions with host plants. *Curr. Opin. Insect Sci.* 9, 56–61. doi: 10.1016/j.cois.2015.02.010
- Tabashnik, B. E., Brévault, T., and Carrière, Y. (2013). Insect resistance to Bt crops: lessons from the first billion acres. *Nat. Biotechnol.* 31, 510–521. doi: 10.1038/nbt.2597
- Tamiru, A., Khan, Z. R., and Bruce, T. J. A. (2015). New directions for improving crop resistance to insects by breeding for egg induced defence. *Curr. Opin. Insect Sci.* 9, 51–55. doi: 10.1016/j.cois.2015.02.011
- Tian, J. C., Yao, J., Long, L. P., Romeis, J., and Shelton, A. M. (2015). Bt crops benefit natural enemies to control non-target pests. *Sci. Rep.* 5:16636. doi: 10.1038/srep16636
- Tingey, W. M., Leigh, T. F., and Hyer, A. H. (1973). *Lygus* bug resistant cotton. *California Agric.* 27, 8–9.
- Trichilo, P. J., and Leigh, T. F. (1986). Predation on spider mite eggs by the Western flower thrip, *Frankliniella occidentalis* (Thysanoptera:Thripidae), an opportunist in a cotton agroecosystem. *Environ. Entomol.* 15, 821–825. doi: 10.1093/ee/15.4.821
- Wäckers, F. L. (2005). “Suitability of (extra-)floral nectar, pollen, and honeydew as insect food sources,” in *Plant-Provided Food for Carnivorous Insects: A Protective Mutualism and its Applications*, eds F. L. Wäckers, P. C. J. van Rijn, and J. Bruun (Cambridge: Cambridge University Press), 17–74.
- Walker, G. P., and Natwick, E. T. (2006). Resistance to silverleaf whitefly, *Bemisia argentifolii* (Hem., Aleyrodidae), in *Gossypium thurberi*, a wild cotton species. *J. Appl. Entomol.* 130, 429–436.
- Wang, Y. P., Cheng, X., Shan, Q. W., Zhang, Y., Liu, J. X., Gao, C. X., et al. (2014). Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. *Nat. Biotechnol.* 32, 947–951. doi: 10.1038/nbt.2969
- Wendel, J. F., and Cronn, R. C. (2001). Polyploidy and the evolutionary history of cotton. *Adv. Agron.* 78, 139–186. doi: 10.1016/S0065-2113(02)78004-8
- Wendel, J. F., and Grover, C. E. (2015). “Taxonomy and evolution of the cotton genus, *Gossypium*,” in *Cotton*, 2nd Edn, ed. R. G. P. D. D. Fang (Madison, WI: American Society of Agronomy, Inc.), 25–44.
- Whitehouse, M. E. A., Wilson, L. J., and Constable, G. A. (2007). Target and non-target effects on the invertebrate community of Vip cotton, a new insecticidal transgenic. *Aust. J. Agric. Res.* 58, 273–285. doi: 10.1071/AR06100
- Whitehouse, M. E. A., Wilson, L. J., Davies, A. P., Cross, D., Goldsmith, P., Thompson, A., et al. (2014). Target and nontarget effects of novel “triple-stacked” Bt-transgenic cotton 1: canopy arthropod communities. *Environ. Entomol.* 43, 218–241. doi: 10.1603/EN13167
- Whitehouse, M. E. A., Wilson, L. J., and Fitt, G. P. (2005). A comparison of arthropod communities in transgenic Bt and conventional cotton in Australia. *Environ. Entomol.* 34, 1224–1241. doi: 10.1093/ee/34.5.1224
- Williams, J. L., Ellers-Kirk, C., Orth, R. G., Gassmann, A. J., Head, G., Tabashnik, B. E., et al. (2011). Fitness cost of resistance to bt cotton linked with increased gossypol content in pink bollworm larvae. *PLoS ONE* 6:e21863. doi: 10.1371/journal.pone.0021863
- Wilson, L., Downes, S., Khan, M., Whitehouse, M., Baker, G., Grundy, P., et al. (2013). IPM in the transgenic era: a review of the challenges from emerging pests in Australian cotton systems. *Crop Pasture Sci.* 64, 737–749.
- Wilson, L. J. (1993). Spider mites (Acar: Tetranychidae) affect yield and fiber quality of cotton. *J. Econ. Entomol.* 86, 566–585. doi: 10.1093/jee/86.2.566
- Wilson, L. J. (1994a). Plant-quality effect on life-history parameters of the two-spotted spider mite (Acar: Tetranychidae) on cotton. *J. Econ. Entomol.* 87, 1665–1673. doi: 10.1093/jee/87.6.1665
- Wilson, L. J. (1994b). Resistance of okra-leaf cotton genotypes to two-spotted spider mites (Acar: Tetranychidae). *J. Econ. Entomol.* 87, 1726–1735. doi: 10.1093/jee/87.6.1726
- Wilson, L. J., Bauer, L. R., and Lally, D. A. (1998). Effect of early season insecticide use on predators and outbreaks of spider mites (Acar:Tetranychidae) in cotton. *Bull. Entomol. Res.* 88, 477–488. doi: 10.1017/S000748530004222X
- Wilson, L. J., Bauer, L. R., and Walter, G. H. (1996). ‘Phytophagous’ thrips are facultative predators of two-spotted spider mites (Acar:Tetranychidae) on cotton in Australia. *Bull. Entomol. Res.* 86, 297–305. doi: 10.1017/S0007485300052597
- Wilson, L. J., and Sadras, V. O. (1998). “Host plant resistance in cotton to spider mites,” in *International Congress of Acarology*, eds R. B. Halliday, D. E. Walter, H. C. Proctor, R. A. Norton, and M. J. Colloff (Clayton, VIC: CSIRO Publishing), 314–327.
- Wilson, L. J., Sadras, V. O., Heimoana, S. C., and Gibb, D. (2003). How to succeed by doing nothing: cotton compensation after simulated early season pest damage. *Crop Sci.* 43, 2125–2134. doi: 10.2135/cropsci2003.2125
- Wink, M. (1988). Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Genet.* 75, 225–233. doi: 10.1007/BF00303957
- Wu, K., and Guo, Y. (2003). Influences of *Bacillus thuringiensis* Berliner cotton planting on population dynamics of the cotton aphid *Aphis gossypii* glover, in Northern China. *Environ. Entomol.* 32, 312–318. doi: 10.1603/0046-225X-32.2.312
- Yue, Z., Liu, X., Zhou, Z., Hou, G., Hua, J., and Zhao, Z. (2016). Development of a novel-type transgenic cotton plant for control of cotton bollworm. *Plant Biotechnol. J.* doi: 10.1111/pbi.12534 [Epub ahead of print].
- Zeilinger, A. R., Olson, D. M., and Andow, D. A. (2011). Competition between stink bug and heliothis caterpillar pests on cotton at within-plant spatial scales. *Entomol. Exp. Appl.* 141, 59–70. doi: 10.1111/j.1570-7458.2011.01165.x
- Zha, W. J., Peng, X. X., Chen, R. Z., Du, B., Zhu, L. L., and He, G. C. (2011). Knockdown of midgut genes by dsRNA-transgenic plant-mediated RNA interference in the Hemipteran insect *Nilaparvata lugens*. *PLoS ONE* 6:e20504. doi: 10.1371/journal.pone.0020504
- Zhang, J., Fang, H., Zhou, H., Hughs, S. E., and Jones, D. C. (2013). Inheritance and transfer of Thrips resistance from Pima cotton to Upland cotton. *J. Cotton Sci.* 16, 163–169.
- Zhang, J., Idowu, O. J., Wedegaertner, T., and Hughs, S. E. (2014a). Genetic variation and comparative analysis of thrips resistance in glandless and glanded cotton under field conditions. *Euphytica* 199, 373–383. doi: 10.1007/s10681-014-1137-x
- Zhang, J., Khan, S. A., Hasse, C., Ruf, S., Heckel, D. G., and Bock, R. (2015). Full crop protection from an insect pest by expression of long double-stranded RNAs in plastids. *Science* 347, 991–994. doi: 10.1126/science.1261680
- Zhang, J., Percy, R. G., and McCarty, J. C. Jr. (2014b). Introgression genetics and breeding between Upland and pima cotton: a review. *Euphytica* 198, 1–12. doi: 10.1007/s10681-014-1094-4
- Zhang, T. Z., Hu, Y., Jiang, W. K., Fang, L., Guan, X. Y., Chen, J. D., et al. (2015). Sequencing of allotetraploid cotton (*Gossypium hirsutum* L. acc. TM-1) provides a resource for fiber improvement. *Nat. Biotechnol.* 33, 531–537. doi: 10.1038/nbt.3207
- Zhu, Q. H., Zhang, J., Liu, D., Stiller, W., Zhang, Z., Llewellyn, D., et al. (2015). Integrated mapping and characterization of the gene underlying the okra leaf trait in *Gossypium hirsutum* L. *J. Exp. Bot.* 67, 763–774. doi: 10.1093/jxb/erv494

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Exploitation of Diversity within Crops—the Key to Disease Tolerance?

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Tolerance, defined as the ability of a crop to maintain yield in the presence of disease, is a difficult characteristic to measure, and its component traits are generally undefined. It has been studied as a characteristic of plant genotypes grown singly or in monoculture crop stands. However, it is similarly valid as a characteristic of ecosystems, or mixtures / inter-cropping in crops and this paper seeks to evaluate theoretical and practical aspects of tolerance in this context. Focusing on cereals and fungal pathogens, consideration is given to the process of yield formation, the impact of disease on yield, and how tolerance might be assessed in monocultures. Variation in tolerance traits in monocultures and how such plants might interact in mixtures is considered; specifically the expression of tolerance in mixtures and how plants with contrasting tolerance traits in monocultures combine. Having focused on disease, further consideration is given to the impact of and on other microbial species in the crop environment. Finally the practical approaches that could be adopted to identify and assess the main traits responsible for expressing tolerance are addressed. These focus on the dynamic nature of plant–plant and plant-microbe interactions particularly in response to both biotic and abiotic stress out with the range of optimal or normal crop evaluation environments. It is proposed that by using more extreme factor parameter values in mixed crop evaluation environments the key traits affecting tolerance will be identified.

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INTRODUCTION

Tolerance of disease may be defined as the ability of a crop to maintain yield in the presence of disease (Schafer, 1971; Bingham and Newton, 2009). That crops differ in their disease tolerance has been recognized for many years, but recently there has been renewed interest in identifying the traits and associated mechanisms that underlie these differences so that tolerance may be increased through crop improvement or agronomic practice (Parker et al., 2004; Bingham and Topp, 2009; Bingham et al., 2009; Bancal et al., 2015). Several factors have prompted this interest. Focusing on cereals, only partial host resistance is available for many important plant pathogens and evolution of pathogen insensitivity to fungicides erodes their effectiveness in disease control. Improved tolerance is thus viewed as a complimentary approach to disease management because it will minimize the impact of disease on yield in cases where epidemics cannot be controlled fully by resistance mechanisms or the application of fungicides. Tolerance is also considered to be a potentially durable form of disease management, unlike disease resistance and fungicides, since it is expected to place little or no selection pressure for resistance on pathogen populations.

To date, traits (see Terminology in **Box 1**) and mechanisms that confer disease tolerance have been investigated for crops grown as monocultures of relatively uniform, genetically similar individuals. Tolerance can be studied at the organ or plant level too, but the focus here will remain the crop as a primary aim of this paper is to identify the traits that are expressed in the field crop context and not necessarily in other contexts. With respect to disease this may be critically important as disease epidemics are a constant threat in genetically uniform crops (Finckh et al., 2000), but in climax ecosystems they are the exception. Increasing the genetic diversity within cropping systems through the use of variety or species mixtures offers a number of potential advantages not only in terms of restricting disease development, but also increasing yield stability and resilience to abiotic stress and delivering other ecosystem services including greater biodiversity (Schöb et al., 2015). Plant-plant interactions are more complex in genetically diverse populations and may involve replacement, facilitation and niche complementarity effects (Brooker et al., 2016). Little consideration has been given to the nature of disease tolerance in mixtures and thus it is not known whether the methods for quantifying tolerance and identifying influential morphological and physiological traits that have been developed for monocultures are appropriate for use in variety or species mixtures. In this paper the concept of disease tolerance is reviewed briefly as developed for genetically uniform crops and the nature of plant-plant interactions in genetically diverse populations, before exploring whether putative tolerance traits identified for monocultures can be exploited in mixtures.

YIELD FORMATION AND THE IMPACT OF DISEASE

Crop yield (Y) can be quantified in terms of the amount of photosynthetically active radiation (PAR) incident upon the crop (I), the fraction of the PAR that is intercepted by green tissue (f), the efficiency with which the energy from PAR is converted into dry matter radiation use efficiency (RUE) and the fraction of the total above ground biomass that is allocated to the harvested parts the harvest index (HI; Monteith, 1977; Reynolds et al., 2005; Bingham et al., 2009; Murchie et al., 2009).

$$Y = I \times f \times RUE \times HI \quad (1)$$

Equation (1) has been used as the basis for analysing variation in yield in response to geographical location, seasonal variations in weather, abiotic and biotic stresses including fungal disease

(Johnson, 1987; Waggoner, 1990; Gaunt, 1995; Paveley et al., 2001; Bingham et al., 2007a,b). Disease may reduce crop growth by reducing radiation interception and RUE (Johnson, 1987; Bingham and Topp, 2009), although for a number of pathosystems the major effect appears to be the reduction in radiation interception with smaller or negligible effects observed on RUE (Rabbinge et al., 1985; Van Oijen, 1990; Robert et al., 2004). Depending on the timing of the disease epidemic, radiation interception by healthy (green) tissue can be reduced by effects of pathogens on leaf growth or healthy leaf area duration.

Disease, i.e., symptoms (see Terminology in **Box 1**), does not necessarily equate to microbial infection as infection is often symptomless. Infection can result in several types of trophic relationships including beneficial or mutualistic relationships such as rhizobium-legume interactions. In this paper the focus is mostly on microbes described loosely as pathogens from an anthropocentric perspective because they produce symptoms. However, used in this context the term pathogen is misleading as it obscures two essential attributes of these plant-microbe interactions that are relevant to consideration of tolerance. Firstly, the interactions can be either parasitic or pathogenic and secondly, they can transition between these states (Newton et al., 2010b). Indeed they can transition with the mutualistic state too and this will be considered later. Examples of diseases resulting from infection by microbes that are normally in the parasitic state are the cereal rusts and powdery mildews, where damage is caused primarily by loss of assimilates to the fungus and loss of active green leaf area from fungal structures mostly associated with sporulation. Also described as biotrophic interactions, the assimilate drain can be an active process where the fungus manipulates host metabolism and the net result is accelerated leaf senescence. Examples of pathogenic interactions are diseases caused by *Botrytis cinerea* and *Sclerotinia sclerotiorum*. Also described as necrotrophs, toxins are used to actively kill host tissue to render it accessible as a substrate for microbial growth. Some microbes may occupy either of these states (or the mutualistic state) at different times in their lifecycle with respect to the host plant and are often described as hemi-biotrophs. *Ramularia collo-cygni* and *Rhynchosporium commune* on barley are good examples of microbes that transition between states during their life cycle. They grow asymptotically within tissues for considerable periods but following certain triggers they produce toxins and visible symptoms (Newton et al., 2010b). In some, but not all, pathosystems changes in host metabolism can precede the development of visible symptoms (Scholes and Rolfe, 2009). At present it is not known whether the asymptomatic

BOX 1 | TERMINOLOGY

Disease: The visual expression of microbial challenge to plants, i.e., the symptoms. Symptoms can be varied but often show a high degree of correlation with loss of green leaf area. Disease does not necessarily equate to microbial infection as infection is often symptomless.

(Plant) trait: A genetically determined characteristic or condition. (Based on The American Heritage® Science Dictionary Copyright ©2002, published by Houghton Mifflin.) Traits may be physical, such as plant height or leaf shape, or they may be behavioral, such as rapid growth and late-flowering, or biochemical such as a disease resistance and salt tolerance. Traits typically result from the combined action of several genes, though some traits are expressed by a single gene.

Trait modifier: Any environmental or genetic factor that influences the expression of a trait, for example temperature or agrochemical treatment.

Trait complex: A set of interacting traits that can be measured together in one or more ways. A good example of a trait complex (/ complex trait) is yield that could be measured simply by weight, or divided into sub-classes and weighed etc.

infection incurs a metabolic cost to the plant, but clearly whether this occurs and when the transition to the symptomatic state takes place will have implications for yield formation as well as the measurement of tolerance. This is because biotrophic and necrotrophic infection can lead to a range physiological changes related to leaf carbon metabolism, including increased rates of respiration, reduced rates of net photosynthesis, alterations in stomatal conductance and chlorophyll concentrations and reductions in the amounts and activities of Calvin-Benson cycle enzymes (Roberts and Walters, 1988; Murray and Walters, 1992; Prats et al., 2006).

Tissue death associated with lesion development by either necrotrophic or hemi-biotrophic pathogens results in a loss of green area and some shrinkage of the leaf surface. The parasitic interactions too lead to premature loss of green leaf area. As symptomatic tissue continues to intercept and absorb a significant fraction of the incident PAR, the amount of radiation intercepted by healthy tissue is correspondingly reduced. The effects of disease on carbon metabolism described above can also reduce the efficiency of conversion of energy from absorbed PAR into dry matter production. In crop growth analysis, RUE is usually quantified from the slope of the relationship between above ground biomass gain and cumulative radiation interception (Bingham et al., 2007a,b). Thus, any effect of disease observed on RUE will be the net outcome of its effects on canopy photosynthesis, respiration and biomass partitioning between roots and shoot.

The impact of reductions in radiation interception and RUE on yield will depend on how disease influences assimilate partitioning and the source-sink balance of the crop. HI is measured at harvest as the final expression of dry matter allocation, but is determined over the course of the crop life cycle. It is influenced by the effects of genotype, crop management and environmental factors (including disease) on the relative growth of photosynthesizing (source) and yield bearing (sink) organs and the deposition and subsequent remobilization of temporary storage reserves. In determinate crops such as wheat and barley, vegetative growth prior to flowering determines the size of canopy produced and the number and potential storage capacity of grains. The number of grains is determined by the production and survival of tillers and the production, survival and fertilization of spikelets or florets. The potential storage capacity of grains has been related to the size of the carpel at flowering and the number of endosperm cells produced shortly after fertilization. The periods of tiller and spikelet/floret mortality and differentiation and growth of the carpel coincide with the phase of rapid stem extension and there is evidence that these processes are influenced by availability of assimilate during this time. Stem water soluble carbohydrate reserves are also deposited as the stem extends. Timing is critical. Thus, early disease epidemics which develop prior to flowering can simultaneously restrict both source (canopy healthy area and deposition of stem soluble carbohydrate reserves) and grain sink capacity (numbers and storage capacity of grains). Late disease epidemics, on the other hand, restrict assimilate availability for grain filling by reducing canopy healthy area and post-flowering photosynthesis. The negative effects of late disease on grain filling

may be buffered by the remobilization of temporary storage reserves.

Not all periods of the crop lifecycle are equally sensitive to abiotic or biotic stress (Ney et al., 2013). In cereals, stress that develops around flowering can be especially damaging to yield because of the irreversible reduction in grain sink capacity that can occur. For example in maize, water stress at flowering can result in the abortion of embryos and a permanent reduction in kernel number. The effect is associated with a reduction in photo-assimilate supply to the ear and can be prevented, in part, by the exogenous supply of sucrose (Zinselmeier et al., 1999). In summary, differences in these mechanisms that together affect yield will have different implications for tolerance to disease.

DISEASE TOLERANCE IN MONOCULTURES

Traits that enable radiation interception, RUE and dry matter partitioning to be maintained in spite of disease will minimize yield loss and hence confer tolerance of disease. Therefore, there are many potential tolerance traits that may operate at a range of organizational levels from the organ through to the crop (Ney et al., 2013). In addition, whether or not a particular trait or trait combination is identified as contributing to tolerance will depend on the techniques used to quantify disease and its relationship with yield (Bingham et al., 2009). Candidate traits conferring tolerance and the issues surrounding the measurement of tolerance have been discussed in detail elsewhere in the context of crop monocultures (Bingham and Newton, 2009; Bingham et al., 2009; Ney et al., 2013) and thus only a brief overview is given here.

The impact of fungal infection on net photosynthetic rates within an infected leaf can vary with both the pathosystem and location of the tissue relative to the disease lesion. There is some evidence of an increase in rate in symptomless regions of diseased leaves (Last, 1963; Habeshaw, 1984), although a reduction is a more common observation (Martin, 1986; Bastiaans, 1991; Scholes and Rolfe, 1995). Similarly, increased rates of photosynthesis in non-infected leaves of diseased plants have also been reported (Roberts and Walters, 1986; Rooney and Hoard, 1989; Murray and Walters, 1992). There have been few attempts to quantify the extent of intra-specific variation in these responses, although there is some limited evidence that intra-specific variation exists in the response of wheat leaves to septoria leaf blotch (Zuckerman et al., 1997). An increase in photosynthetic rate in apparently healthy tissue, made in response to the development of disease elsewhere on the plant, could lead to tolerance by compensating for the loss of healthy tissue and thus maintaining yield. However, for any particular pathosystem it would need to be established that the increase is indeed compensatory and results in carbon fixation that is used to support yield formation rather than just the biosynthesis of defense compounds (Tiffin, 2000). Morphological plasticity is another mechanism by which plants might restore photosynthetic capacity in response to defoliation. Although most widely documented for plants defoliated by herbivory,

reductions in the allocation of biomass to root growth relative to shoots and an increase in leaf area ratio have also been observed in several pathosystems involving foliar disease (Walters and Ayres, 1981; Paul and Ayres, 1986; Rooney and Hoad, 1989).

It has been postulated that cereals whose grain storage capacity (sink capacity) is small relative to their ability to supply grains with photosynthate during grain filling (source capacity) will be relatively tolerant of post-flowering disease (Gaunt, 1995; Bingham et al., 2009). There is evidence that the yield of many crops is sink-limited (Borrás et al., 2004), but that the extent of the source-sink imbalance varies widely between sites and years (Bingham et al., 2007a,b). This would suggest that tolerance of post-flowering disease might also vary widely between crops. Carbon assimilates for grain filling come from concurrent photosynthesis and the remobilization of temporary storage reserves, although the contribution of the latter differs between species. Intra-specific variation in the concentration of water soluble carbohydrate reserves in wheat has been reported, prompting speculation that genotypes with large reserves will be more tolerant of disease (Foulkes et al., 2002). However, direct evidence to support this has not yet been found.

Modeling of canopy photosynthesis in diseased crops suggests that canopy size and architecture are traits that may influence tolerance (Bingham and Topp, 2009). Large canopies and canopies with a relatively high light extinction coefficient were found to be relatively more tolerant of disease especially if disease was located in the lower canopy. This is because in those canopies most of the incident light is intercepted by the upper leaf layers and the lower-most leaves contribute little to canopy photosynthesis (Bingham and Topp, 2009).

QUANTIFYING TOLERANCE VARIATION

As many of the potential mechanisms conferring tolerance operate at the canopy level, measurements are generally made in field experiments (Parker et al., 2004; Foulkes et al., 2006; Bancal et al., 2015). Achieving equivalent disease severity across a range of genotypes is almost impossible under field conditions and so an approach is adopted in which disease severity is varied over a defined range by inoculation or by using fungicides as necessary. Tolerance can then be quantified as the change in yield per unit change in disease severity. The most common measurement of disease severity has been the Area Under the Disease Progress Curve (AUDPC) which integrates disease severity over time (Kramer et al., 1980; Newton et al., 1998). However, measurements of AUDPC provide no indication of the amount of healthy tissue remaining. As the relationship between canopy area and radiation interception is non-linear, variation in canopy size and hence residual green (healthy) area can have an appreciable effect on the reduction in crop growth or yield under a given disease severity (Bingham and Topp, 2009). Canopy growth is sensitive to variations in soil, climatic and crop management factors and this may contribute to the large variation observed in AUDPC-yield loss relationships and designations of tolerance for varieties across sites and seasons (Kramer et al., 1980; Johnson, 1987; Waggoner and Berger, 1987;

Newton et al., 1998, 2000). In order to minimize this problem in wheat and provide a more robust estimate of genotypic variation in tolerance across environments, post-anthesis healthy area duration has been used as a surrogate for disease severity as it links more directly with radiation capture (Parker et al., 2004; Ney et al., 2013).

Characteristics of plant-microbial interactions and host traits that might influence the designation of tolerance by modifying disease-yield loss relationships are categorized in a hierarchical way in **Table 1**: (1) asymptomatic and symptomatic microbial challenges resulting in differential effects on yield loss relationships by inoculum pressure / pathogen challenge and disease symptom expression variability; (2) yield compensation, facilitation and competition responses to disease and plant developmental responses; (3) protocol effects including the effects carried over from previous crop treatments, seed health or environments (epi-genetic) and of fungicide mode-of-action types favoring germplasm differentially either through direct physiological responses or differential effects on asymptomatic microbial infections / challenges. Most of these traits also show interaction with: (4) plant developmental stage, nutrients, environment / weather, abiotic stress etc., some of which might be expressed in terms of yield sensitivity, for example response to site fertility affecting varieties differentially (Finlay and Wilkinson, 1963).

As disease tolerance is defined and measured in terms of visible disease severity or a surrogate, the effects of asymptomatic microbial infection on plant growth and yield are particularly important. These may be classified as parasitic, mutualistic/beneficial or pathogenic and each state may be associated with different physiological interactions and therefore effects on host metabolic processes resulting in different effects on tolerance. Furthermore, for many plant-microbe interactions these interactions are dynamic and transition through a lifecycle. Hence these are divided into: (1a) asymptomatic challenge, either parasitic or mutualistic / beneficial, and (1b) symptomatic which is largely synonymous with pathogenic challenges (**Table 1**). The latter result in either hypersensitive resistance with minimal symptoms, some form of partial or non-hypersensitive resistance, or susceptibility. In addition to visual and other biomass assessment methods, defining molecular mechanism and specific gene expression profiling will be highly informative. The different response types will differ in expression levels of some pathways, for example lower defense pathway expression in non-pathogens. Equating these to energy or assimilate cost would have great potential for correlation with yield response. In molecular terms pathogen and non-pathogen responses are usefully classified as Pathogen-Associated Molecular Patterns (PAMPS) and Microbe-Associated Molecular Patterns (MAMPS) respectively (Newman et al., 2013). However, within each group, inoculum pressure will show its own dynamic interaction and is affected by the ability of each host to support sporulation. Sporulation can occur whether visible symptoms are present or not (Newton et al., 2010b) though it is likely to be greater in pathogenic interactions. Some varieties are likely to be carrying different microbial loads, not necessarily pathogens though. For example, the old cultivar Igri carries a different microbial population from most other

TABLE 1 | Groupings and types of mechanisms or factors that might impact disease / yield loss relationships in plant communities.

Group	Factors and mechanism	Impact on yield
(1a) Microbial asymptomatic infection	Parasitic Mutualistic/beneficial	-- -/+
(1b) Pathogen microbial challenge	Hypersensitive resistance (HR) Partial and non-HR resistance Susceptibility	-- -- -- -- -- --
(2) Developmental response to plant or microbial interaction / challenge	Compensation growth Facilitation response Competition response	++ ++ -/+
(3) Protocol effects	Previous crop legacies (e.g., microbial inoculum / anti-microbial substances) Plant physiological legacies (vigor etc.) Epigenetic legacies on plant physiology / gene expression Direct fungicide / agronomic treatment effects on plant physiology Indirect fungicide / agronomic treatment effects on microbial challenges Assessment methodologies	- -/+ -/+ + -/+ -/+ -/+
(4) Environmental modifiers of 1–3 above	Nutrient availability Weather / climate Abiotic stress (cold / drought / salt etc.) Soil (root stress, nutrient availability etc.)	-- -- -- --

Impact on yield scale from very negative to positive (– – –, – – –, – –, –, +) is arbitrary and dependent on appropriate measurement method for validation.

winter barleys (Gravouil, 2012). Germplasm identified with traits that affect the potential untreated yield loss may be due to fewer biotic interactions that cause induction of defense when this is not necessary, or selection for detrimental rather than beneficial microbial phylloplane populations.

The consequences of the microbial interactions are expressed in the second group (**Table 1**) that impacts yield loss relationships, i.e., the developmental response to plant or microbial interaction or challenge. Whilst these processes operate in monocultures (i.e., self-competition), their importance will be discussed more in the context of diversity.

Many apparent tolerance traits are responses to particular attributes of the experimental or growing protocols used, our third group (**Table 1**). These need some careful consideration if methodologies for detecting tolerance are to be developed. The rationale for good crop rotation practice is to maintain soil health described in terms of soil physical and microbial structure, nutrients and pathogens. These can include practices that induce shifts in the microbial spectrum including promotion of root exudates with anti-microbial properties. However, soil microbes are crucial not only to soil processes that then affect plant growth, but also many induce plant responses directly. The most studied are classed as Induced Systemic Resistance (ISR) whereby microbes such as *Pseudomonas* species induce specific defense pathways that make above-ground parts of the plant resistant to many pathogens (Kuc, 2001). Induction of resistance has energetic cost that must be considered in the overall defense strategy of the plant and will therefore impact

yield loss relationships. A good rotation keeps all these things in balance or within an acceptable range. However, when they are out of balance tolerance traits may be easier to identify (see below).

Another possible factor that may influence tolerance is a plant physiological legacy such as vigor. This could be simply related to seed resources such as endosperm size or composition. They could be also epigenetic legacies on plant physiology or gene expression and evidence is accumulating rapidly that these may be very common (Walters and Paterson, 2012; Pastor et al., 2013). The mechanisms are beginning to be identified together with the genetic loci controlling them (Luna et al., 2014). As these genes respond to environmental triggers, demonstrating their effect and relationship to tolerance is difficult but potentially very important both for agronomic management and financial benefit.

Assessing the effects of agronomic treatments such as the application of fungicides is often not as simple as determining the reduction in pathogens and subsequent disease. A fungicide application has effects on plants due to (1) the physical spray / formulation / adjuvant composition, and (2) mode of action, and each of these will impact both (a) the microbial population composition and (b) plant metabolic processes. The net result again affects apparent tolerance characteristics. For example prothioconazole and pyraclostrobin increase grain number in spring barley in the absence of disease whereas chlorothalonil did not (Bingham et al., 2014). Biostimulants, whether specific products, the indirect effects of resistance elicitors or indirect

effects of certain fungicide modes of action are even more likely to impact yield loss relationships and are another example of where molecular analyses of gene expression could be very helpful in understanding mechanisms (Lyon et al., 2014).

The fourth group (**Table 1**) are the modifiers of tolerance such as nutrient availability, the day-to-day weather, the general climate, abiotic stress such as cold, drought, salination, temperature shocks, wind, soil physical characteristics as well as the microbial composition referenced above causing root physical stress and affecting water and nutrient availability. The effects of all such factors can be profiled in many ways, not least gene expression. Wind, rain and other touch treatments for example, affect overall plant growth form and health and subsequently the plant's ability to respond to other challenges (Braam and Davis, 1990). There are many common stress-response genes and biochemical pathways and these are key to what might describe as healthy or normal plants (Newton et al., 2012b).

The importance of understanding what mechanisms are operating in plant-microbial interactions is to identify whether consequential changes in yield will affect visible disease symptoms and therefore the classical definition of tolerance.

PLANT-PLANT AND PLANT-PATHOGEN INTERACTIONS IN MIXED PLANT POPULATIONS

Clearly different cultivars can be classified as having different expressions of the factors and mechanisms that affect tolerance. Therefore, their accurate and appropriate assessment is necessary to determine whether their combined expression in mixtures is additive or synergistic. The component combinations that contribute most beneficially to tolerance in mixtures can be dissected-out. Facilitative plant-plant interactions are "positive, non-trophic interactions that occur between physiologically independent plants and that are mediated through changes in the abiotic environment or through other organisms" (Brooker et al., 2008). It is widely recognized and demonstrated that heterogeneous plant communities produce more total biomass than monocultures (Newton et al., 2009; Schöb et al., 2015). The interaction of two or more crop species growing together and co-existing for a time can result in more efficient resource use through niche differentiation and complementarity. This reduces negative competitive interactions through reduced niche overlap but also enables enhanced resource availability through direct facilitation, for example the secretion by some crop species of substances such as organic acids and phosphatases to increase P availability in acidic soils or N transfer from nitrogen-fixing legumes to companion species (summarized from Brooker et al., 2016). There can be more general effects too such as hydraulic lift causing increased water availability to all the plant community (Prieto et al., 2012). Brooker et al. (2016) also cite pollinator attraction and protection from pests and similar effects below-ground through increasing plant biomass or diversity enhancing the density or diversity of beneficial soil microbes.

In **Table 1** the interactions are classified as compensation, facilitation or competition but microbes are also a component of all these interactions, be they in the rhizosphere or the phyllosphere. The dynamics of pathogen populations and heterogeneous plants have been investigated in many studies and often characterized by population modulating characteristics. One of the best-known benefits resulting from enhanced niche complementarity through indirect facilitation is disease and pest control. The diverse components within the crop contribute in several ways to reducing overall pest and disease incidence, specifically (1) dilution of susceptible individuals or preferred hosts, (2) the barrier effect of resistant individuals, (3) induction of resistance in individuals neighboring infected plants (Chin and Wolfe, 1984), (4) changes in vegetation structure and microclimate affecting infection processes and (5) providing a more heterogeneous resource supply that supports a higher abundance and diversity of natural enemies of crop pests (i.e., associational resistance; Gunton, 2011; Letourneau et al., 2011). These processes operate at both inter- and intra-specific levels (Newton et al., 2009; Kiær et al., 2012). The first two processes are physical spatial effects whilst the others are physiological and biochemical effects and are dependent on the challenging organism's mode of pathogenicity or parasitology, population structure, plant architecture, development stage and physiology, and of course many environmental variables. Furthermore, where defense mechanism are induced there can be a metabolic cost so the trade-off against potential loss must be positive. Such effects are compounded in polycyclic diseases when pathogen inoculum pressure is reduced at each cycle. Such pest and disease resistance effects are examples of facilitation. However, these effects on disease are most obvious when there is a moderate pathogen challenge on the crop because they can be swamped by too much inoculum (Newton et al., 2002).

DISEASE TOLERANCE IN MIXTURES

Few attempts have been made to quantify the contribution of disease tolerance *per se* to the productivity of crop mixtures. In principle, individual genotypes in mixed populations might differ in their inherent tolerance via mechanisms discussed above that operate at the organ and plant level, although the expression of tolerance may conceivably be modified by external factors such as nutrition, solar radiation and plant-plant interactions (**Table 1**). If it is assumed that external factors have a minor influence or that each genotype is affected equally, then the tolerance of the mixture would be expected to be the same as the average of the tolerance of the individual components. For traits that operate at the crop level, on the other hand, such as canopy size and architecture (Ney et al., 2013), their influence on disease tolerance of the mixture will depend on the interactions between individuals and the spatial arrangements of leaves and disease within the canopy (Bingham and Topp, 2009). Where genotypes differ in their disease resistance, if the more susceptible genotypes have their leaves positioned lower in the canopy than the resistant ones, the impact of disease on canopy photosynthesis will be minimized and tolerance favored. The converse would be the case

if the susceptible genotypes are the tallest and disease epidemics develop in the upper canopy.

Further, in mixed populations of plants with differing disease resistance, negative competitive interactions are likely to occur as a result of niche overlap. Here disease developing on one or more components could shift the competitive balance in favor of the non-diseased components leading to stability of productivity of the population. If this is measured in terms of biomass production or yield per unit of disease over time it can be viewed as tolerance and would be equivalent to compensatory adjustments in assimilation or growth of new organs in a monoculture. In this case tolerance of the population is not dependent on maximizing tolerance of the individual genotypes within the mixture. Indeed if the dominant genotype is disease tolerant, then competition with other components may be maintained in spite of the disease and thus adjustments in growth of subordinate components reduced. The overall effect, however, would be one of tolerance within the mixture.

This concept begs the question—is there any value in seeking to maximize the tolerance of individual genotypes, if tolerance can be achieved with mixtures of genotypes with contrasting/complimentary disease resistance? In other words, tolerance in mixed populations comprises an additional set of traits and mechanisms from tolerance in self-populations. Other factors must be considered when trying to answer this question. The extent of the tolerance in a mixture will depend on the capacity of subordinate genotypes to increase their yield. However, a relief of competition and increase in resource capture by subordinate genotypes may not necessarily lead to an equivalent increase in yield if the plant is sink-limited and at a developmental stage at which it cannot increase its sink capacity in response to the increased resource availability. Potentially this is likely to be more of an issue when the mixed populations are composed of different species with contrasting resource use efficiencies in their formation of yield and the economic value of their harvested parts, as may be the case in some intercrops. Thus, protecting the yield of the most resource efficient and highest value component of the mixture through effective disease resistance and tolerance of the individual component may be more beneficial than relying on partial compensation for yield loss to disease within the mixture from other less efficient and lower value components. Similarly, if the yield advantage of a mixture in the absence of disease is dependent on facilitation mechanisms there may be merit in protecting the facilitator component by maximizing its individual disease tolerance or resistance so that the facilitation is sustained.

Yield loss relationships tend to fit a range of regression relationships that may compound multiple simultaneous relationships, some of which change behavior upon attaining thresholds. Mechanistically this is likely as inoculum-disease relationships often have such thresholds, classically expressed in quorum-sensing with bacterial diseases but expressed more incrementally in fungal diseases. The plant defense responses similarly have thresholds that must be exceeded before, for example, cell death processes are triggered as these are irreversible and costly to the plant. This may be reflected in the high cost of powdery mildew resistance caused by

Blumeria graminis f.sp. *hordei* and conferred by the *mlo* gene in barley as this is characterized by very early or fast recognition and response from a mutant regulatory gene (Piffanelli et al., 2002). Major gene resistance to Septoria Leaf Blight caused by *Zymoseptoria tritici* in wheat is similarly costly when effective and may have a mechanistic explanation also (Brown, 2002). In both cases these represent non-tolerance traits analogous to trait over-expression.

In mixtures losses in more diseased components will be compensated for partially by the less damaged components. However, other interactions between components may be contributing more to the mixture advantage through enhanced resource capture rather than compensating for loss. This is often true as even when there is a strong correlation between component number in the mixture and disease reduction that is reflected in yield, the same correlation is clear in the absence of disease suggesting that this the non-disease control interactions are dominant (Newton et al., 1997). Therefore, mixture advantage would be expected to be greater if this is the case when surplus resources are available such as under higher fertilizer rates. This is what is often found in practice (Newton et al., 2012a). The candidate traits are those that enable more of the available resources to be captured and/or for more time. This is clearly shown when contrasting canopy types are combined such as those expressing either, neither or both of the two common dwarfing genes in spring barley. Whereas, normally combinations of elite spring barley genotypes show small gains in the order of 0–3% above the mean of their components, 10% was achieved with three-component mixtures of these contrasting canopy type components (Newton et al., 2004).

EXPLOITATION OF DIVERSITY

Crops are communities of plants bred and grown in self-competition in a monoculture crop typical of much intensive agriculture. The fundamental approach is to express all desirable traits to their optimum state to produce ever improving yields. Diversity is exploited in this process, but only by selecting strong or extremes of desirable trait expressions. This approach is very successful for many traits so there is a tendency to assume that it will be successful in general and therefore applied to all traits. However, this may be a fundamentally flawed assumption for other traits, especially plant interactions with other complex communities of other organisms, particularly pathogen interactions but microbial interactions in general for the reasons outlined above.

Disease-reducing traits, whether specific resistance, non-specific resistance, or factors that affect infection and subsequent disease development, may have varying levels of expression. These can be classified as strong or weak expression such as classical major gene resistance and partial resistance to cereal rusts respectively. In a cultivar mixture the effects of either may increase with mixture proportion, but the maximum effect of the strong expression trait will be greater than the same proportion of the weak expression trait. These are represented by the straight diagonal lines in **Figure 1**. However, this also

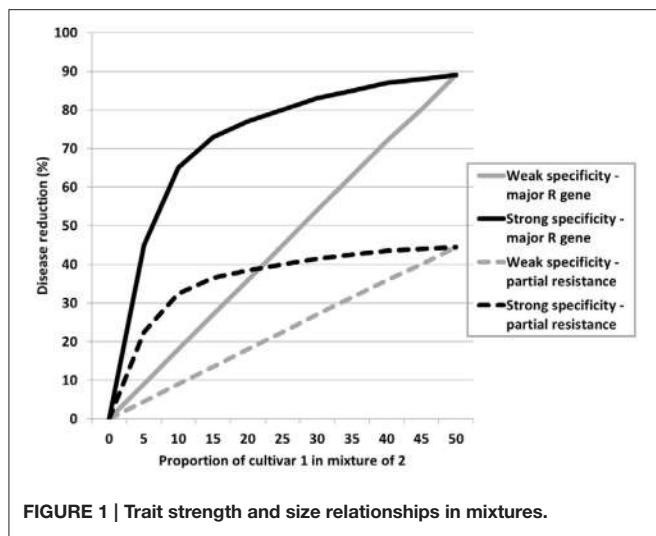


FIGURE 1 | Trait strength and size relationships in mixtures.

assumes specialism, i.e., each component cultivar expresses effective resistance only against a proportion of the pathogen population. Where this specificity is strong a small proportion of either cultivar will have a disproportionately large effect as represented by the curved lines in **Figure 1**. This is supported by both experimental data (Newton and Guy, unpublished data) and modeling (Mikaberidze et al., 2015). Generalizing this, if it is assumed that specificity strength is generally a measure of trait strength and major gene or partial resistance equates to the magnitude of the trait expression overall (size), then this can be applied to other traits to help design mixtures and predict outcomes. Thus, strong traits can be exploited in small proportions whatever their overall or maximum expression might be. Strong can be interpreted also as traits with contrasting expressions. Thus, canopy types such as tall, semi-prostrate, erectoid and double-dwarf conferred by the combinations of two dwarfing genes referred to in the last section are very strong and contrasting trait expressions and indeed combinations have strong positive interactions greater than the weighted means of their components in terms of yield benefit (Newton et al., 2004).

Before moving on to discuss these community plant-pathogen interactions more, It should be acknowledge also that the ideal trait assembly forming a very superior crop plant is rare and that by assembling different crop cultivars with different and complementary traits, overall crop performance can be enhanced. Elite germplasm developed and exploited under optimal agronomic and environmental conditions generally offers few opportunities for exploiting complementarity, be it through competition or facilitation, as most traits have very similar expressions. In any single year and on individual sites, single cultivars are likely to be the top performers, but it is unlikely that any one cultivar will be top on all sites and in all years. Under real farm conditions that are seldom uniformly optimal and across the years, heterogeneous assemblies of elite cultivars are likely to out-perform the mean of the components grown separately (Finckh et al., 2000; Newton et al., 2009; Kiær et al., 2012). However, the greater opportunities may come from associations with other crop species where many traits have

strong or highly contrasting expressions and the opportunities for complementation are much greater.

CROP DIVERSITY FROM THE MICROBIAL PERSPECTIVE

Very little is known about the non-pathogenic microbial component of these heterogeneous plant communities in the phyllosphere, though it is known that they enhance microbial diversity in the rhizosphere (Johnson et al., 1992; Lawrence et al., 2012). Another dimension can be added to this, that of pathogen/parasite-non pathogen interactions as these represent a complex spectrum of interactions ranging from hyper-parasitism (Kiss, 1997) to mutualism where the disease is caused or exacerbated by a microbial complex (Newton and Toth, 1999). However, focusing on the plant response, whether beneficial resources are supplied or damage is caused, plants respond to enhance their fecundity in ecological terms, though this may be distorted in crops (Newton et al., 2010a).

The focus on defense against disease is often driven from a highly anthropocentric rather than ecological point-of-view. Disease is assumed to be caused by pathogens and a classical “arms race” approach is often used to describe defense strategies. However, understanding the nature of plant-microbial interactions in a more ecological framework often leads to a more sustainable “soft power” or diplomatic approach. Disease is simply a particular outcome of a plant-microbe interaction with specific spatial and temporal parameters. In an ecological context the same plant and microbe may also exhibit mutualistic or parasitic interaction at other times or places. Overall both plant and microbe are likely to benefit from their association, but at any one time the balance may be skewed strongly toward one or the other. Essentially the relationships between plants and microbes are dynamic. However, in the case of crop plants where the economic yield component has been greatly enhanced. This presents a large substrate to the microbial community with a narrow range of expressions of plant defense mechanisms which is normally to the microbe’s advantage. Even then the association may be either pathogenic or parasitic depending whether the host is actively damaged using necrosis-inducing mechanisms such as Botrytis infection on lettuce, or simply drained of resources, the rust pathogens on cereals being a classic example of the latter (Browder, 1985). Pathogen communities often generate a reservoir of trait variation that can overcome plant defenses. However, a single genotype host generally has only a narrow range of expressions of defense and the only back-up defense is with replacement genotypes from the plant breeders. In a community of plants the back-up is in the plant community that is being constantly challenged and selected.

As plants in dynamic association deliver community benefits through competition and facilitation, so too microbes work in association to more effectively interact with their host. Examples of this are found in complex microbial infections where one organism may be the apparent “causal agent” but disease symptoms are the expression of several working together for mutual benefit, again through competition and facilitation

(Dewey et al., 1999). Microbes also deliver benefits to the overall plant-microbial interaction for both partners through component dynamic mutualist-pathogen-parasite interactions, i.e., competition and facilitation (Newton et al., 2010b). Put these together and a complex web of interactions is assembled comprising many, varied and dynamic competition and facilitation relationships.

MEASURING TOLERANCE IN MIXTURES

Tolerance in mixtures is potentially more complex and uses different mechanisms compared with monocultures, thus the task of identifying the contribution of individual components to tolerance and their response to modifying factors represents as a considerable challenge. Nevertheless, a greater understanding of tolerance and its contribution to resource use efficiency and yield stability of mixtures would allow a more rational approach (greater element of crop system design) to exploitation of crop diversity in disease management.

Tolerance is the combination or sum of several traits and their combination in plant communities, so how should their importance be ranked and how can their parameter range be calibrated or profiled? Using molecular biology terminology, the best strategy might be to use knock-outs and/or over-expression of key traits of factors that influence them. Only when particular traits expressions are removed or exaggerated will their contribution to the composite tolerance trait be strongly expressed and measurable, i.e., when the system is out of balance or unstable.

An example of over-expression is the effect of inoculum pressure and fertilizer on tolerance designations in spring barley (Newton et al., 2000). It was not possible to identify barley genotypes that were consistently tolerant across all trial

conditions. However, there was good agreement between the both low and high fertilizer conditions under high inoculum pressure and there was also good agreement between the low fertilizer conditions under both low and high inoculum pressure. There was also good agreement between high inoculum + high fertilizer and low inoculum + low fertilizer, in other words the more contrasting or over-/under-expression conditions resulted in stronger expression of the tolerance composite trait.

A second example involving inoculum pressure and tolerance is the effect on mixture efficacy. As noted above (Table 1), group 2 heterogeneous plant communities generally increase biomass production and decrease disease. Group 1b pathogenic and non-pathogenic biotic challenges balances the cost of defense with these interactions. However, whilst under high inoculum pressure mixtures consistently reduced relative disease less, an increased yield response did not necessarily follow (Newton et al., 2002). This is likely because the pathogen control effects in mixtures were not the dominant interaction leading to enhanced yield in these trials.

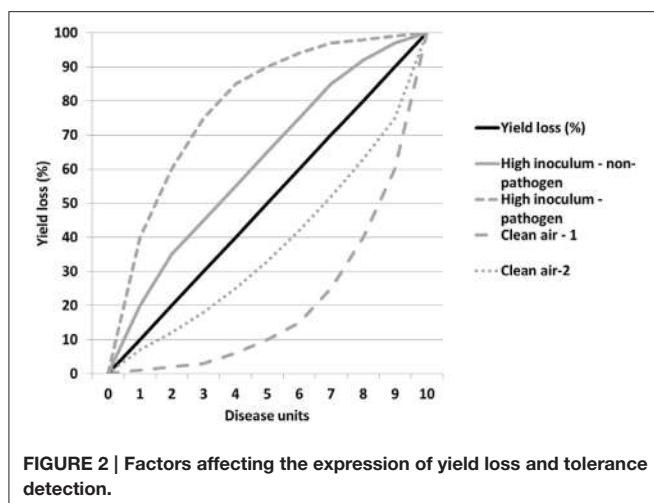
Designing “over-expression” and “knock-out” treatments that might be used to parameterize the expression of tolerance traits will be difficult from many points-of-view. The first will be designing the comparator. Although this should be “optimal” conditions, all conditions are in fact compromises and plants need to be exposed to a range of both biotic and abiotic conditions to grow “normally” and therefore arbitrary norms should be defined. Some parameters that might be manipulated experimentally could be over-expressed or strongly under-expressed / knocked-out, bearing in mind that they will likely have consequences for other parameters (Table 2). For example, providing a nutrient in excess or deficiency will likely affect uptake of other nutrients both directly and indirectly. Nevertheless, these conditions may help identify groups of germplasm with common trait expressions

TABLE 2 | “Over-expression” and “knock-out” treatments that might be used to identify factors that affect tolerance traits in plant communities.

Trait group	“Knock-out”	“Over-expression”	Comparator
Microbial challenge—airborne inoculum	Clean air; disinfected environment; inert microbe-free growing medium	Heavy / frequent inoculation; multiple species microbial challenges above- and below-ground, with pathogen / non-pathogen	“Optimal” ^a controlled environment; “normal” ^a field environment
Microbial challenge – waterborne inoculum	Clean water	High spore/mycelial concentration inoculation; multiple species microbial challenges above- and below-ground, with pathogen / non-pathogen	“Optimal” ^a controlled environment with low inoculum treatment; “normal” field environment
Water	Drought	Waterlogging	Field capacity
Temperature	Low / high mean	Heat / cold shock	“Optimal” controlled environment
Nutrient	Series of single and multiple nutrient deficiencies	Series of single and multiple nutrients in excess	“Optimal” fertilizer
Crop protectants / stimulants	Range of fungicide modes of action	Resistance elicitors and biostimulants with and without pathogen challenge ^b	Standard crop agronomic protocol or clean environment
Light	Low level light; short daylength	High intensity, wavelength-specific treatments combinations; long daylength	“Optimal” light in controlled environment or field
Atmosphere	Low CO ₂ concentration	High CO ₂ concentration, high ozone concentration	“Normal” atmospheric composition

^aArbitrary comparison or reference level.

^bPriming response only expressed with subsequent pathogen challenge.



behaving similarly as potential component traits of tolerance in mixtures.

The relationship between yield loss and disease may not be always linear, perhaps especially toward the extremes (Madden et al., 1981). Even if we assume it is, how knock-out and over-expression of influencing traits will affect this relationship may vary. **Figure 2** shows how a regression might change its slope positively or negatively in response to heavy inoculum pressure or the absence of any air-borne challenge compared with the norm. These relationships may equally fit a non-linear regression where the more extreme levels of disease have disproportionate effects, for example where plant defenses are triggered above certain inoculum thresholds resulting in a cost and risk to plant fecundity. Such a novel approach to identifying and characterizing tolerance using more extreme factor parameter values in evaluation environments should facilitate identification of key traits affecting tolerance, especially in crop mixtures where the dynamics are otherwise too complex to do so by more mechanistic means.

CONCLUSIONS

Given the complexity of the interactions in mixtures and the effects of modifiers on expression of tolerance, applying concepts

REFERENCES

- Bancal, P., Bancal, M. O., Collin, F., and Gouache, D. (2015). Identifying traits leading to tolerance of wheat to *Septoria tritici* blotch. *Field Crops Res.* 180, 176–185. doi: 10.1016/j.fcr.2015.05.006
- Bastiaans, L. (1991). The ratio between virtual and visual lesion size as a measure to describe reduction in leaf photosynthesis of rice due to leaf blast. *Phytopathology* 81, 611–615. doi: 10.1094/Phyto-81-611
- Bingham, I. J., Blake, J., Foulkes, M. J., and Spink, J. (2007a). Is barley yield in the UK sink limited? I. Post-anthesis radiation interception, radiation use efficiency and source-sink balance. *Field Crops Res.* 101, 198–211. doi: 10.1016/j.fcr.2006.11.005
- Bingham, I. J., Blake, J., Foulkes, M. J., and Spink, J. (2007b). Is barley yield in the UK sink limited? II. Factors affecting potential grain size. *Field Crops Res.* 101, 212–220. doi: 10.1016/j.fcr.2006.11.004
- Bingham, I. J., and Newton, A. C. (2009). “Crop tolerance of foliar pathogens: possible mechanisms and potential for exploitation,” in *Disease Control in Crops – Biological and Environmentally Friendly Approaches*, ed D. Walters (Chichester, UK: Wiley-Blackwell), 142–161. doi: 10.1002/9781444312157.ch7
- Bingham, I. J., and Topp, C. F. E. (2009). Potential contribution of selected canopy traits to the tolerance of foliar disease by spring barley. *Plant Pathol.* 58, 1010–1020. doi: 10.1111/j.1365-3059.2009.02137.x
- Bingham, I. J., Walters, D. R., Foulkes, M. J., and Paveley, N. D. (2009). Crop traits and the tolerance of wheat and barley to foliar disease. *Ann. Appl. Biol.* 154, 159–173. doi: 10.1111/j.1744-7348.2008.00291.x
- Bingham, I. J., Young, C. S., Bounds, P., and Paveley, N. D. (2014). How do fungicides increase yield of spring barley when

developed for monocultures to mixtures may not identify the traits responsible. It may be better to consider resilience of the system as a whole and not to adopt only the reductionist approach of trying to improve tolerance through trait selection in monocultures. Resilience would encompass restricting disease development and enhancing yield stability of the mixture rather than focusing on the tolerance traits of individual components.

Whether a crop mixture is more tolerant than a monoculture is the outcome of many plant and microbe community dynamic responses operating under a range of biotic and abiotic challenges. Such variable conditions are a normal part of the environment and required for normal plant development, but it is the extremes conditions, both high and low, that reveal the traits most influential on plant community tolerance. It is unlikely therefore that tolerance can be assessed or selected under normal field trial conditions where treatments tend toward the optimal. Furthermore, it is on-farm performance where conditions are more often sub-optimal where tolerance can be best exploited and therefore where the traits most favored need to be identified and optimized.

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The author confirms being the main contributor of this work and approved it for publication.

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- disease is low or absent? *Proc. Crop Protect. North. Br.* 2014, 77–82.
- Borrás, L., Slafer, G. A., and Otegui, M. E. (2004). Seed dry weight response to source–sink manipulations in wheat, maize and soybean: a quantitative reappraisal. *Field Crops Res.* 86, 131–146. doi: 10.1016/j.fcr.2003.08.002
- Braam, J., and Davis, R. W. (1990). Rain-, wind-, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis*. *Cell* 60, 257–364. doi: 10.1016/0092-8674(90)90587-5
- Brooker, R. W., Maestre, F. T., Callaway, R. M., Lortie, C. L., Cavieres, L. A., Kunstler, G., et al. (2008). Facilitation in plant communities: the past, the present, and the future. *J. Ecol.* 96, 18–34. doi: 10.1111/j.1365-2745.2007.01295.x
- Brooker, R. W., Karley, A. J., Newton, A. C., Pakeman, R. J., and Schöb, C. (2016). Facilitation and sustainable agriculture: a mechanistic approach to reconciling crop production and conservation. *Funct. Ecol.* 30, 98–107. doi: 10.1111/1365-2435.12496
- Brown, J. K. M. (2002). Yield penalties of disease resistance in crops. *Curr. Opin. Plant Biol.* 5, 339–344. doi: 10.1111/1365-2435.12496
- Browder, L. E. (1985). Parasite: host: environment specificity in the cereal rusts. *Ann. Rev. Phytopath.* 23, 201–222. doi: 10.1146/annurev.py.23.090185.001221
- Chin, K. M., and Wolfe, M. S. (1984). The spread of *Erysiphe graminis* f. sp. hordei in mixtures of barley varieties. *Plant Pathol.* 33, 89–100. doi: 10.1111/j.1365-3059.1984.tb00592.x
- Dewey, F. M., Wong, Y., Seery, R., Hollins, T. W., and Gurr, S. J. (1999). Bacteria associated with *Stagonospora (Septoria) nodorum* increase pathogenicity of the fungus. *New Phytol.* 144, 489–497. doi: 10.1046/j.1469-8137.1999.00542.x
- Finckh, M. R., Gacek, E. S., Goyeau, H., Lannou, C., Merz, U., Mundt, C. C., et al. (2000). Cereal variety and species mixtures in practice, with emphasis on disease resistance. *Agron. Plant Gen. Br.* 20, 813–837. doi: 10.1051/agro:2000177
- Finlay, K. W., and Wilkinson, G. N. (1963). The analysis of adaptation in a plant breeding programme. *Austral. J. Agric. Res.* 14, 742–754. doi: 10.1071/AR9630742
- Foulkes, M. J., Scott, R. K., and Sylvester-Bradley, R. (2002). The ability of wheat cultivars to withstand drought in UK conditions: formation of grain yield. *J. Agric. Sci.* 138, 153–169. doi: 10.1017/s0021859601001836
- Foulkes, M. J., Paveley, N. D., Worland, A., Welham, S. J., Thomas, J., and Snape, J. W. (2006). Major genetic changes in wheat with potential to affect disease tolerance. *Phytopathology* 96, 680–688. doi: 10.1094/PHYTO-96-0680
- Gaunt, R. E. (1995). The relationship between plant disease severity and yield. *Ann. Rev. Phytopathol.* 33, 119–144. doi: 10.1146/annurev.py.33.090195.001003
- Gravouil, C. (2012). *Identification of the Barley Phyllosphere and Characterisation of Manipulation Means of the Bacteriome Against Leaf Scald and Powdery Mildew*. Ph.D Thesis, University of Nottingham.
- Gunton, R. M. (2011). Integrating associational resistance into arable weed management. *Agric. Ecosys. Environ.* 142, 129–136. doi: 10.1016/j.agee.2011.05.022
- Habeshaw, D. (1984). “Effects of pathogens on photosynthesis,” in *Plant Diseases: Infection Damage and Loss*, eds R. K. S. Wood and G. J. Jellis (Oxford, UK: Blackwell Scientific Publications), 63–72.
- Johnson, K. B. (1987). Defoliation, disease and growth: a reply. *Phytopathology* 77, 1495–1497.
- Johnson, N. C., Copeland, P. J., Crookston, R. K., and Pfleger, F. L. (1992). Mycorrhizae: possible explanation for yield decline with continuous corn and soybean. *Agron. J.* 84, 387. doi: 10.2134/agronj1992.00021962008400030007x
- Kiær, L. P., Skovgaard, Ib. M., and Østergaard, H. (2012). Effects of inter-varietal diversity, biotic stresses and environmental productivity on grain yield of spring barley variety mixtures. *Euphytica* 185, 123–138. doi: 10.1007/s10681-012-0640-1
- Kiss, L. (1997). Genetic diversity in Ampelomyces isolates, hyperparasites of powdery mildew fungi, inferred from RFLP analysis of the rDNA ITS region. *Mycol. Res.* 101, 1073–1080. doi: 10.1017/S0953756297003705
- Kramer, T., Gildemacher, B. H., van der Ster, M., and Parlevliet, J. E. (1980). Tolerance of spring barley cultivars to leaf rust. *Euphytica* 29, 209–216. doi: 10.1007/BF00025116
- Kuć, J. (2001). Concepts and direction of induced systemic resistance in plants and its application. *Eur. J. Plant Pathol.* 107, 7–12. doi: 10.1023/A:1008718824105
- Last, F. T. (1963). Metabolism of barley leaves inoculated with *Erysiphe graminis* Marchal. *Ann. Bot.* 27, 685–690.
- Lawrence, D., Fiegna, F., Behrends, V., Bundy, J. G., Phillimore, A. B., Bell, T., et al. (2012). Species interactions alter evolutionary responses to a novel environment. *PLoS Biol.* 10:e1001330. doi: 10.1371/journal.pbio.1001330
- Letourneau, D., Armbrecht, I., Salguero Rivera, B., Lerma, J. M., Jimenez Carmona, E., Constanza Daza, M., et al. (2011). Does plant diversity benefit agroecosystems? A synthetic review. *Ecol. Appl.* 21, 9–21. doi: 10.1890/09-2026.1
- Luna, E., López, A., Kooiman, J., and Ton, J. (2014). Role of NPR1 and KYP in long-lasting induced resistance by β-aminobutyric acid. *Front. Plant Sci.* 5:184. doi: 10.3389/fpls.2014.00184
- Lyon, G. D., Newton, A. C., and Walters, D. R. (2014). “Induced resistance in crop protection: the future, drivers and barriers,” in *Induced Resistance for Plant Defence: A Sustainable Approach to Crop Protection*, 2nd Edn, eds D. Walters, G. D. Lyon and A. C. Newton (Oxford, UK: Blackwell Science), 316–325.
- Madden, L. V., Pennypacker, S. P., Antle, C. E., and Kingsolver, C. H. (1981). A loss model for crops. *Phytopathology* 71, 685–689. doi: 10.1094/Phyto-71-685
- Martin, P. J. (1986). Gaseous exchange studies of barley leaves infected with *Rhynchosporium secalis* (Oudem). *Phys. Mol. Plant Pathol.* 28, 3–14. doi: 10.1016/S0048-4059(86)80003-0
- Mikaberidze, A., McDonald, B. A., and Bonhoeffer, S. (2015). Developing smarter host mixtures to control plant disease. *Plant Pathol.* 64, 996–1004. doi: 10.1111/ppa.12321
- Monteith, J. L. (1977). Climate and the efficiency of crop production in Britain. *Philos. Trans. R. Soc. Lond.* 281, 277–294. doi: 10.1098/rstb.1977.0140
- Murchie, E. H., Pinto, M., and Horton, P. (2009). Agriculture and the new challenges for photosynthesis research. *New Phytol.* 181, 532–552. doi: 10.1111/j.1469-8137.2008.02705.x
- Murray, D., and Walters, D. R. (1992). Increased photosynthesis and resistance to rust infection in upper, uninfected leaves of rusted broad bean (*Vicia faba* L.). *New Phytol.* 120, 235–242. doi: 10.1111/j.1469-8137.1992.tb05659.x
- Newman, M. A., Sundelin, T., Nielsen, J. T., and Erbs, G. (2013). MAMP (microbe-associated molecular pattern) triggered immunity in plants. *Front. Plant Sci.* 4:139. doi: 10.3389/fpls.2013.00139
- Newton, A. C., Ellis, R. P., Hackett, C. A., and Guy, D. C. (1997). The effect of component number on *Rhynchosporium secalis* infection and yield in mixtures of winter barley cultivars. *Plant Pathol.* 46, 930–938. doi: 10.1046/j.1365-3059.1997.d01-83.x
- Newton, A. C., Thomas, W. T. B., Guy, D. C., and Gaunt, R. (1998). The interaction of fertiliser treatment with tolerance to powdery mildew in spring barley. *Field Crops Res.* 55, 45–56. doi: 10.1016/S0378-4290(97)00096-8
- Newton, A. C., and Toth, I. K. (1999). Helper bacteria and pathogenicity assessments. *New Phytol.* 144, 385–386. doi: 10.1046/j.1469-8137.1999.00527.x
- Newton, A. C., Guy, D. C., Gaunt, R. E., and Thomas, W. T. B. (2000). The effect of powdery mildew inoculum pressure and fertiliser level on disease tolerance in spring barley. *J. Plant Dis. Prot.* 107, 67–73.
- Newton, A. C., Guy, D. C., Nadziak, J., and Gacek, E. (2002). The effect of inoculum pressure, germplasm selection and environment on spring barley cultivar mixtures efficacy. *Euphytica* 125, 325–335. doi: 10.1023/A:1016052121581
- Newton, A. C., Swanston, J. S., and Guy, D. (2004). “Enhanced durability and utility of genes for resistance by deployment in cultivar mixtures,” in *Proceedings Molecular Plant-Microbe Interactions XI*, (St Petersburg), 240–243.
- Newton, A. C., Begg, G., and Swanston, J. S. (2009). Deployment of diversity for enhanced crop function. *Ann. Appl. Biol.* 154, 309–322. doi: 10.1111/j.1744-7348.2008.00303.x
- Newton, A. C., Gravouil, C., and Fountaine, J. M. (2010a). Managing the ecology of foliar pathogens: ecological tolerance in crops. *Ann. Appl. Biol.* 157, 343–359. doi: 10.1111/j.1744-7348.2010.00437.x
- Newton, A. C., Fitt, B. D. L., Atkins, S. D., Walters, D. R., and Daniell, T. (2010b). Pathogenesis, mutualism and parasitism in the trophic space of microbe-plant interactions. *Trends Microbiol.* 18, 365–373. doi: 10.1016/j.tim.2010.06.002
- Newton, A. C., Guy, D. C., Bengough, A. G., Gordon, D. C., McKenzie, B. M., Sun, B., et al. (2012a). Soil tillage effects on the efficacy of cultivar and their mixtures in winter barley. *Field Crops Res.* 128, 91–100. doi: 10.1016/j.fcr.2011.12.004

- Newton, A. C., Torrance, L., Holden, N., Toth, I., Cooke, D. E. L., Blok, V., et al. (2012b). Climate change and defence against pathogens in plants. *Adv. Appl. Microbiol.* 81, 89–132. doi: 10.1016/B978-0-12-394382-8.00003-4
- Ney, B., Bancal, M. O., Bancal, P., Bingham, I. J., Foulkes, J., Gouache, D., et al. (2013). Crop architecture and crop tolerance to fungal diseases and insect herbivory. Mechanisms to limit crop losses. *Eur. J. Plant Pathol.* 135, 561–580. doi: 10.1007/s10658-012-0125-z
- Parker, S. R., Welham, S., Paveley, N. D., Foulkes, J., and Scott, R. K. (2004). Tolerance of septoria leaf blotch in winter wheat. *Plant Pathol.* 53, 1–10. doi: 10.1111/j.1365-3059.2004.00951.x
- Pastor, V., Luna, E., Maunch-Mani, B., Ton, J., and Flors, V. (2013). Primed plants do not forget. *Environ. Exp. Bot.* 94, 46–56. doi: 10.1016/j.envexpbot.2012.02.013
- Paul, N. D., and Ayres, P. G. (1986). The effects of infection by rust (*Puccinia lagenophorae* Cooke) on the growth of groundsel (*Senecio vulgaris* L.) cultivated under a range of nutrient concentrations. *Ann. Bot.* 58, 321–331.
- Paveley, N. D., Sylvester-Bradley, R., Scott, R. K., Craigon, J., and Day, W. (2001). Steps in predicting the relationship of yield on fungicide dose. *Phytopathology* 91, 708–716. doi: 10.1094/PHYTO.2001.91.7.708
- Piffanelli, P., Zhou, F., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., et al. (2002). The barley MLO modulator of defense and cell death is responsive to biotic and abiotic stress stimuli. *Plant Physiol.* 129, 1076–1085. doi: 10.1104/pp.010954
- Prats, E., Gay, A. P., Mur, L. A. J., Thomas, B. J., and Carver, T. L. W. (2006). Stomatal lock-open, a consequence of epidermal cell death, follows transient suppression of stomatal opening in barley attacked by *Blumeria graminis*. *J. Exp. Bot.* 57, 2211–2226. doi: 10.1093/jxb/erj186
- Prieto, I., Armas, C., and Pugnaire, F. I. (2012). Water release through plant roots: new insights into its consequences at the plant and ecosystem level. *New Phytol.* 193, 830–841. doi: 10.1111/j.1469-8137.2011.04039.x
- Rabbinge, R., Jorritsma, I. T. M., and Schans, J. (1985). Damage components of powdery mildew in winter wheat. *Neth. J. Plant Pathol.* 91, 235–247. doi: 10.1007/BF01997967
- Reynolds, M. P., Pellegrineschi, A., and Skovmand, B. (2005). Sink-limitation to yield and biomass: a summary of some investigations in spring wheat. *Ann. Appl. Biol.* 146, 39–49. doi: 10.1111/j.1744-7348.2005.03100.x
- Roberts, A. M., and Walters, D. R. (1986). Stimulation of photosynthesis in uninfected leaves of rust-infected leeks. *Ann. Bot.* 56, 893–896.
- Roberts, A. M., and Walters, D. R. (1988). Photosynthesis in discrete regions of leek infected with rust, *Puccinia allii*. *New Phytol.* 110, 371–376. doi: 10.1111/j.1469-8137.1988.tb00274.x
- Robert, C., Bancal, M.-O., Nicolas, P., Lannou, C., and Ney, B. (2004). Analysis and modelling of effects of leaf rust and *Septoria tritici* blotch on wheat growth. *J. Exp. Bot.* 55, 1079–1094. doi: 10.1093/jxb/erh108
- Rooney, J. M., and Hoad, G. V. (1989). Compensation in growth and photosynthesis of wheat (*Triticum aestivum* L.) following early inoculations with *Septoria nodorum* (Berk.) Berk. *New Phytol.* 113, 513–521. doi: 10.1111/j.1469-8137.1989.tb00363.x
- Schafer, J. F. (1971). Tolerance to plant disease. *Ann. Rev. Phytopathol.* 9, 235–252. doi: 10.1146/annurev.py.09.090171.001315
- Scholes, J. D., and Rolfe, S. A. (1995). How do biotrophic pathogens affect the photosynthetic metabolism of their hosts? *Asp. Appl. Biol.* 42, 91–99.
- Schöb, C., Kerle, S., Karley, A. J., Morcillo, L., Pakeman, R. J., Newton, A. C., et al. (2015). Intra-specific genetic and composition modify species-level diversity-productivity relationships. *New Phytol.* 205, 720–730. doi: 10.1111/nph.13043
- Scholes, J. D., and Rolfe, S. A. (2009). Chlorophyll fluorescence imaging as a tool for understanding the impact of fungal diseases on plant performance: a phenomics perspective. *Funct. Plant Biol.* 36, 880–892. doi: 10.1071/FP09145
- Tiffin, P. (2000). Mechanisms of tolerance to herbivore damage: what do we know? *Evol. Ecol.* 14, 523–536. doi: 10.1023/A:1010881317261
- Van Oijen, M. (1990). Photosynthesis is not impaired in healthy tissue of blighted potato plants. *Neth. J. Plant Pathol.* 96, 55–63. doi: 10.1007/BF02005129
- Waggoner, P. E. (1990). “Assembling and using models of epidemics,” in *Epidemics of Plant Disease. Mathematical Analysis and Modeling*, 2nd Edn., ed J. Kranz (Berlin; Heidelberg; New York, NY; Springer), 230–260.
- Waggoner, P. E., and Berger, R. D. (1987). Defoliation, disease and growth. *Phytopathology* 77, 393–398.
- Walters, D. R., and Ayres, P. G. (1981). Growth and branching pattern of roots of barley infected with powdery mildew. *Ann. Bot.* 47, 159–162.
- Walters, D. R., and Paterson, L. (2012). Parents lend a helping hand to their offspring in plant defence. *Biol. Lett.* 8, 871–873. doi: 10.1098/rsbl.2012.0416
- Zinselmeier, C., Jeong, B.-R., and Boyer, J. S. (1999). Starch and the control of kernel number in maize at low water potential. *Plant Physiol.* 121, 25–35. doi: 10.1104/pp.121.1.25
- Zuckerman, E., Eshel, A., and Eyal, Z. (1997). Physiological aspects related to tolerance of spring wheat cultivars to *Septoria tritici* blotch. *Phytopathology* 87, 60–65. doi: 10.1094/PHYTO.1997.87.1.60

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Silicon: Potential to Promote Direct and Indirect Effects on Plant Defense Against Arthropod Pests in Agriculture

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Silicon has generally not been considered essential for plant growth, although it is well recognized that many plants, particularly Poaceae, have substantial plant tissue concentrations of this element. Recently, however, the International Plant Nutrition Institute [IPNI] (2015), Georgia, USA has listed it as a “beneficial substance”. This reflects that numerous studies have now established that silicon may alleviate both biotic and abiotic stress. This paper explores the existing knowledge and recent advances in elucidating the role of silicon in plant defense against biotic stress, particularly against arthropod pests in agriculture and attraction of beneficial insects. Silicon confers resistance to herbivores via two described mechanisms: physical and biochemical/molecular. Until recently, studies have mainly centered on two trophic levels; the herbivore and plant. However, several studies now describe tri-trophic effects involving silicon that operate by attracting predators or parasitoids to plants under herbivore attack. Indeed, it has been demonstrated that silicon-treated, arthropod-attacked plants display increased attractiveness to natural enemies, an effect that was reflected in elevated biological control in the field. The reported relationships between soluble silicon and the jasmonic acid (JA) defense pathway, and JA and herbivore-induced plant volatiles (HIPVs) suggest that soluble silicon may enhance the production of HIPVs. Further, it is feasible that silicon uptake may affect protein expression (or modify proteins structurally) so that they can produce additional, or modify, the HIPV profile of plants. Ultimately, understanding silicon under plant ecological, physiological, biochemical, and molecular contexts will assist in fully elucidating the mechanisms behind silicon and plant response to biotic stress at both the bi- and tri-trophic levels.

Keywords: herbivore, HIPV, effector proteins, insect-plant interactions, trophic interactions, resistance mechanisms, omics, systems biology

INTRODUCTION

Silicon and the Soil

Silicon is the second most abundant element, after oxygen, in the Earth's crust and in the soil solution (Epstein, 1994). It is mainly present in the soil solution in the form of silicic acid, H_4SiO_4 , since this is the only form of water-soluble silicon. Soil concentrations typically range from 0.1 to 0.6 mM (Epstein, 1994). This concentration range is similar to that of major inorganic nutrients including potassium, calcium, and sulfate in the soil solution (Epstein, 1972). Several factors influence soil silicon availability to plants, including soil type, parent material, land use, organic matter, temperature, soil pH, and texture (Liang et al., 1994; Alexandre et al., 1997; Struyf et al., 2010; Cornelis et al., 2011; Han et al., 2011; Miles et al., 2014; Anda et al., 2015).

Silicon and Plants

Silicon is taken up by plants via the transpiration stream (i.e., passive uptake) and is transported from the roots to the shoots as monosilicic acid, where it is deposited as solid, amorphous, hydrated plant silica ($\text{SiO}_2 \cdot n\text{H}_2\text{O}$; Jones and Handreck, 1967). Once deposited, silicon is not remobilized (Raven, 1983). Silicon is transported in the plant through the xylem via apoplastic transport (Raven, 1983) and must remain in solution (i.e., remain unpolymerized) during this passage; however the mechanisms preventing polymerization are not well understood (Epstein, 1994). Active silicon uptake is exhibited by some plant species including rice *Oryza sativa* L. (Takahashi et al., 1990; Henriet et al., 2006; Liang et al., 2006), as is rejective uptake (i.e., uptake at rates lower than passive; Takahashi et al., 1990). The existence of these processes indicates that, in some plant taxa at least, plant silicon levels are actively manipulated. Selection pressure for the evolution of active silicon uptake and metabolism is evident in the beneficial effects of silicon to plants under abiotic and biotic stress. However, silicon has not generally been recognized as an essential plant nutrient, though recently the International Plant Nutrition Institute [IPNI] (2015), Georgia, USA listed silicon as a "beneficial substance" (International Plant Nutrition Institute [IPNI], 2015).

The positive effects of silicon against abiotic and biotic stress are not always obvious since the extent of silicon accumulation differs among plant species and cultivars (Deren, 2001; Mitani and Ma, 2005; Keeping and Reynolds, 2009). Terrestrial plants have tissue concentrations of silicon, ranging from 1 to 15% dry weight (Epstein, 1994), with a very irregular distribution among the plant kingdom (Epstein, 1999). In agricultural systems, silicon is applied as a crop protection treatment and this is the major focus of this review. Major crops that respond to silicon application include some monocotyledonous plants such as rice, maize, *Zea mays* L., and wheat, *Triticum aestivum* L., that actively absorb and accumulate high amounts of silicon, together with some dicotyledonous crops such as cotton (*Gossypium hirsutum* L.), soybean [*Glycine max* (L.) Merr.], some vegetables (e.g., cucurbits) and fruit crops (e.g., tomato (*Lycopersicon esculentum* Mill.) that accumulate silicon through specific transporters

(Liang et al., 2015). While it is well documented that sugarcane responds strongly to silicon fertilization, active absorption of silicon has not been demonstrated and an active transporter has not yet been found. More recently, high-throughput sequencing and easier access to genomic data has enabled accurate determination of the ability of a plant to accumulate silicon, based on its genetic predisposition (Liang et al., 2015).

Until the discovery of specific genes involved in silicon uptake, silicon accumulation in plants was little understood. These silicon transporter genes, influx and efflux (LSi1 and LSi2, respectively), responsible for silicon uptake by the roots were first described in rice (Ma et al., 2006, 2007). Homologs are now reported in barley, *Hordeum vulgare* L., maize, and wheat (Chiba et al., 2009; Mitani et al., 2009a,b; Montpetit et al., 2012), with pumpkin, *Cucurbita moschata*, Poir. the first dicot to record a gene encoding a silicon influx transporter, LSi1 (Mitani et al., 2011) and two efflux transporters, CmLSi2-1 and CmLSi2-2 (Mitani-Ueno et al., 2011) followed by two putative influx silicon transporter genes (GmNIP2-1 and GmNIP2-2) in soybean (Deshmukh et al., 2013) and cucumber (CSiT-1, CSiT-2; Wang et al., 2015). An influx transporter has also been identified in the primitive plant, horsetail, *Equisetum arvense* L. (Gégoire et al., 2012). A silicon influx transporter, LSi6, present in the root tips, leaf sheaths and leaf blades has also been identified in several graminaceous species, including rice, and is responsible for xylem unloading of silicon (Yamaji et al., 2008).

Silicon and Stress

The beneficial effects of silicon application on plant growth and crop yield are well documented (for a recent review see Guntzer et al., 2012), but it is in the mitigation of both abiotic and biotic plant stresses, where the application of silicon demonstrates its real potential (Keeping and Reynolds, 2009). Notably, biochemical or molecular responses (and frequently growth/yield responses) due to silicon fertilization, are usually not apparent unless in the presence of a biotic (or abiotic) stressor. Studies have shown resistance to a range of abiotic stress factors including drought and salinity stress, heavy metal toxicity, excess nitrogen and phosphorous, and lodging (for a recent review see Liang et al., 2015). Biotic stressors may come in the form of plant pathogens, including fungi, bacteria, viruses, and animals (vertebrate and arthropod herbivores). Defense against biotic stress, has centered around two main mechanisms, mechanical (physical), and biochemical or molecular.

There is a dominance of work on fungal pathogens, compared with other disease-causing agents. Those fungal pathogens defined as biotrophic or hemibiotrophic, including the powdery mildews and blast fungus (*Magnaporthe grisea* (T.T. Hebert) M.E. Barr), appear to be better controlled by silicon than are necrotrophs (Liang et al., 2015). The reasons for this are increasingly becoming apparent, with a recent study showing that while silicon contributes to *Arabidopsis* defense priming following pathogen infection, that silicon will confer protection even when priming is altered, indicating other mechanisms may be involved (Vivancos et al., 2015). Evidence suggests that silicon may interfere with effector proteins released by these pathogens,

permitting the plant to mount better defense reactions (Vivancos et al., 2015). Other work has confirmed the role of silicon in priming plants in plant–pathogen interactions (Fauteux et al., 2005; Chain et al., 2009; Van Bockhaven et al., 2013). It is thought that the work on silicon and effector proteins may assist in developing a unifying theory around the mode of action of silicon in alleviating biotic stresses (Vivancos et al., 2015). A recent, comprehensive review of silicon and plant–pathogen interactions in agriculture is provided by Liang et al. (2015).

Vertebrate herbivores are probably the least studied biotic stressors, against which silicon provides some protection, and research in this area has largely focused on natural ecological systems. We briefly review this field because it has some relevance to arthropod pests given that plant defenses are at the heart of the phenomenon. The majority of studies have been on field voles, *Microtus agrestis* L (Rodentia: Cricetidae), showing reductions in the body weight and growth rate of juveniles and adults when fed on silicon-treated grasses (Massey and Hartley, 2006; Massey et al., 2008). Recent laboratory work demonstrated that grasses employ several defense strategies against *M. agrestis* including silicon, endophytes, and secondary metabolites (Huitu et al., 2014). It is hypothesized that induction of silicon-based plant defense in response to herbivore damage may influence rodent population cycles (Massey et al., 2008). In sites where *M. agrestis* population density was high, silica levels in the leaves of their food plant, collected several months later were also high and vole populations afterward declined, while population density increased where vole population density was initially low and silicon levels were also low (Massey et al., 2008). A key food species, *Deschampsia cespitosa* L., of *M. agrestis* exhibits a delayed defensive response to grazing by increasing silica concentrations (Reynolds et al., 2012). Further, the authors presented theoretical modeling that predicts that this response alone could lead to population cycles observed in *M. agrestis* and in other graminivorous rodent populations, where populations that reach sufficiently high densities can induce silica defenses in their food source.

Studies on the root vole, *Microtus oeconomus* (Pallas, 1776), have shown that changes in the silicon content of tussock sedges may be induced by variations in vole population densities (Wieczorek et al., 2015). However, no correlation was shown between the silicon content in the faeces of *M. oeconomus* and survival rate (Wieczorek et al., 2015). A very recent study in Poland demonstrated that the amount of silica in plants, fed upon by voles, leaves a traceable record in their dental microwear textures, and that these differ through different phases of vole population cycles (Calandra et al., 2016). The authors hypothesize that the high quantity of phytoliths, produced due to intense grazing in peak years, can result in malocclusion and other dental abnormalities, and may explain how these silicon-based plant defenses contribute to population crashes. Silicon-treated wheat plants showed enhanced resistance to feeding by the wild rabbit (*Oryctolagus cuniculus* L.), a major vertebrate pest of cereals in the United Kingdom (Cotterill et al., 2007). Further, severe, potentially lethal feeding damage due to rabbit browsing, was reduced in silicon-treated wheat by over 50%. Feeding preference in sheep (*Ovis aries* L.), in response to silicon availability, did not

differ within a grass species; however, there were differences in the bite rate and feeding preference between grass species, with these differences more obvious in silicon-treated plants (Massey et al., 2009). Further, silicon influenced grass preference less in palatable species, compared to less desirable species, an effect that appeared to be due to the most palatable species containing relatively little silicon even after supplementation, and being less tough (Massey et al., 2009).

Numerous studies have shown enhanced resistance of plants treated (soil and/or foliar application) with silicon to insect herbivores and other arthropods, including folivores (Korndorfer et al., 2004; Redmond and Potter, 2006; Massey et al., 2007; Han et al., 2015), borers (Kvedaras and Keeping, 2007; Kvedaras et al., 2007a,b, 2009; Hou and Han, 2010; Keeping et al., 2013; Vilela et al., 2014), phloem (Correa et al., 2005; Goussain et al., 2005; He et al., 2015) and xylem feeders (Yoshihara et al., 1979), mites (Nikpay and Nejadian, 2014) and nematodes (Silva et al., 2015). However, there is no consistent evidence for silicon having a greater effect in any particular feeding guild or taxon (Keeping and Kvedaras, 2008). The vast majority of studies are at two trophic levels, with few studies at the third trophic level (Reynolds et al., 2009; Gurr and Kvedaras, 2010; Kvedaras et al., 2010). A comprehensive review of earlier work on the role of silicon against herbivorous insects was provided by Reynolds et al. (2009).

This paper explores the more recent advances in the role of silicon in ameliorating the effects of biotic stress, particularly that caused by arthropods from agricultural systems, and the response of their natural enemies, together with the mechanisms involved in bi- and tri-trophic interactions. We also review literature relating to the effects of silicon on plant pathogens where this helps illustrate underlying mechanisms of plant defense that may have relevance to arthropods. Understanding the role and function of silicon against arthropod pests, will ultimately enable us to optimize the use of this element in the context of sustainable agriculture.

BI-TROPHIC INTERACTIONS

Silicon fertilization of plants has proven to be effective in controlling insect herbivores and other arthropods. Indeed, silicon application has become a routine practice in rice production in some countries, including Japan, where a silicon fertilizer was first applied to any crop worldwide (Ma and Takahashi, 2002). In agricultural systems, silicon is typically applied to the soil, or as a foliar spray to the vegetation. It is feasible that foliar application of silicon can have an effect on arthropods, e.g., via surface pH or osmotic effects. However, there is now considerable evidence, notably in fungal systems, that soil applied silicon leads to significantly more silicon accumulation in plant tissues, than foliar applications and produces much better results against biotic stressors (Liang et al., 2005, 2015; Guével et al., 2007; Dallagnol et al., 2015). Details of the mechanisms underlying silicon-mediated plant resistance against biotic stress are increasingly becoming clear, with an increase in the number of publications in this area in recent years.

Physical Mechanisms

An increased physical barrier produced by silicon deposition beneath leaf cuticles has long been considered to represent a major component underlying silicon-mediated plant resistance to insect pests. Silicon deposition contributes to increased rigidity and abrasiveness of plant tissues, thereby forming a mechanical barrier and reducing their palatability and digestibility to both vertebrate (Massey and Hartley, 2006, 2009) and invertebrate herbivores (Goussain et al., 2005; Kvedaras et al., 2007a; Massey and Hartley, 2009). Increased abrasiveness of leaves due to silicon deposition reduces food quality for herbivores and may cause wear of herbivore mouthparts, which further reduces feeding efficiency and growth rates (Massey and Hartley, 2009). Conversely, using a simple method to determine mandibular wear (Smith et al., 2007), it was shown that although there was a trend for increased wear in *Eldana saccharina* larvae that developed on silicon-treated sugarcane, the ability of larvae to renew their mandibles at each moult probably allows them to compensate for increased wear (Kvedaras et al., 2009). Finely ground wollastonite (CaSiO_3) in artificial diets at rates of up to 3.3% silicon had no significant effect on larval growth of *Helicoverpa armigera* (Hübner; Lepidoptera: Noctuidae) and *Helicoverpa punctigera* Wallengren, suggesting that silicon may not be directly deleterious to insects via ingestion and other mechanisms may be involved in silicon-mediated plant resistance (Stanley et al., 2014). It should be noted, however, that by grinding the silicon, this has likely removed potential abrasive attributes, in addition to the potential effects of soluble-silicon-induced plant defenses.

Using energy-dispersive X-ray (EDX) and X-ray mapping, it was shown that the pattern of silicon deposition in sugarcane, especially at the internode and root band, is likely the reason (at least, in part) for enhanced resistance of silicon-treated sugarcane to penetration and feeding by *E. saccharina* at these sites (Keeping et al., 2009). Further, epidermal silicon was higher in the control (i.e., no silicon treatment), *E. saccharina* resistant cultivar, than the susceptible control cultivar, suggesting that such differences in silicon-mediated resistance exist to a large extent due to the varying ability of cultivars to deposit silicon within the stalk epidermis (Keeping et al., 2009), thus preventing *E. saccharina* penetration (Kvedaras and Keeping, 2007). A more recent study using scanning electron microscopy and EDX compared four grass species, and showed that spine and phytolith morphology both within and between species may be more important than leaf silicon concentration in determining the abrasiveness and/or digestibility of leaves and thus the effectiveness of anti-herbivore defense (Hartley et al., 2015). The authors showed that all the grasses tested were able to deposit new types of silicon-based structures when silicon supply was increased. These changes were particularly evident when the leaves were mechanically damaged; however, damage in the absence of additional silicon did not produce such structures (Hartley et al., 2015).

Biochemical/Molecular Mechanisms

McNaughton and Tarrants (1983) were the first to show induction of silica. They showed that plants growing in

a more heavily mammal-grazed grassland in the Serengeti, Tanzania, accumulated more silica in their leaf blades relative to plants from a less heavily grazed site, and blade silica content was higher when plants were defoliated, suggesting that silification is an inducible defense against mammalian herbivores. Massey et al. (2007) demonstrated in a laboratory study, that feeding by both a mammal, *M. agrestis* and an insect, *Schistocerca gregaria* Forskal (Orthoptera: Acrididae) led to increased levels of silica in grass leaves. Other recent studies on arthropods have demonstrated that silicon-mediated anti-herbivore defense is both inducible and allelochemical-mediated (Gomes et al., 2005; Kvedaras et al., 2010; Costa et al., 2011) and these effects can complement the physical effects described above, leading to impaired feeding, growth, and development (Figure 1).

Increasing evidence shows that silicon treatment increases transcript levels of defense-related genes, thereby enhancing the activities of plant defensive enzymes (Liang et al., 2003; Cai et al., 2008; Rahman et al., 2015) leading to increased accumulation of defensive compounds, such as phenolics, phytoalexins, and momilactones (Fawe et al., 1998; Rodrigues et al., 2004; Rémus-Borel et al., 2005). Gomes et al. (2005) showed that the addition of silicon strongly enhanced wheat resistance to greenbug *Schizaphis graminum* (Rondani; Hemiptera: Aphididae). Further, silicon pre-treatment increased the activities of the defensive enzymes peroxidase, polyphenoloxidase, and phenylalanine ammonia lyase. In particular, silicon facilitated the strongest resistance if wheat plants had previously been infested with aphids. Chérif et al. (1994) found that silicon-treated cucumber plants show increased activity of the enzymes peroxidase, polyphenoloxidase, β -1,3 glucanase, and chitinase in response to infection by pathogens. Perennial ryegrass (*Lolium perenne* L.) grown in silicon-amended soil exhibited greater activity of peroxidase and polyphenoloxidase, higher levels of several phenolic acids, including chlorogenic acid and flavonoids, and enhanced expression levels of genes encoding phenylalanine ammonia lyase (PALa and PALb) and lipoxygenase (LOXa) in response to infection by *Magnaporthe oryzae* (T.T. Hebert) M.E. Barr (Rahman et al., 2015). Histological and ultrastructural analyses revealed that silicon mediates active localized cell defenses, and epidermal cells of silicon-treated plants displayed specific defense reactions including papilla formation, production of callose, and accumulation of glycosilated phenolics in response to pathogen infection by the fungus *Blumeria graminis* f. sp. *tritici* (DC.) Speer (Bélanger et al., 2003). Silicon-mediated brown spot resistance in rice plants is independent of the classic immune hormones, salicylic acid and jasmonic acid (JA; Van Bockhaven et al., 2015). Conversely, silicon mounted rice resistance to the brown spot fungus *Cochliobolus miyabeanus* (Ito and Kurabayashi) Dastur, by interfering with the production and/or action of fungal ethylene, prevents the fungus from suppressing the rice innate immune system (Van Bockhaven et al., 2015).

Pre-treatment with certain chemicals or previous biotic stressor may provoke a specific physiological state in plants called “priming” (Fauteux et al., 2006; Hao et al., 2012;

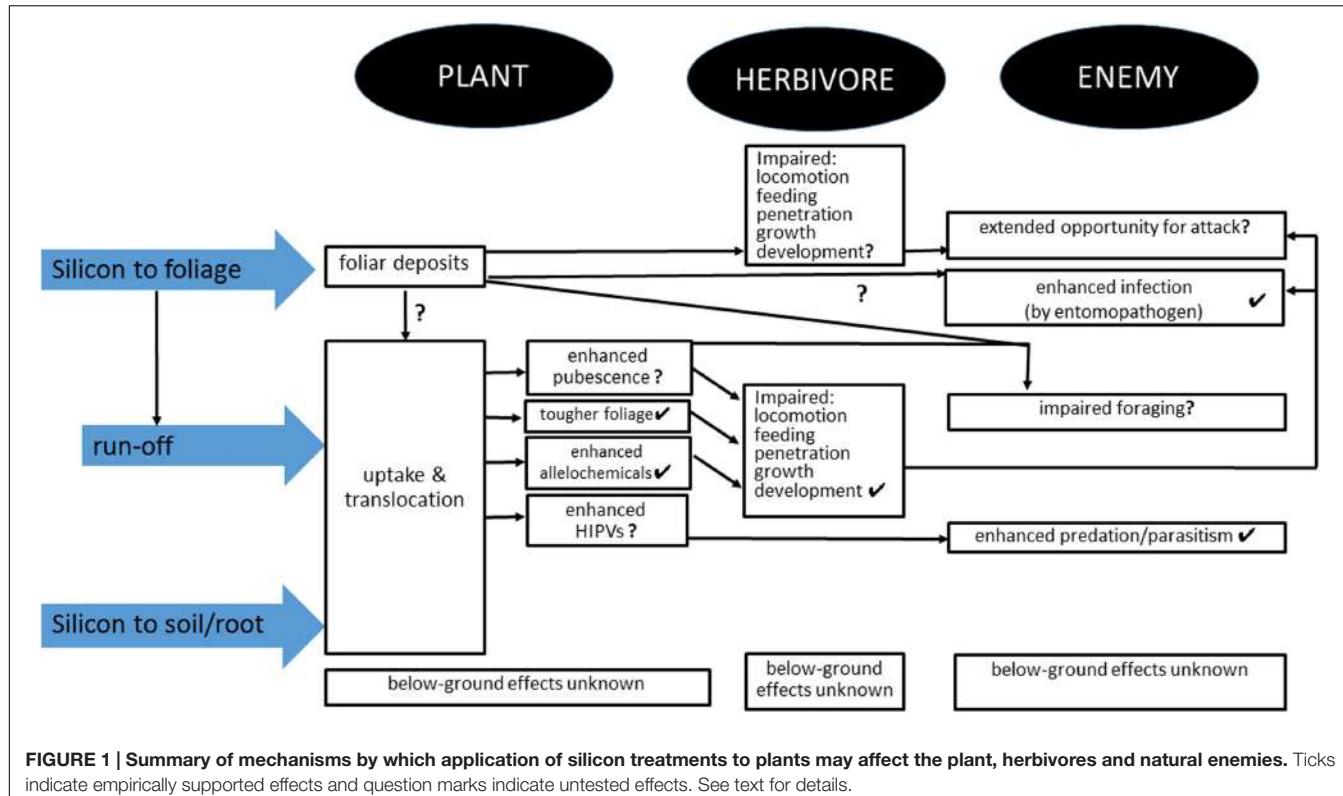


FIGURE 1 | Summary of mechanisms by which application of silicon treatments to plants may affect the plant, herbivores and natural enemies. Ticks indicate empirically supported effects and question marks indicate untested effects. See text for details.

Worrall et al., 2012; Aimé et al., 2013). Primed plants are thus physiologically prepared to induce quicker and/or stronger defense responses upon subsequent attack, providing plants with a more effective means to respond to challenges (Ton et al., 2006; Jung et al., 2009; Slaughter et al., 2012; Ye et al., 2013). A recent study demonstrates that silicon is able to prime jasmonate-mediated defense responses and rice defense against a chewing herbivore, the rice leaffolder, *Cnaphalocrocis medinalis* (Lepidoptera: Pyralidae; (Ye et al., 2013)). More interestingly, activation of jasmonate signaling in turn promotes silicon accumulation in rice leaves, indicating a strong interaction between silicon and jasmonate in rice defense against insect herbivores. Some recent studies have shown that silicon can also prime plants for alleviating biotic stress imposed by pathogens (Ghareeb et al., 2011; Rahman et al., 2015). Vivancos et al. (2015) showed that priming is also an important mechanism of silicon-mediated resistance of *Arabidopsis thaliana* (L.) Heynh. against powdery mildew caused by *Golovinomyces cichoracearum* (DC.). Further, this work has also revealed that silicon may interfere with effector proteins released by such biotrophic pathogens, suggesting that mechanisms other than salicylic acid-dependent plant defense priming are involved (Vivancos et al., 2015). It has been suggested that priming of plant defense responses, alterations in phytohormone homeostasis, and interaction with defense signaling components are all potential mechanisms involved in regulating silicon-triggered resistance responses (Van Bockhaven et al., 2013). Silicon has also been demonstrated to prime plants for resistance against abiotic stresses (Ahmed et al., 2013). Research on silicon-mediated herbivore resistance lags

far behind that on silicon-mediated disease resistance. Further studies are needed to determine the exact nature of silicon-primed anti-herbivore defense and indeed other mechanisms that may play a role in plant resistance to biotic stressors. For example, effectors that modulate plant defenses have also been identified in the saliva of insects (for a review see Hogenhout and Bos, 2011) and it is feasible that a similar mechanism proposed for plant pathogens, also operates for insects, although this remains to be elucidated.

Recent developments regarding the understanding of molecular mechanisms controlling silicon accumulation and the discovery of silicon transporters have enabled a ready ability to classify a plant as Si-competent, or not. This will enable a better understanding of the role of silicon in several fundamental aspects of ecology concerning plant fitness under stress (Deshmukh and Bélanger, 2015).

TRI-TROPHIC INTERACTIONS

Natural enemies of herbivores can be important in the management of agricultural pest species. Evidence for this includes the wide literature on biological control using predators, parasitoids and entomopathogens. In this section we consider what is currently the least thoroughly investigated aspect of plant-silicon-herbivore interactions: the mechanisms by which the application of silicon compounds may affect the impact of natural enemies on herbivores.

Entomopathogenic Microorganisms

Entomopathogens are increasingly used in arthropod pest management. However, as this approach uses applications of live organisms rather than chemicals, as in conventional insecticide use, particular attention needs to be given to maximizing the viability and impact of the treatment on the target pest. In work with the fungus *Beauveria bassiana* (Bals.-Criv.) Vuill., 1912, potassium silicate was added to nutrient solutions applied to plant roots seven days after inoculation with spider mite, *Tetranychus urticae* Koch (Gatarayiha et al., 2010). Potassium silicate alone did not kill the pest mites, but when used at the higher rates, equivalent to 80 and 160 mg of pure silicon per liter, pest mortality caused by *B. bassiana* was up to 92%. The authors of that study hypothesized that silicon application primed biochemical defenses in the plants (see above) which interfered with the feeding of mites making them more susceptible to the entomopathogen (Figure 1).

Predators

Of particular relevance to the possible effects of silicon on non-entomopathogenic natural enemies is a study of induced defense in rice (Ye et al., 2013). This study, employing rice mutant lines in which genes for jasmonate synthesis or jasmonate perception were silenced, showed a strong interaction between soil-applied silicon and JA in defense against insect herbivores. This involved priming of JA-mediated defense responses by silicon and the promotion of silicon accumulation by JA (Ye et al., 2013). While that work did not extend to considering natural enemies it is significant for third trophic level effects because it identified a relationship between silicon and JA. Silicon is translocated within plants in the form of monosilicic acid, Si(OH)₄ which is reported as an elicitor for systemic stress signals including JA (Fauteux et al., 2005). JA, in turn, is the primary signaling pathway that is activated by chewing herbivores leading to herbivore-induced plant volatiles (HIPV) production (Dicke et al., 1999, 2009).

The first published study of the effects of silicon on plant defense in which HIPV-mediated effects has been the focus was in cucumber (Kvedaras et al., 2010). That work demonstrated that soil-applied silicon enhanced the attraction of the predator *Dicranolaius bellulus* (Guerin-Meneville; Coleoptera: Melyridae) to *Helicoverpa armigera* (Hubner; Lepidoptera: Noctuidae) infested cucumber plants in a Y-tube olfactometer bioassay. Further, a small-scale field trial, using *H. armigera* eggs affixed to potted cucumber plants, before they were placed in a field plot of lucerne, showed that increased biological control by "wild" predators was significantly higher for soil-applied, silicon-treated plants than for control plants (Kvedaras et al., 2010; Figure 2). The authors hypothesized that this was due to a change in the plant volatile profile (HIPVs) produced by cucumber plants when attacked by an herbivore. Additional studies to measure and identify the compounds produced by pest-infested silicon-treated and untreated cucumber plants are worthwhile. Similar work on grapevines has yielded preliminary evidence for volatile-mediated defenses to promote predator attraction to pest-infested plants (Connick, 2011). A study of the volatiles produced by grapevines infested by the Lepidoptera pest, grapevine moth

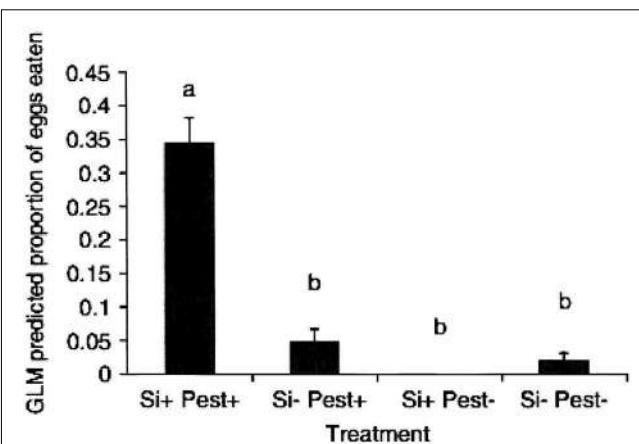


FIGURE 2 | The effect of prior treatment with potassium silicate (silicon+) and infestation with 10 *H. armigera* larvae/plant (pest+) on the proportion of prey eggs removed from potted cucumber plants over a 24-h period when exposed to predators in the field. ($N = 4$), columns with differing letters differ (LSD test, $P = 0.05$). (Reproduced with permission from Kvedaras et al., 2010).

Phalaenoides glycinae (Lewin; Lepidoptera: Noctuidae) found that soil applied potassium silicate had profound effects. Seven volatile compounds emitted from *P. glycinae*-infested grapevines were identified and *n*-heptadecane found to be produced in significant amounts only by silicon-treated plants. Cis-thio rose oxide production, in contrast, was significantly lower in silicon-treated grapevines. A second study in that thesis found that the attractiveness of grapevines infested with the lightbrown apple moth (*Epiphyas postvittana* (Walker; Lepidoptera: Tortricidae) was positively correlated with plant foliar tissue concentration of silicon (Connick, 2011).

The impact of natural enemies on herbivores may be enhanced by mechanisms other than induced, indirect defenses based on HIPVs. By extending development time, and particularly the period over which neonate larvae feed on the exterior of plants before being able to penetrate the plant cuticle and commence mining or boring, herbivores are exposed to a higher risk of attack by predators. Delayed penetration was evident in a study of sugarcane borer, *E. saccharina* (Kvedaras and Keeping, 2007). Massey and Hartley (2006) reported similar findings for *Spodoptera exempta* Walker feeding on grass with high silicon levels. Many natural enemies forage for prey by locomotion over the foliar surface, so the practice of applying silicon treatments to the above ground plant parts could have physical or chemical effects on natural enemy foraging (Figure 1). Examples of recent studies that included treatments with foliar applied silicon are Dalastra et al. (2011) and de Assis et al. (2012, 2013), and in the latter of those studies, there was no effect of foliar treatments to potato plants on predatory beetles, although the plants treated with silicic acid were less preferred by defoliators. Further work needs to test for the strength of such effects on a wider range of natural enemy taxa.

Foraging of predators may also be affected by foliar pubescence, especially glandular trichomes. The latter produce

irritant, toxic and adhesive liquid secretions from the tips that can provide high levels of protection from foliar-associated herbivores (Gurr and McGrath, 2002) but can also affect natural enemies (Simmons and Gurr, 2004, 2005). When subject to herbivores, plants have the capacity to regenerate new leaves that exhibit enhanced densities of trichomes, an induced defense that is under the control of JA (Yoshida et al., 2009). This form of induced defense is remarkable in taking place over days rather than the timespan of hours as in the case of induced production of semiochemical volatiles. This phenomenon has relevance to the interplay between silicon and plant defense because plant-available silicon influences the JA signaling pathway (Ye et al., 2013). Accordingly, the phenomenon of herbivore-attacked plants producing more hirsute foliage is another form of plant defense that we hypothesize may be amplified by silicon pre-treatment (**Figure 1**).

Not only might plant-available silicon promote the density of trichomes on young foliage, work on deposition patterns of silica in the leaf epidermis suggests that the bases of trichomes is a major site in cucumber (Samuels et al., 1991a,b), while in the grasses *D. cespitosa* and *Festuca ovina* L., silica was particularly evident in the tips of spines under control conditions, but was distributed throughout the spine and the leaf surface when silicon fertilized (Hartley et al., 2015). The epicarp hairs present on the mature caryopses of the four cereals, barley, oats, rye, and wheat (Bennett and Parry, 1981) are also important silicon deposition sites, particularly in the tips of hairs where it is most likely to promote adverse effects on herbivores including – potentially – human consumers of grain products (Parry et al., 1984). It remains to be tested whether the potentially adverse effects of trichomes on predators are exacerbated by silicon supplementation and the extent to which any such effects are offset by stronger effects on herbivores.

Among studies of the effects of silicon on pests that do consider third trophic level effects, these tend to use designs that are not well suited to detecting the full range of possible mechanisms that may operate. An example is work by Moraes et al. (2004), with the lacewing *Chrysoperla externa* Steinmann in which wheat aphid (*Schizaphis graminum* (Rondani; Hemiptera: Aphididae) prey were removed from the test plants before being exposed to the predators. Since predators were not exposed to plants or their volatiles, they would have been unable to detect HIPV-mediated effects, though effects related to prey quality could be assessed.

A major limit on our current understanding of the effects of silicon on natural enemies is the apparent absence of studies on below-ground effects. Many arthropod pests cause important damage to plant roots so studies of how silicon might promote natural enemies such as predacious beetle larvae and entomopathogenic nematodes would be valuable.

Parasitoids

Of the three types of natural enemies, parasitoids are the least well studied in relation to plant available silicon, though many of the comments made above, for established and possible effects on predators (**Figure 1**), will apply to parasitoids. Of particular significance is the wealth of evidence for HIPVs

attracting parasitoids to pest-infested plants (Dicke et al., 2009). The only study with silicon-treated and un-treated plants in which a parasitoid was considered is that by Moraes et al. (2004) with *Aphidius colemani* Viereck (Hymenoptera: Aphidiidae). Unfortunately, this confined wasps to narrowly spaced wheat plants and, because it used non-choice conditions, would not have allowed HIPV-mediated effects to be apparent.

HOW “OMICS” SUPPORT PLANT DEFENSE STUDIES?

To understand how the addition of silicon to a plant’s environment can improve plant defense, the plant as a whole must be considered through global analysis of the major responsive components of the DNA, RNA, proteins, and metabolites which are then holistically viewed using bioinformatics (**Figure 3**).

While system-wide analysis has long been applied to plants, their application to analyzing plant defense has been limited (Chen et al., 2005; Giri et al., 2006; Thivierge et al., 2010; Lewandowska-Gnatowska et al., 2011; Duceppe et al., 2012; Timbo et al., 2014) and analyzing silicon’s role even more so. Numerous reductionist experiments targeting specific proteins or enzymes have shown that silicon treatment induces plant defensive enzymes (Liang et al., 2003; Cai et al., 2008), leading to the accumulation of defensive compounds and metabolites (Fawe et al., 1998; Rodrigues et al., 2004).

But the power of -omics approaches lies in its non-targeted nature, allowing the unearthing of unexpected changes. Transcriptome analysis represents the only -omic analysis of silicon’s effects, with a study on challenged *A. thaliana* showing silicon treatment causes a decrease in primary metabolism that allows a more efficient defense response (Fauteux et al., 2006). A similar analysis was also conducted on rice (Ye et al., 2013), as indicated above. Recent work has sought to establish the “Prime-ome”, or the mechanism behind how a plant defends itself or is in a “primed state” to rapidly respond to attack by insects and microbial pathogens (Balmer et al., 2015). Not surprisingly, the available -omics scale data shows that the plant’s response depends on the priming inducer and the pathogen, which is also observed in defense against arthropods (Balmer et al., 2015). Silicon’s role in defense against herbivores remains vastly understudied by -omics methodologies which would reveal the role of, as yet, untargeted molecules, including proteins and metabolites, through global analysis.

Transcriptomics alone is insufficient to understand an organism’s phenotype (Barah and Bones, 2015) as it is the proteome and metabolome that provide the molecular mechanisms that allow a plant to defend itself (Oliveira et al., 2014). While proteomics and metabolomics are rapidly maturing fields, they are still limited by the issues of throughput and the depth of proteome and metabolome coverage due to the dynamic range of concentration of the molecules present (Jorge et al., 2015). The abundance of proteins can vary by 7–10 orders of magnitude (Ly and Wasinger, 2008; Zubarev, 2013) and the existence of a proteoform is often reported by the detection of

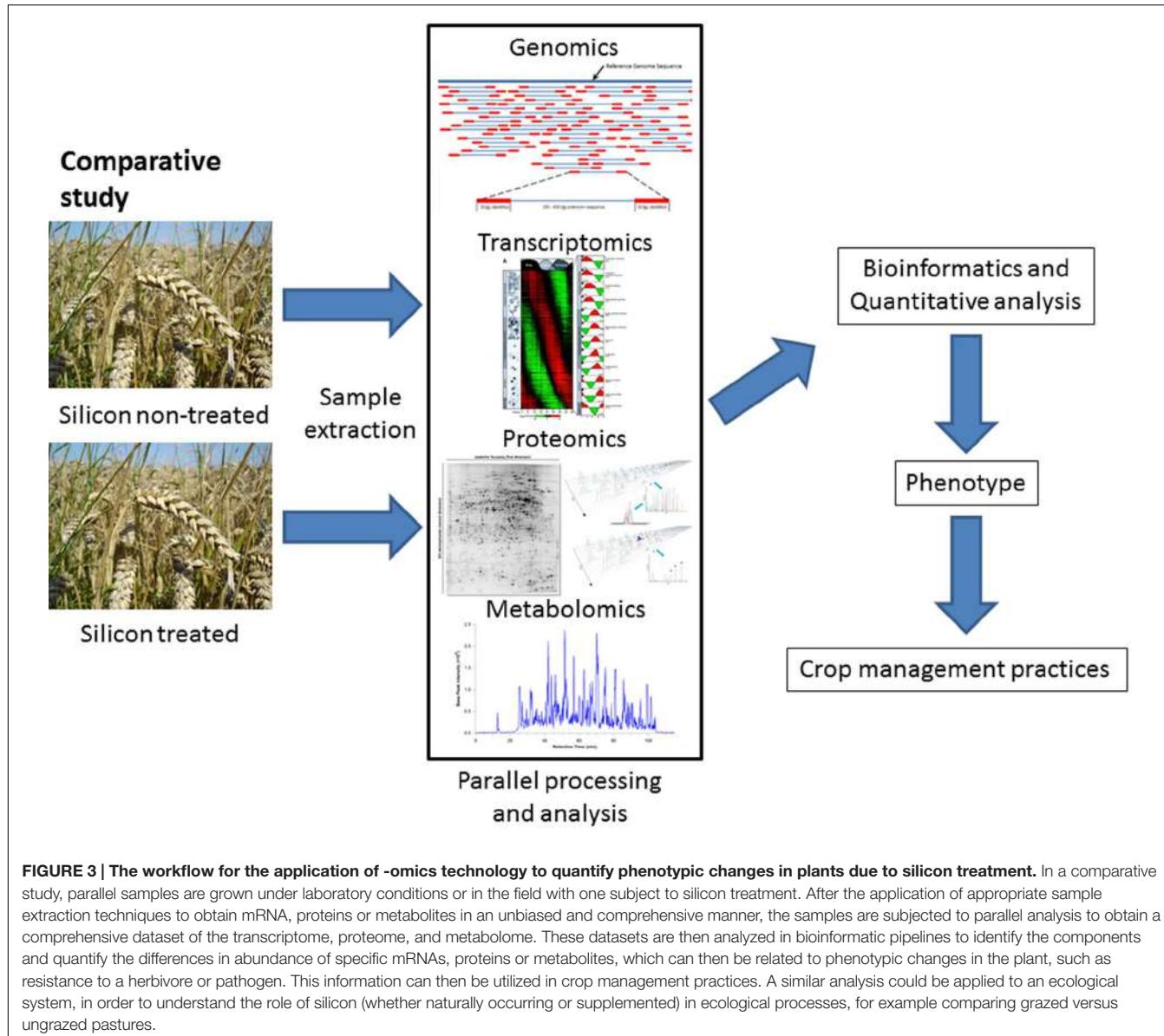


FIGURE 3 | The workflow for the application of -omics technology to quantify phenotypic changes in plants due to silicon treatment. In a comparative study, parallel samples are grown under laboratory conditions or in the field with one subject to silicon treatment. After the application of appropriate sample extraction techniques to obtain mRNA, proteins or metabolites in an unbiased and comprehensive manner, the samples are subjected to parallel analysis to obtain a comprehensive dataset of the transcriptome, proteome, and metabolome. These datasets are then analyzed in bioinformatic pipelines to identify the components and quantify the differences in abundance of specific mRNAs, proteins or metabolites, which can then be related to phenotypic changes in the plant, such as resistance to a herbivore or pathogen. This information can then be utilized in crop management practices. A similar analysis could be applied to an ecological system, in order to understand the role of silicon (whether naturally occurring or supplemented) in ecological processes, for example comparing grazed versus ungrazed pastures.

only a single peptide (Mallick et al., 2007). Without an equivalent of PCR utilized in genomics and transcriptomics, the only way to reliably detect and quantify the abundance of low copy number proteins is to start with more material (Zubarev, 2013) and fractionate the proteins to isolate those of high abundance from the rest (Stasyk and Huber, 2004; Righetti et al., 2005; Ly and Wasinger, 2011). The same logic applies to metabolites but in both cases the number of fractions requiring analysis increases.

In the case of proteomics, fractionation of intact proteins reduces this increase compared to “shotgun” peptide-centric methods while retaining the option of utilizing 2D-PAGE as a further fractionation and quantification method (Coorsen and Yergey, 2015). To determine plant defense responses as a result of silicon treatment, 2D-PAGE has the distinct advantage of quantifying protein abundance changes prior to

identification. This is contrary to LC/MS/MS methodologies where identification of peptides and their assignment to a protein isoform needs to be performed prior to quantitation. Thus, 2D-PAGE can decrease the number of samples requiring analyses by mass spectrometry (MS), freeing valuable instrument time. In proteomics, the issue of throughput is being addressed somewhat by faster instrument scan speeds (Richards et al., 2015), the adoption of ultra high-pressure chromatography (Kocher et al., 2011; Thakur et al., 2011) and data-independent acquisition (DIA) techniques in LC/MS/MS (Huang et al., 2015). DIA methodologies have also been applied to measure nitrogen flux and metabolism (Ullmann-Zeunert et al., 2012) indicating that DIA could have application in quantitative metabolomics, in order to assess how changes in the levels of specific metabolites can be related to observed plant defensive phenotypes.

CONCLUSIONS AND FUTURE DIRECTIONS

There is now considerable literature supporting the role of silicon as a physical defense mechanism, and a growing number of published works on the role of silicon-mediated biochemical defense. However, there are few references on the role of silicon in tri-trophic interactions.

Research should focus on understanding the relative importance of both physical and biochemical defence and how (if) this differs between herbivores. A meta-analysis of the literature would be valuable to discern if silicon has a greater effect in certain feeding guilds or taxons. Understanding the interaction between silicon and the plant defense pathways, and if there is a similar mechanism acting against insects, and pathogens, will also be paramount, as there is a wealth of literature on silicon/pathogen interactions that can inform arthropod work.

Future researchers need to address the lack of knowledge on below-ground effects of silicon application to plants on predators. There is a more general dearth of knowledge on how silicon might alter root toughness and chemical defenses. There is also a need to test for the effects of foliar deposits from foliar applied silicon on natural enemy foraging and impact. Work also needs to consider the possibility that changing the plant surface, by denser or more robust trichomes, may have negative effects on natural enemy foraging (Figure 1). More generally, workers need to consider the effects of silicon under field conditions (something done quite extensively for mammals in natural ecological systems) and be less reliant on greenhouse and laboratory studies, especially those that make it impossible for natural enemy mediated effects on herbivores to be apparent. Finally, there are currently no published studies of the effects of silicon on HIPV production but such work is known to be underway. If strong evidence is forthcoming for effects on the blend of HIPVs, this will add impetus to the need for greater attention to be given to the third trophic level in studies of silicon on plant defenses.

REFERENCES

- Ahmed, M., Kamran, A., Asif, M., Qadeer, U., Ahmed, Z. I., and Goyal, A. (2013). Silicon priming: a potential source to impart abiotic stress tolerance in wheat: a review. *Aust. J. Crop Sci.* 7, 484–491.
- Aimé, S., Alabouvette, C., Steinberg, C., and Olivain, C. (2013). The endophytic strain *Fusarium oxysporum* Fo47: a good candidate for priming the defense responses in tomato roots. *Mol. Plant Microbe Interact.* 26, 918–926. doi: 10.1094/MPMI-12-12-0290-R
- Alexandre, A., Meunier, J. D., Colin, F., and Koud, J. M. (1997). Plant impact on the biogeochemical cycle of silicon and related weathering processes. *Geochim. Cosmochim. Acta* 61, 677–682. doi: 10.1016/s0016-7037(97)00001-x
- Anda, M., Suryani, E., Husnain, and Subardja, D. (2015). Strategy to reduce fertilizer application in volcanic paddy soils: nutrient reserves approach from parent materials. *Soil Tillage Res.* 150, 10–20. doi: 10.1016/j.still.2015.01.005
- Balmer, A., Pastor, V., Gamir, J., Flors, V., and Mauch-Mani, B. (2015). The ‘prime-ome’: towards a holistic approach to priming. *Trends Plant Sci.* 20, 443–452. doi: 10.1016/j.tplants.2015.04.002

Using system-wide analysis or -omics technologies would permit us to not only understand silicon's role in the production of defense-related compounds, but in the production of HIPVs, in addition to the associated energy costs to the plant. This could potentially inform the manipulation of plants to minimize herbivory and maximize the impact of natural enemies.

Modern approaches of transcriptomics, proteomics, metabolomics, and transgenic mutants will serve as powerful tools for dissecting the underlying mechanism/s involved in silicon and plant defense. In an era when sustainable pest management is receiving more attention than ever before, due largely to restrictions or the withdrawal of toxic pesticides, because of their negative impacts on human and environmental health, silicon treatment should be more widely considered and tested as a pest management option.

AUTHOR CONTRIBUTIONS

OR and GG developed the concept, drafted, and critically revised the manuscript. MP and RZ drafted and critically revised the manuscript.

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- Barah, P., and Bones, A. M. (2015). Multidimensional approaches for studying plant defence against insects: from ecology to omics and synthetic biology. *J. Exp. Bot.* 66, 479–493. doi: 10.1093/jxb/eru489
- Bélanger, R., Benhamou, N., and Menzies, J. (2003). Cytological evidence of an active role of silicon in wheat resistance to powdery mildew (*Blumeria graminis* f. sp. *tritici*). *Phytopathology* 93, 402–412. doi: 10.1094/PHYTO.2003.93.4.402
- Bennett, D. M., and Parry, D. W. (1981). Electron-probe microanalysis studies of silicon in the epicarp hairs of the caryopses of *Hordeum sativum* Jess., *Avena sativa* L., *Secale cereale* L. and *Triticum aestivum* L. *Ann. Bot.* 48, 645–654.
- Cai, K., Gao, D., Luo, S., Zeng, R., Yang, J., and Zhu, X. (2008). Physiological and cytological mechanisms of silicon-induced resistance in rice against blast disease. *Physiol. Plant.* 134, 324–333. doi: 10.1111/j.1399-3054.2008.01140.x
- Calandra, I., Zub, K., Szafranska, P. A., Zalewski, A., and Merceron, G. (2016). Silicon-based plant defences, tooth wear and voles. *J. Exp. Biol.* 219, 501–507. doi: 10.1242/jeb.134890
- Chain, F., Cote-Beaulieu, C., Belzile, F., Menzies, J. G., and Belanger, R. R. (2009). A comprehensive transcriptomic analysis of the effect of silicon on wheat plants under control and pathogen stress conditions. *Mol. Plant Microbe Interact.* 22, 1323–1330. doi: 10.1094/MPMI-22-11-1323

- Chen, H., Wilkerson, C. G., Kuchar, J. A., Phinney, B. S., and Howe, G. A. (2005). Jasmonate-inducible plant enzymes degrade essential amino acids in the herbivore midgut. *Proc. Natl. Acad. Sci. U.S.A.* 102, 19237–19242. doi: 10.1073/pnas.0509026102
- Chérif, M., Asselin, A., and Bélanger, R. (1994). Defense responses induced by soluble silicon in cucumber roots infected by *Pythium* spp. *Phytopathology* 84, 236–242. doi: 10.1094/Phyto-84-236
- Chiba, Y., Mitani, N., Yamaji, N., and Ma, J. F. (2009). HvLsi1 is a silicon influx transporter in barley. *Plant J.* 57, 810–818. doi: 10.1111/j.1365-313X.2008.03728.x
- Connick, V. J. (2011). *The Impact of Silicon Fertilisation on the Chemical Ecology of Grapevine, Vitis vinifera Constitutive and Induced Chemical Defences Against Arthropod Pests and Their Natural Enemies*. Ph.D. thesis, Charles Sturt University, Albury–Wodonga, NSW.
- Coorsen, J., and Vergey, A. (2015). Proteomics is analytical chemistry: fitness-for-purpose in the application of top-down and bottom-up analyses. *Proteomes* 3, 440–453. doi: 10.3390/proteomes3040440
- Cornelis, J. T., Delvaux, B., Georg, R. B., Lucas, Y., Ranger, J., and Opfergelt, S. (2011). Tracing the origin of dissolved silicon transferred from various soil-plant systems towards rivers: a review. *Biogeosciences* 8, 89–112. doi: 10.5194/bg-8-89-2011
- Correa, R. S. B., Moraes, J. C., Auad, A. M., and Carvalho, G. A. (2005). Silicon and acibenzolar-s-methyl as resistance inducers in cucumber, against the whitefly *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) Biotype B. *Neotrop. Entomol.* 34, 429–433. doi: 10.1590/S1519-566X2005000300011
- Costa, R. R., Moraes, J. C., and DaCosta, R. R. (2011). Feeding behaviour of the greenbug *Schizaphis graminum* on wheat plants treated with imidacloprid and/or silicon. *J. Appl. Entomol.* 135, 115–120. doi: 10.1111/j.1439-0418.2010.01526.x
- Cotterill, J. V., Watkins, R. W., Brennan, C. B., and Cowan, D. P. (2007). Boosting silica levels in wheat leaves reduces grazing by rabbits. *Pest. Manag. Sci.* 63, 247–253. doi: 10.1002/ps.1302
- Dalastra, C., Campos, A. R., Fernandes, F. M., Martins, G. L. M., and Campos, Z. R. (2011). Silicon as a resistance inducer controlling the silvering thrips *Enneothrips flavens* Moulton, 1941 (Thysanoptera: Thripidae) and its effects on peanut yield. *Ciênc. Agrotecnol.* 35, 531–538. doi: 10.1590/S1413-70542011000300014
- Dallagnol, L. J., Rodrigues, F. A., Pascholati, S. F., Fortunato, A. A., and Camargo, L. E. A. (2015). Comparison of root and foliar applications of potassium silicate in enhancing post-infection defences of melon against powdery mildew. *Plant Pathol.* 64, 1085–1093. doi: 10.1111/ppa.12346
- de Assis, F., Moraes, J. C., Silveira, L., Francoso, J., Nascimento, A. M., and Antunes, C. (2012). Inducers of resistances in potato and its effects on defoliators and predatory insects. *Rev. Colomb. Entomol.* 38, 30–34.
- de Assis, F. A., Moraes, J. C., Auad, A. M., and Coelho, M. (2013). The effects of foliar spray application of silicon on plant damage levels and components of larval biology of the pest butterfly *Chlosyne lacinia* saundersii (Nymphalidae). *Int. J. Pest Manag.* 59, 128–134. doi: 10.1080/09670874.2013.779049
- Deren, C. W. (2001). “Plant genotype, silicon concentration, and silicon-related responses” in *Silicon in Agriculture*, eds L. E. Datnoff, G. H. Snyder, and G. H. Korndorfer (Amsterdam: Elsevier Science), 149–158.
- Deshmukh, R., and Bélanger, R. (2015). Molecular evolution of aquaporins and silicon influx in plants. *Funct. Ecol.* doi: 10.1111/1365-2435.12570
- Deshmukh, R. K., Vivancos, J., Guerin, V., Sonah, H., Labbe, C., Belzile, F., et al. (2013). Identification and functional characterization of silicon transporters in soybean using comparative genomics of major intrinsic proteins in *Arabidopsis* and rice. *Plant Mol. Biol.* 83, 303–315. doi: 10.1007/s11103-013-0087-3
- Dicke, M., Gols, R., Ludeking, D., and Posthumus, M. A. (1999). Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25, 1907–1922. doi: 10.1023/A:1020942102181
- Dicke, M., van Loon, J. J. A., and Soler, R. (2009). Chemical complexity of volatiles from plants induced by multiple attack. *Nat. Chem. Biol.* 5, 317–324. doi: 10.1038/nchembio.169
- Duceppe, M. O., Cloutier, C., and Michaud, D. (2012). Wounding, insect chewing and phloem sap feeding differentially alter the leaf proteome of potato, *Solanum tuberosum* L. *Proteome Sci.* 10, 73. doi: 10.1186/1477-5956-10-73
- Epstein, E. (1972). *Mineral Nutrition of Plants: Principles and Perspectives*. New York, NY: Wiley.
- Epstein, E. (1994). The anomaly of silicon in plant biology. *Proc. Natl. Acad. Sci. U.S.A.* 91, 11–17. doi: 10.1073/pnas.91.1.11
- Epstein, E. (1999). Silicon. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 641–664. doi: 10.1146/annurev.arplant.50.1.641
- Fauteux, F., Chain, F., Belzile, F., Menzies, J. G., and Bélanger, R. R. (2006). The protective role of silicon in the *Arabidopsis*-powdery mildew pathosystem. *Proc. Natl. Acad. Sci. U.S.A.* 103, 17554–17559. doi: 10.1073/pnas.0606330103
- Fauteux, F., Rémus-Borel, W., Menzies, J. G., and Bélanger, R. R. (2005). Silicon and plant disease resistance against pathogenic fungi. *FEMS Microbiol. Lett.* 249, 1–6. doi: 10.1016/j.femsle.2005.06.034
- Fawe, A., Abou-Zaid, M., Menzies, J. G., and Bélanger, R. R. (1998). Silicon-mediated accumulation of flavonoid phytoalexins in cucumber. *Phytopathology* 88, 396–401. doi: 10.1094/PHYTO.1998.88.5.396
- Gatarayiha, M. C., Laing, M. D., and Miller, R. M. (2010). Combining applications of potassium silicate and *Beauveria bassiana* to four crops to control two spotted spider mite, *Tetranychus urticae* Koch. *Int. J. Pest Manag.* 56, 291–297. doi: 10.1080/09670874.2010.495794
- Ghareeb, H., Bozsó, Z., Ott, P. G., Repenning, C., Stahl, F., and Wydra, K. (2011). Transcriptome of silicon-induced resistance against *Ralstonia solanacearum* in the silicon non-accumulator tomato implicates priming effect. *Physiol. Mol. Plant Pathol.* 75, 83–89. doi: 10.1016/j.pmp.2010.11.004
- Giri, A. P., Wunsche, H., Mitra, S., Zavala, J. A., Muck, A., Svatos, A., et al. (2006). Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. VII. Changes in the plant's proteome. *Plant Physiol.* 142, 1621–1641. doi: 10.1104/pp.106.088781
- Gomes, F. B., Moraes, J. C. D., Santos, C. D. D., and Goussain, M. M. (2005). Resistance induction in wheat plants by silicon and aphids. *Sci. Agric.* 62, 547–551.
- Goussain, M. M., Prado, E., and Moraes, J. C. (2005). Effect of silicon applied to wheat plants on the biology and probing behaviour of the greenbug *Schizaphis graminum* (Rond.) (Hemiptera: Aphididae). *Neotrop. Entomol.* 34, 807–813. doi: 10.1590/S1519-566X2005000500013
- Grégoire, C., Rémus-Borel, W., Vivancos, J., Labbe, C., Belzile, F., and Bélanger, R. R. (2012). Discovery of a multigene family of aquaporin silicon transporters in the primitive plant *Equisetum arvense*. *Plant J.* 72, 320–330. doi: 10.1111/j.1365-313X.2012.05082.x
- Guével, M. H., Menzies, J. G., and Bélanger, R. R. (2007). Effect of root and foliar applications of soluble silicon on powdery mildew control and growth of wheat plants. *Euro. J. Plant Pathol.* 119, 429–436. doi: 10.1007/s10658-007-9181-1
- Guntzer, F., Keller, C., and Meunier, J.-D. (2012). Benefits of plant silicon for crops: a review. *Agron. Sustain. Dev.* 32, 201–213. doi: 10.1007/s13593-011-0039-8
- Gurr, G. M., and Kvedaras, O. L. (2010). Synergizing biological control: scope for sterile insect technique, induced plant defences and cultural techniques to enhance natural enemy impact. *Biol. Control* 52, 198–207. doi: 10.1016/j.bioc.2009.02.013
- Gurr, G. M., and McGrath, D. (2002). Foliar pubescence and resistance to potato moth, *Phthorimaea operculella*, in *Lycopersicon hirsutum*. *Entomol. Exp. Appl.* 103, 35–41. doi: 10.1023/A:1019819722203
- Han, W. X., Fang, J. Y., Reich, P. B., Woodward, F. I., and Wang, Z. H. (2011). Biogeography and variability of eleven mineral elements in plant leaves across gradients of climate, soil and plant functional type in China. *Ecol. Lett.* 14, 788–796. doi: 10.1111/j.1461-0248.2011.01641.x
- Han, Y., Lei, W., Wen, L., and Hou, M. (2015). Silicon-mediated resistance in a susceptible rice variety to the rice leaf folder, *Cnaphalocrocis medinalis* Guenée (Lepidoptera: Pyralidae). *PLoS ONE* 10:0120557. doi: 10.1371/journal.pone.0120557
- Hao, Z., Fayolle, L., van Tuinen, D., Chatagnier, O., Li, X., Gianinazzi, S., et al. (2012). Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defence gene responses in grapevine. *J. Exp. Bot.* 63, 3657–3672.
- Hartley, S. E., Fitt, R. N., McLarnon, E. L., and Wade, R. N. (2015). Defending the leaf surface: intra- and inter-specific differences in silicon deposition in grasses in response to damage and silicon supply. *Front. Plant Sci.* 6:35. doi: 10.3389/fpls.2015.00035

- He, W., Yang, M., Li, Z., Qiu, J., Liu, F., Qu, X., et al. (2015). High levels of silicon provided as a nutrient in hydroponic culture enhances rice plant resistance to brown planthopper. *Crop Protect.* 67, 20–25. doi: 10.1016/j.cropro.2014.09.013
- Henriet, C., Draye, X., Oppitz, I., Swennen, R., and Delvaux, B. (2006). Effects, distribution and uptake of silicon in banana (*Musa spp.*) under controlled conditions. *Plant Soil* 287, 359–374. doi: 10.1007/s11104-006-9085-4
- Hogenhout, S. A., and Bos, J. I. (2011). Effector proteins that modulate plant-insect interactions. *Curr. Opin. Plant Biol.* 14, 422–428. doi: 10.1016/j.pbi.2011.05.003
- Hou, M., and Han, Y. (2010). Silicon-mediated rice plant resistance to the asiatic rice borer (lepidoptera: crambidae): effects of silicon amendment and rice varietal resistance. *J. Econ. Entomol.* 103, 1412–1419. doi: 10.1603/ec09341
- Huang, Q., Yang, L., Luo, J., Guo, L., Wang, Z., Yang, X., et al. (2015). SWATH enables precise label-free quantification on proteome-scale. *Proteomics* 15, 1215–1223. doi: 10.1002/pmic.201400270
- Huitu, O., Forbes, K. M., Helander, M., Julkunen-Tiitto, R., Lambin, X., Saikkonen, K., et al. (2014). Silicon, endophytes and secondary metabolites as grass defenses against mammalian herbivores. *Front. Plant Sci.* 5:478. doi: 10.3389/fpls.2014.00478
- International Plant Nutrition Institute [IPNI] (2015). *Nutri-Facts. Silicon. No. 14.* Available at: [http://www.ipni.net/publication/nutrifacts-na.nsf/0/A7B4AB4D35C153BF85257ECE006E0E34/\\$FILE/NutriFacts-NA-14.pdf](http://www.ipni.net/publication/nutrifacts-na.nsf/0/A7B4AB4D35C153BF85257ECE006E0E34/$FILE/NutriFacts-NA-14.pdf) [accessed April 4, 2016].
- Jones, L. H. P., and Handreck, K. A. (1967). Silica in soils, plants and animals. *Advan. Agron.* 19, 107–149. doi: 10.1016/S0065-2113(08)60734-8
- Jorge, T. F., Rodrigues, J. A., Caldana, C., Schmidt, R., van Dongen, J. T., Thomas-Oates, J., et al. (2015). Mass spectrometry-based plant metabolomics: metabolite responses to abiotic stress. *Mass Spectrom. Rev.* doi: 10.1002/mas.21449 [Epub ahead of print].
- Jung, H. W., Tschaplinski, T. J., Wang, L., Glazebrook, J., and Greenberg, J. T. (2009). Priming in systemic plant immunity. *Science* 324, 89–91. doi: 10.1126/science.1170025
- Keeping, M. G., and Kvedaras, O. L. (2008). Silicon as a plant defence against insect herbivory: response to massey, ennos and hartley. *J. Anim. Ecol.* 77, 631–633. doi: 10.1111/j.1365-2656.2008.01380.x
- Keeping, M. G., Kvedaras, O. L., and Bruton, A. G. (2009). Epidermal silicon in sugarcane: cultivar differences and role in resistance to sugarcane borer *Eldana saccharina*. *Environ. Exp. Bot.* 66, 54–60. doi: 10.1016/j.envexpbot.2008.12.012
- Keeping, M. G., Meyer, J. H., and Sewpersad, C. (2013). Soil silicon amendments increase resistance of sugarcane to stalk borer *Eldana saccharina* walker (Lepidoptera: Pyralidae) under field conditions. *Plant Soil* 363, 297–318. doi: 10.1007/s11104-012-1325-1
- Keeping, M. G., and Reynolds, O. L. (2009). Silicon in agriculture: new insights, new significance and growing application. *Ann. Appl. Biol.* 155, 153–154. doi: 10.1111/j.1744-7348.2009.00358.x
- Kocher, T., Swart, R., and Mechtler, K. (2011). Ultra-high-pressure RPLC hyphenated to an LTQ-Orbitrap velos reveals a linear relation between peak capacity and number of identified peptides. *Anal. Chem.* 83, 2699–2704. doi: 10.1021/ac103243t
- Korndorfer, A. P., Cherry, R., and Nagata, R. (2004). Effect of calcium silicate on feeding and development of tropical sod webworms (Lepidoptera: Pyralidae). *Flor. Entomol.* 87, 393–395. doi: 10.1653/0015-4040(2004)087[0393:EOCSOF]2.0.CO;2
- Kvedaras, O. L., An, M., Choi, Y. S., and Gurr, G. M. (2010). Silicon enhances natural enemy attraction and biological control through induced plant defences. *Bull. Entomol. Res.* 100, 367–371. doi: 10.1017/S0007485309990265
- Kvedaras, O. L., Byrne, M. J., Coombes, N. E., and Keeping, M. G. (2009). Influence of plant silicon and sugarcane cultivar on mandibular wear in the stalk borer *Eldana saccharina*. *Agric. For. Entomol.* 11, 301–306. doi: 10.1111/j.1461-9563.2009.00430.x
- Kvedaras, O. L., and Keeping, M. G. (2007). Silicon impedes stalk penetration by the borer *Eldana saccharina* in sugarcane. *Entomol. Exp. Appl.* 125, 103–110. doi: 10.1111/j.1570-7458.2007.00604.x
- Kvedaras, O. L., Keeping, M. G., Goebel, F. R., and Byrne, M. J. (2007a). Larval performance of the pyralid boree *Eldana saccharina* walker and stalk damage in sugarcane: influence of plant silicon, cultivar and feeding site. *Int. J. Pest Manag.* 53, 183–195. doi: 10.1080/09670870601110956
- Kvedaras, O. L., Keeping, M. G., Goebel, R., and Byrne, M. (2007b). Water stress augments silicon-mediated resistance of susceptible sugarcane cultivars to the stalk borer, *Eldana saccharina* (Lepidoptera: Pyralidae). *Bull. Entomol. Res.* 97, 175–183. doi: 10.1017/S0007485307004853
- Lewandowska-Gnatowska, E., Johnston, M. L., Antoine, W., Szczegielniak, J., Muszynska, G., and Miernyk, J. A. (2011). Using multiplex-staining to study changes in the maize leaf phosphoproteome in response to mechanical wounding. *Phytochemistry* 72, 1285–1292. doi: 10.1016/j.phytochem.2011.01.030
- Liang, Y., Chen, Q., Liu, Q., Zhang, W., and Ding, R. (2003). Exogenous silicon (Si) increases antioxidant enzyme activity and reduces lipid peroxidation in roots of salt-stressed barley (*Hordeum vulgare* L.). *J. Plant Physiol.* 160, 1157–1164. doi: 10.1078/0176-1617-01065
- Liang, Y., Hua, H., Zhu, Y.-G., Zhang, J., Cheng, C., and Roemheld, V. (2006). Importance of plant species and external silicon concentration to active silicon uptake and transport. *New Phytol.* 172, 63–72. doi: 10.1111/j.1469-8137.2006.01797.x
- Liang, Y., Nikolic, M., Belanger, R., Haijun, G., and Song, A. (2015). *Silicon in Agriculture. From Theory to Practice.* Dordrecht: Springer.
- Liang, Y. C., Ma, T. S., Li, F. J., and Feng, Y. J. (1994). Silicon availability and response of rice and wheat to silicon in calcareous soils. *Commun. Soil Sci. Plant Anal.* 25, 2285–2297. doi: 10.1080/00103629409369189
- Liang, Y. C., Sun, W. C., Si, J., and Römhild, V. (2005). Effects of foliar- and root-applied silicon on the enhancement of induced resistance to powdery mildew in *Cucumis sativus*. *Plant Pathol.* 54, 678–685. doi: 10.1111/j.1365-3059.2005.01246.x
- Ly, L., and Wasinger, V. C. (2008). Peptide enrichment and protein fractionation using selective electrophoresis. *Proteomics* 8, 4197–4208. doi: 10.1002/pmic.200701088
- Ly, L., and Wasinger, V. C. (2011). Protein and peptide fractionation, enrichment and depletion: tools for the complex proteome. *Proteomics* 11, 513–534. doi: 10.1002/pmic.201000394
- Ma, J. F., and Takahashi, E. (2002). *Soil, Fertilizer, and Plant Silicon Research in Japan.* Amsterdam: Elsevier.
- Ma, J. F., Tamai, K., Yamaji, N., Mitani, N., Konishi, S., Katsuhabara, M., et al. (2006). A silicon transporter in rice. *Nature* 440, 688–691. doi: 10.1038/nature04590
- Ma, J. F., Yamaji, N., Mitani, N., Tamai, K., Konishi, S., Fujiwara, T., et al. (2007). An efflux transporter of silicon in rice. *Nature* 448, 209–U212. doi: 10.1038/nature05964
- Mallick, P., Schirle, M., Chen, S. S., Flory, M. R., Lee, H., Martin, D., et al. (2007). Computational prediction of proteotypic peptides for quantitative proteomics. *Nat. Biotechnol.* 25, 125–131. doi: 10.1038/nbt1275
- Massey, F. P., Ennos, A. R., and Hartley, S. E. (2007). Herbivore specific induction of silica-based plant defences. *Oecologia* 152, 677–683. doi: 10.1007/s00442-007-0703-5
- Massey, F. P., and Hartley, S. E. (2006). “Experimental demonstration of the antiherbivore effects of silica in grasses: impacts on foliage digestibility and vole growth rates,” in *Proceedings of the Royal Society of London Royal Society*, London, 2299–2304.
- Massey, F. P., and Hartley, S. E. (2009). Physical defences wear you down: progressive and irreversible impacts of silica on insect herbivores. *J. Anim. Ecol.* 78, 281–291. doi: 10.1111/j.1365-2656.2008.01472.x
- Massey, F. P., Massey, K., Ennos, A. R., and Hartley, S. E. (2009). Impacts of silica-based defences in grasses on the feeding preferences of sheep. *Basic Appl. Ecol.* 10, 622–630. doi: 10.1016/j.baae.2009.04.004
- Massey, F. P., Smith, M. J., Lambin, X., and Hartley, S. E. (2008). Are silica defences in grasses driving vole population cycle? *Biol. Lett.* 4, 419–422. doi: 10.1098/rsbl.2008.0106
- McNaughton, S. J., and Tarrants, J. L. (1983). Grass leaf silicification: natural selection for an inducible defense against herbivores. *Proc. Natl. Acad. Sci. U.S.A.* 80, 790–791. doi: 10.1073/pnas.80.3.790
- Miles, N., Manson, A. D., Rhodes, R., van Antwerpen, R., and Weigel, A. (2014). Extractable silicon in soils of the South African sugar industry and relationships with crop uptake. *Commun. Soil Sci. Plant Anal.* 45, 2949–2958. doi: 10.1080/00103624.2014.956881

- Mitani, N., Chiba, Y., Yamaji, N., and Ma, J. F. (2009a). Identification and characterization of maize and barley Lsi2-Like silicon efflux transporters reveals a distinct silicon uptake system from that in rice. *Plant Cell* 21, 2133–2142. doi: 10.1101/tpc.109.067884
- Mitani, N., and Ma, J. F. (2005). Uptake system of silicon in different plant species. *J. Exp. Bot.* 56, 1255–1261. doi: 10.1093/jxb/eri121
- Mitani, N., Yamaji, N., Ago, Y., Iwasaki, K., and Ma, J. F. (2011). Isolation and functional characterization of an influx silicon transporter in two pumpkin cultivars contrasting in silicon accumulation. *Plant J.* 66, 231–240. doi: 10.1111/j.1365-313X.2011.04483.x
- Mitani, N., Yamaji, N., and Ma, J. F. (2009b). Identification of maize silicon influx transporters. *Plant Cell Physiol.* 50, 5–12. doi: 10.1093/pcp/pcn110
- Mitani-Ueno, N., Yamaji, N., and Ma, J. F. (2011). Silicon efflux transporters isolated from two pumpkin cultivars contrasting in Si uptake. *Plant Signal. Behav.* 6, 991–994. doi: 10.4161/psb.6.7.15462
- Montpetit, J., Vivancos, J., Mitani-Ueno, N., Yamaji, N., Remus-Borel, W., Belzile, F., et al. (2012). Cloning, functional characterization and heterologous expression of TaLsi1, a wheat silicon transporter gene. *Plant Mol. Biol.* 79, 35–46. doi: 10.1007/s11103-012-9892-3
- Moraes, J. C., Goussain, M. M., Basagli, M. A. B., Carvalho, G. A., Ecole, C. C., and Sampaio, M. V. (2004). Silicon influence on the tritrophic interaction: wheat plants, the greenbug *Schizaphis graminum* (Rondani) (Hemiptera: Aphididae), and its natural enemies, *Chrysoperla externa* (Hagen) (Neuroptera: Chrysopidae) and *Aphidius colemani* viereck (Hymenoptera: Aphidiidae). *Neotrop. Entomol.* 33, 619–624.
- Nikpay, A., and Nejadian, E. S. (2014). Field applications of silicon-based fertilizers against sugarcane yellow mite *Oligonychus sacchari*. *Sugar Technol.* 16, 319–324. doi: 10.1007/s12355-013-0276-z
- Oliveira, B. M., Coorssen, J. R., and Martins-de-Souza, D. (2014). 2DE: the phoenix of proteomics. *J. Proteom.* 104, 140–150. doi: 10.1016/j.jprot.2014.03.035
- Parry, D. W., Hodson, M. J., Sangster, A. G., Jones, W. C., and Neill, C. H. (1984). Some recent advances in studies of silicon in higher plants [and discussion]. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 304, 537–549. doi: 10.1098/rstb.1984.0045
- Rahman, A., Wallis, C., and Uddin, W. (2015). Silicon induced systemic defense responses in perennial ryegrass against infection by *Magnaporthe oryzae*. *Phytopathology* 105, 748–757. doi: 10.1094/PHYTO-12-14-0378-R
- Raven, J. A. (1983). The transport and function of silicon in plants. *Biol. Rev.* 58, 179–207. doi: 10.1111/j.1469-185X.1983.tb00385.x
- Redmond, C. T., and Potter, D. A. (2006). Silicon fertilization does not enhance creeping bentgrass resistance to cutworms and white grubs. *Appl. Turfgrass Sci.* 6, 1–7.
- Rémus-Borel, W., Menzies, J. G., and Bélanger, R. R. (2005). Silicon induces antifungal compounds in powdery mildew-infected wheat. *Physiol. Mol. Plant Pathol.* 66, 108–115. doi: 10.1016/j.pmp.2005.05.006
- Reynolds, J. H., Lambin, X., Massey, F. P., Reidinger, S., Sherratt, J. A., Smith, M. J., et al. (2012). Delayed induced silica defences in grasses and their potential for destabilising herbivore population dynamics. *Oecologia* 170, 445–456. doi: 10.1007/s00442-012-2326-8
- Reynolds, O. L., Keeping, M. G., and Meyer, J. H. (2009). Silicon-augmented resistance of plants to herbivorous insects: a review. *Ann. Appl. Biol.* 155, 171–186. doi: 10.1111/j.1744-7348.2009.00348.x
- Richards, A. L., Hebert, A. S., Ulbrich, A., Bailey, D. J., Coughlin, E. E., Westphal, M. S., et al. (2015). One-hour proteome analysis in yeast. *Nat. Protoc.* 10, 701–714. doi: 10.1038/nprot.2015.040
- Righetti, P. G., Castagna, A., Antonioli, P., and Boschetti, E. (2005). Prefractionation techniques in proteome analysis: the mining tools of the third millennium. *Electrophoresis* 26, 297–319. doi: 10.1002/elps.200406189
- Rodrigues, F. A., McNally, D. J., Datnoff, L. E., Jones, J. B., Labbe, C., Benhamou, N., et al. (2004). Silicon enhances the accumulation of diterpenoid phytoalexins in rice: a potential mechanism for blast resistance. *Phytopathology* 94, 177–183. doi: 10.1094/PHYTO.2004.94.2.177
- Samuels, A. L., Glass, A. D. M., Ehret, D. L., and Menzies, J. G. (1991a). Distribution of silicon in cucumber leaves during infection by powdery mildew fungus (*Sphaerotheca fuliginea*). *Can. J. Bot.* 69, 140–146. doi: 10.1139/b91-020
- Samuels, A. L., Glass, A. D. M., Ehret, D. L., and Menzies, J. G. (1991b). Mobility and deposition of silicon in cucumber plants. *Plant Cell Environ.* 14, 485–492. doi: 10.1111/j.1365-3040.1991.tb01518.x
- Silva, R. V., de Lima Oliveira, R. D. A., da Silva Ferreira, P., Castro, D. B., and Rodrigues, F. A. (2015). Effects of silicon on the penetration and reproduction events of *Meloidogyne exigua* on coffee roots. *Bragantia* 74, 196–199. doi: 10.1590/1678-4499.360
- Simmons, A. T., and Gurr, G. M. (2004). Trichome-based host plant resistance of *Lycopersicon* species and the biocontrol agent *Mallada signata*: are they compatible? *Entomol. Exp. Appl.* 113, 95–101. doi: 10.10111/j.0013-8703.2004.00210.x
- Simmons, A. T., and Gurr, G. M. (2005). Trichomes of *Lycopersicon* species and their hybrids: effects on pests and natural enemies. *Agric. For. Entomol.* 7, 265–276. doi: 10.1111/j.1461-9555.2005.00271.x
- Slaughter, A., Daniel, X., Flors, V., Luna, E., Hohn, B., and Mauch-Mani, B. (2012). Descendants of primed *Arabidopsis* plants exhibit resistance to biotic stress. *Plant Physiol.* 158, 835–843. doi: 10.1104/pp.111.191593
- Smith, M. T., Kvedaras, O. L., and Keeping, M. G. (2007). A novel method to determine larval mandibular wear of the African stalk borer, *Eldana saccharina* walker (Lepidoptera: Pyralidae). *Afr. Entomol.* 15, 204–208. doi: 10.4001/1021-3589-15.1.204
- Stanley, J. N., Baqir, H. A., and McLaren, T. I. (2014). Effect on larval growth of adding finely ground silicon-bearing minerals (wollastonite or olivine) to artificial diets for *Helicoverpa* spp. (Lepidoptera: Noctuidae). *Austral Entomol.* 53, 436–443. doi: 10.1111/aen.12086
- Stasyk, T., and Huber, L. A. (2004). Zooming in: fractionation strategies in proteomics. *Proteomics* 4, 3704–3716. doi: 10.1002/pmic.200401048
- Struyf, E., Smis, A., Van Damme, S., Garnier, J., Govers, G., Van Wesemael, B., et al. (2010). Historical land use change has lowered terrestrial silica mobilization. *Nat. Commun.* 1:129. doi: 10.1038/ncomms1128
- Takahashi, E., Ma, J. F., and Miyake, Y. (1990). The possibility of silicon as an essential element for higher plants. *J. Agric. Food Chem.* 2, 99–122. doi: 10.1016/j.bbagen.2013.11.021
- Thakur, S. S., Geiger, T., Chatterjee, B., Bandilla, P., Frohlich, F., Cox, J., et al. (2011). Deep and highly sensitive proteome coverage by LC-MS/MS without prefractionation. *Mol. Cell. Proteom.* 10:M110003699. doi: 10.1074/mcp.M110.003699
- Thivierge, K., Prado, A., Driscoll, B. T., Bonneil, E., Thibault, P., and Bede, J. C. (2010). Caterpillar- and salivary-specific modification of plant proteins. *J. Proteome Res.* 9, 5887–5895. doi: 10.1021/pr100643m
- Timbo, R. V., Hermes-Lima, M., Silva, L. P., Mehta, A., Moraes, M. C., and Paula, D. P. (2014). Biochemical aspects of the soybean response to herbivory injury by the brown stink bug *Euschistus heros* (Hemiptera: Pentatomidae). *PLoS ONE* 9:e109735. doi: 10.1371/journal.pone.0109735
- Ton, J., Pieterse, C. M., and Van Loon, L. (2006). “The relationship between basal and induced resistance in *Arabidopsis*,” in *Multigenic and Induced Systemic Resistance in Plants*, eds S. Tuzun and E. Bent (New York, NY: Springer), 197–224.
- Ullmann-Zeunert, L., Muck, A., Wielsch, N., Hufsky, F., Stanton, M. A., Bartram, S., et al. (2012). Determination of (1)(5)N-incorporation into plant proteins and their absolute quantitation: a new tool to study nitrogen flux dynamics and protein pool sizes elicited by plant-herbivore interactions. *J. Proteome Res.* 11, 4947–4960. doi: 10.1021/pr300465n
- Van Bockhaven, J., De Vleesschauwer, D., and Hofte, M. (2013). Towards establishing broad-spectrum disease resistance in plants: silicon leads the way. *J. Exp. Bot.* 64, 1281–1293. doi: 10.1093/jxb/ers329
- Van Bockhaven, J., Spíchal, L., Novák, O., Strnad, M., Asano, T., Kikuchi, S., et al. (2015). Silicon induces resistance to the brown spot fungus *Cochliobolus miyabeanus* by preventing the pathogen from hijacking the rice ethylene pathway. *New Phytol.* 206, 761–773. doi: 10.1111/nph.13270
- Vilela, M., Moraes, J. C., Alves, E., Santos-Cividanes, T. M., and Santos, F. A. (2014). Induced resistance to *Diatraea saccharalis* (Lepidoptera: Crambidae) via silicon application in sugarcane. *Rev. Colomb. Entomol.* 40, 44–48.
- Vivancos, J., Labbe, C., Menzies, J. G., and Belanger, R. R. (2015). Silicon-mediated resistance of *Arabidopsis* against powdery mildew involves mechanisms other than the salicylic acid (SA)-dependent defence pathway. *Mol. Plant Pathol.* 16, 572–582. doi: 10.1111/mpp.12213
- Wang, H.-S., Wang, C. Y., Pei-Pei, F., Bin-Fu, B., Tao, L., and Zhu-Jun, Z. (2015). Identification of two cucumber putative silicon transporter genes in *Cucumis sativus*. *J. Plant Growth Regul.* 34, 332–338. doi: 10.1007/s00344-014-9466-5

- Wieczorek, M., Zub, K., Szafranska, P. A., Ksiazek, A., and Konarzewski, M. (2015). Plant-herbivore interactions: silicon concentration in tussock sedges and population dynamics of root voles. *Funct. Ecol.* 29, 187–194. doi: 10.1111/1365-2435.12327
- Worrall, D., Holroyd, G. H., Moore, J. P., Glowacz, M., Croft, P., Taylor, J. E., et al. (2012). Treating seeds with activators of plant defence generates long-lasting priming of resistance to pests and pathogens. *New Phytol.* 193, 770–778. doi: 10.1111/j.1469-8137.2011.03987.x
- Yamaji, N., Mitatni, N., and Ma, J. F. (2008). A transporter regulating silicon distribution in rice shoots. *Plant Cell* 20, 1381–1389. doi: 10.1105/tpc.108.059311
- Ye, M., Song, Y., Long, J., Wang, R., Baerson, S. R., Pan, Z., et al. (2013). Priming of jasmonate-mediated antiherbivore defense responses in rice by silicon. *Proc. Natl. Acad. Sci. U.S.A.* 110, E3631–E3639. doi: 10.1073/pnas.13058548110
- Yoshida, Y., Sano, R., Wada, T., Takabayashi, J., and Okada, K. (2009). Jasmonic acid control of GLABRA3 links inducible defense and trichome patterning in *Arabidopsis*. *Development* 136, 1039–1048. doi: 10.1242/dev.030585
- Yoshihara, T., Sogawa, M., Pathak, B. O., Juliano, B. O., and Sakamura, S. (1979). Soluble silicic acid as a sucking inhibitory substance in rice against the rice brown planthopper (Delphacidae: Homoptera). *Entomol. Exp. Appl.* 26, 314–322. doi: 10.1111/j.1570-7458.1979.tb02932.x
- Zubarev, R. A. (2013). The challenge of the proteome dynamic range and its implications for in-depth proteomics. *Proteomics* 13, 723–726. doi: 10.1002/pmic.201200451

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The Use of Arbuscular Mycorrhizal Fungi to Improve Strawberry Production in Coir Substrate

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Strawberry is an important fruit crop within the UK. To reduce the impact of soil-borne diseases and extend the production season, more than half of the UK strawberry production is now in substrate (predominantly coir) under protection. Substrates such as coir are usually depleted of microbes including arbuscular mycorrhizal fungi (AMF) and consequently the introduction of beneficial microbes is likely to benefit commercial cropping systems. Inoculating strawberry plants in substrate other than coir has been shown to increase plants tolerance to soil-borne pathogens and water stress. We carried out studies to investigate whether AMF could improve strawberry production in coir under low nitrogen input and regulated deficit irrigation. Application of AMF led to an appreciable increase in the size and number of class I fruit, especially under either deficient irrigation or low nitrogen input condition. However, root length colonization by AMF was reduced in strawberry grown in coir compared to soil and Terragreen. Furthermore, the appearance of AMF colonizing strawberry and maize roots grown in coir showed some physical differences from the structure in colonized roots in soil and Terragreen: the colonization structure appeared to be more compact and smaller in coir.

Keywords: strawberry, yield, growing substrate, AMF, coir, Class I yield quality

INTRODUCTION

Strawberry is an important horticultural crop in the UK and is a highly nutritious and important food source. Strawberry accounted for 67% of all soft fruit production worth an estimated £247 million in 2013 (DEFRA, 2015), and this is set to rise significantly over the coming years. Recently, a significant trend in commercial strawberry cropping has been to move away from traditional field cultivation toward production into substrate. Industry estimates that more than 50% of the UK strawberry production is produced in substrates, usually coir (coconut fiber) and mostly under protection (polythene tunnel or glasshouse). This change was intended to mitigate the threat of soil-borne fungal pathogens, principally wilt (*Verticillium dahliae* Kleb). Chemical treatments have been an indispensable tool for controlling soil-borne pathogens; however, several of these treatments are already banned or face an uncertain future due to legislation (Martin, 2003). There are many significant benefits to the adoption of substrates in commercial strawberry cropping, such as to extension of the growing season, increased ease of picking and better control of the crop from

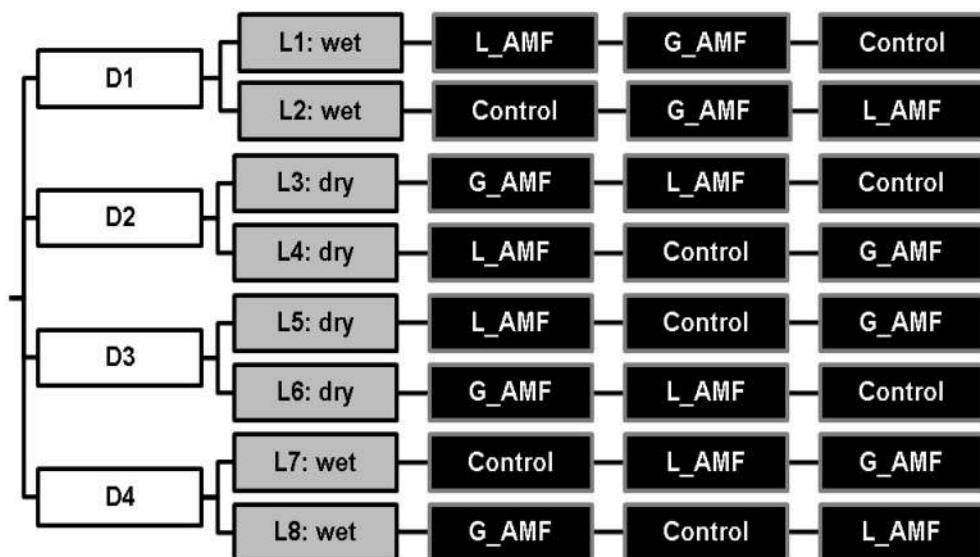


FIGURE 1 | Experimental setup for the study of the effect of AMF inoculation on strawberry in coir. AMF inoculation treatments consisted of either non-inoculated control, Liquid spore suspension of *R. irregularis* (L_AMF) or granular application of commercial inoculum (G_AMF). Two levels of irrigation were included, fully watered (wet), and 60% RDI (dry), along with 2 levels of Nitrogen level, D1 and 2 at the standard commercial rate and D3 and 4 at a 60% reduction of the standard rate. The experiment was repeated three times.

fertigation and pollination regimes. However, this practice relies heavily on high inputs of water and fertigation; these inputs are estimated to be more than doubled compared to a field grown crop, with an increased cost of up to £1800 per Hectare.

Arbuscular mycorrhizal fungi (AMF) penetrate the roots of plants to form a mutualistic symbiotic relationship. Mineral nutrients, mainly phosphorus, nitrogen and water are extracted from the soil via the extensive hyphal network and transferred to the plant. Organic carbon compounds are transferred to the AMF in return. They are known to improve plant nutrient uptake, protect plants from pathogens (Borowicz, 2001; Ismail and Hijri, 2012; Ren et al., 2013) and buffer against adverse environmental conditions, especially drought (Smith et al., 2010; Robinson-Boyer et al., 2015). A number of studies have reported the beneficial effects of mycorrhiza on strawberry plants (Castellanos-Morales et al., 2010) and commercial AMF inoculum has been shown to increase both growth (crowns, roots and leaf area) and tolerance to water stress in micro-propagated strawberry (Borkowska, 2002).

The maintenance of a developed and diverse population of AMF and other soil micro-organisms is important in achieving sustainable agriculture (Jeffries et al., 2003) thus reducing the requirement of such high levels of fertigation. However, products containing AMF are rarely used in commercial agriculture because of (a) difficulties in producing AMF inoculum in large quantities, (b) their variable beneficial effects, and (c) uncertainties in the benefits with added AMF in the presence of resident AMF populations. Substrates such as coir are usually devoid of beneficial microbes such as AMF; thus introducing them into substrate production is more likely to generate benefits.

This paper reports results from three studies on the use of AMF in strawberry production in coir substrate. First, we assessed whether use of AMF in substrate could improve strawberry fruit yield in respect to water stress and nutrient input. This work showed positive effects of the addition of AMF in fruit production despite observing low levels of AMF colonization and compact, immature mycorrhizal structures inside colonized roots of strawberry. Thus, we conducted further experiments to better understand the extent and structure of root colonization in different types of substrate (including soil). Furthermore, to establish if the effects observed on root colonization were limited to strawberry only, we included maize in the experiment as maize is a common, highly mycorrhizal host plant of AMF.

MATERIALS AND METHODS

Effect of AMF Inoculation of Strawberry in Coir

The experimental design was a full factorial design with three factors: AMF inoculation, irrigation and nutrient. For AMF inoculation, there were three treatment levels: negative control with no inoculum added, and application of either granular or liquid formulation of AMF (G_AMF or L_AMF). There were two irrigation regimes: well-watered, to capacity (WW), and regulated deficit irrigation (RDI, 60% of the WW). There were two nutrient input regimes: standard or reduced nitrogen input (60% of the standard). Thus, there were 12 treatments in total (see Figure 1). This experiment was conducted on three separate occasions, with two replicates of each treatment each time.

Agronomical management of strawberry (including dosatron setting, nutrient composition and irrigation) followed previously established protocols (Xu et al., 2013), which were based on current commercial practices. From a combination of visual assessment of water leakage from coir bags and moisture content measurements, estimated using a Delta-T "WET" sensor (Delta-T Devices, Cambridge, UK), the amount of irrigation water was adjusted as necessary via a Galcon irrigation timer. Overall, the volume of irrigation water applied increased gradually over time, reached the maximum at the first week of blossom and thereafter remained at this level, equivalent to 2 L per day per bag.

Irrigation and fertigation were delivered to plants via eight irrigation lines using drippers, two of which were controlled by a separate irrigation controller (dosatron). Each dosatron was randomly allocated to one of the four nutrient and water combinational treatments. Within each irrigation line, there were three replicate bags, each allocated to one of the three AMF treatments. Thus, the experiment was a split plot design—the main plot was the dosatron (two irrigation lines) and the subplot was the individual coir bag. All coir bags (BotaniCoir, England) prior to planting were saturated with water over a period of 2 weeks in order to re-hydrate the coir. Inoculum of AMF was supplied by PlantWorks Ltd, Kent, UK, the granular formulation applied as commercially available "Rootgrow" (*Funneliformis mosseae*, *F. geosporus*, *Claroideoglomus claroideum*, *Glomus microaggregatum*, *Rhizophagus irregularis*), containing propagules of spores, hyphal and root fragments. The liquid application was an *in-vitro* produced preparation of *R. irregularis* DAOM197198 (consisting of sterile water and spores).

Cold-stored (-2°C) runners of cv. Elsanta (Hargreaves Plants, UK) were planted in coir bags. At the time of planting, for the G_AMF treatment, 20 g of granular AMF was placed to a single planting hole before the plant was planted; for L_AMF, a liquid AMF suspension [4 ml estimated to be taken up per plant] was applied to the roots of individual runners and for the control nothing was added. For both G_AMF and L_AMF each plant received ca. 6650 propagules of AMF estimated using MPN analysis (Cochran, 1950). After the onset of flowering a mini hive of bees, *Bombus terrestris*, (Agralan, UK) was introduced to the compartment to pollinate (with the exception of the first replicate experiment). Plants were grown in a GroDome compartment (Unigro, UK) set at 22°C day/ 20°C night with a 16 h day/8 h night cycle with supplementary lighting.

A sample of roots from a number of plants was assessed prior to planting to check for colonization by AMF. Roots were cleared with KOH before being stained using Trypan Blue and assessed microscopically for root length colonization (RLC) using the grid-line intersect method (McGonigle et al., 1990). Colonization was expressed as a percentage of the root colonized by AMF. Ripe fruit were picked regularly (2–3 times weekly). Except for the first experiment, fruit were divided by size into Class I (above 18 mm diameter) & II and weighed separately for individual bags and the number of fruit was recorded. For the first experiment, because of smaller fruit (lack of pollinators), fruit were not divided into Class I or II. After harvesting, fresh weight of individual plants (both above- and below-ground parts) was determined. A composite sample of roots was taken for each coir bag at harvest to check

colonization by AMF. Only fresh, recently formed roots were sampled, and the original runner roots were avoided. Roots were stained and assessed as above.

The Effect of Substrate on Colonization by AMF

Substrate Effect and Time of Inoculation

Maize (cv Thalys, Cotswold Seeds, UK) was used in this experiment since it is known to be highly responsive to AMF and a common host for production of commercial AMF and is used here to study the effect of substrate on root colonization. There were three treatment factors: pre-emergence inoculation with AMF (PreAMF: Yes or No), post-emergence inoculation with AMF (PostAMF: Yes or NO). Substrates compared were Top soil: S, Terragreen: T, coir: C, and peat-free compost: PF. These substrates are commonly used for commercial cropping with the exception of Terragreen (attapulgite clay; OilDry, UK) which is routinely used in the study of AMF, giving a clear indication of "expected" colonization. A randomized block design was used with five blocks. There were two pots per treatment in each block: one for destructive sampling 4 weeks after transplanting, and the other after 10 weeks.

Maize seeds were first soaked in sterile water for 24 h. Multi-cell trays (cell volume 250 ml) were filled with Terragreen. Half of the cells were amended with 10 g of granular AMF inoculum to allow inoculation of seedlings (pre-emergence). One seed was manually sown 2 cm deep per cell. Seedlings for the Pre-AMF inoculation treatment were sampled and checked microscopically for colonization by AMF (as above) and only those seedlings with colonization were retained. Prior to transplanting, a sufficient number of coir bags were thoroughly wetted; coir from these bags was then used to fill pots. Similarly, top soil (ca top 10–15 cm) from a plot at East Malling Research was obtained to fill pots; peat-free compost was purchased commercially (Dobbies, UK). Seedlings were transplanted approximately 2 weeks after sowing. On the day before transplanting, all pots were thoroughly watered to reach the fully-wet state. A planting hole was made in each pot and 10 ml AMF sprinkled into the hole for those pots allocated to the Post-AMF inoculation treatment. Then a single seedling was transplanted to each single pot (4 L). All plants were fed with Vitafeed 102 (Vitax, UK) 1 gL^{-1} every 2 weeks. The height and stem diameter, just above the substrate surface and colonization by AMF (RLC) were assessed destructively 4 and 10 weeks after transplanting. The root samples analyzed at 10 weeks were sampled from two positions on the plants, firstly very close to the inoculation site and secondly from the peripheral roots, and stained and assessed as above.

Effect of Coir Substrate on AMF Colonization

For this study, both maize and strawberry were used to compare colonization by AMF in coir and in Terragreen. Maize seedlings (cv. Jubilee F1, B&Q, UK) were obtained as in the previous experiment, except Terragreen was not amended with inoculum of AMF. Strawberry module plants (cv. Elsanta), produced from tipping in compost, were obtained from a commercial nursery (Hargreaves plants, UK); plants derived in this way have shown

in previous work to be free from colonization by AMF (Xu, unpublished data), although a few plants were tested prior to the experiment to confirm this. Individual plants were transplanted to 1 L pots [one plant per pot]; all plants were inoculated with 20 g of granular inoculum of AMF at the time of transplanting. There were 10 replicate pots per treatment (substrate [Terragreen or coir] and host [strawberry or maize]). A complete randomisation design was used. A standard commercial fertigation scheme of N-P-K = 120-45-176 for strawberry was used to manage the plants (J. Atwood, ADAS, England, per. comm.). Only eight strawberry and four maize plants per substrate (randomly selected from 10 plants in each treatment) were sampled to assess root colonization 10 weeks after transplanting; the amount of vesicles, arbuscules and hyphae were also recorded.

Data Analysis

ANOVA of a split-plot design was applied to strawberry data from all three experiments treating individual experiments as a blocking factor, using GenStat 13 (VSN International, England). In addition to total Class I yield and number of Class I fruit, average individual fruit weight was also analyzed. Fresh plant weight was also used as a co-variate in ANOVA but it did not alter the main results; therefore, only results from ANOVA without the co-variate are presented. Interactions between three factors: AMF, irrigation and nitrogen input were statistically tested. For the data on AMF colonization in different substrates, standard ANOVA were used to compare treatment effect. In all analyses, once ANOVA indicated significant effects of a specific treatment factor or interaction, pairwise comparison was then carried out based on the LSD test. Common diagnostic plots (e.g., q-q plot, residuals-fitted value plot) did not reveal apparent violation of the normality and homoscedasticity assumptions. Hence no transformation was necessary in order to satisfy ANOVA assumptions for fruit yield and RLC data.

RESULTS

AMF on Strawberry Production in Coir

Strawberry plants in all treatments grew normally and there were no visual differences in plant growth between treatments. Fewer and lighter fruit were produced in the first replicate experiment than in the other two experiments, due to less developed fruit from the lack of insect pollination. Plants in each coir bag on average produced 57, 127, and 108 fruit for the 1st, 2nd, and 3rd replicate experiment, respectively; the corresponding average fruit weight was 4.7, 16.8, and 11.3 g. There was a large variation in fruit yield among individual picks in all three replicate experiments but the three AMF treatments followed a similar trend over time (**Figure 2**). AMF-treated strawberries (particularly G_AMF) had increased fruit production in the mid to late harvest period (**Figure 2**).

Because of the lack of bee pollinators, the 1st replicate experiment was excluded from statistical comparisons, hence all the subsequent presentations were from statistical analysis of replicate experiments 2 and 3 (**Table 1**). There were significant differences in the yield [total class I fruit weight; $F_{(2, 24)} = 3.43, P < 0.05$], and number of fruit per

plant [$F_{(2, 24)} = 3.30, P < 0.05$] among AMF treatments. The G_AMF treatment led to higher ($P < 0.05$) yields than the control but not different from the L_AMF treatment (**Figure 3**). There were also no significant differences in yields between the L_AMF and the control treatments. On average the G_AMF and L_AMF treatments led to a greater ($P < 0.05$) number of fruit than the control (**Figure 3**). In both experiments, G_AMF had higher yield than L_AMF, although this difference is not statistically significant. Higher yields and more fruit were obtained in both high nitrogen and well-watered treatments than the low nitrogen and RDI treatments but these differences were not statistically significant (**Table 1**). For both the average fruit weight and plant fresh weight, none of treatments resulted in significant differences (**Table 1**).

All interactions involving any two factors were not statistically significant (**Table 1**). However, the three-way interaction was significant for both the total yield [$F_{(2, 24)} = 4.59, P < 0.05$] and average number of fruit per plant [$F_{(2, 24)} = 7.45, P < 0.01$]. The interactions mainly resulted from the fact that the increase in the yield and number of fruit associated with AMF application was for the high nitrogen input under the deficit irrigation but the low nitrogen input under the wet treatment (**Figure 3**).

Prior to planting, runners were colonized by AMF and the RLC ranged from 20 to 40% for all three experiments. However, after the final harvest, there was almost no colonization (average <1%) found in roots of the control, non-inoculated, plants and the level of AMF colonization found in the roots of the treated plants was low (average <15%) and highly variable among samples; many samples failed to show any colonization. There were no differences between treatments in RLC.

The Effect of Substrate on Colonization by AMF

The Effect of Substrate on RLC of Maize

There was no AMF colonization in non-inoculated plants when assessed 4 or 10 weeks after transplanting. For inoculated plants, RLC at 4 weeks ranged from 0 to 75.0% (with an average of 25.3%); only for five plants were AMF not observed. At 10 weeks, average AMF colonization over all roots, regardless of the position of sampled roots in relation to the inoculation site, was 49.2%; only for a single plant were AMF not observed.

At 4 weeks after transplanting, RLC did not differ significantly between plants inoculated twice at both pre- and post-emergence and those plants only inoculated once (24.4% [both] vs. 25.5% [single]; **Table 2**). In contrast, inoculation during sowing resulted in greater [$F_{(1, 43)} = 5.5, P < 0.05$] RLC than inoculation during transplanting: 30.0 vs. 20.9%. There were large [$F_{(3, 43)} = 19.6, P < 0.001$] differences in RLC between four substrates, accounting for ca. 44% of the total variation. Average RLC for Terragreen was 40.9%, significantly ($P < 0.01$) greater than coir (25.5%), and peat (27.1%), which in turn was greater ($P < 0.001$) than the peat-free substrate (6.8%). In addition, RLC differences between two inoculation timings varied with

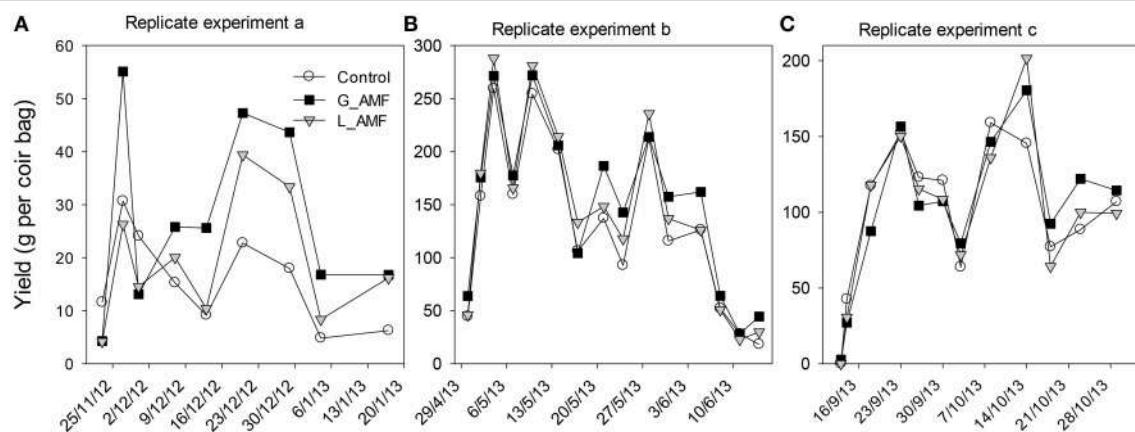


FIGURE 2 | Class I fruit yield (g per coir bag) for each pick date for the three replicate experiments (A–C). Each point was the average of eight individual bags over the four combinations of nitrogen and irrigation treatments. Yield from the first experiment was much lower than the other two because bees were not provided for pollinations.

TABLE 1 | Summary of ANOVA (F-values) of strawberry class I yield in two replicate experiments where granular and liquid AMF products were applied to strawberry plants grown in coir bags.

Terms	Degree of freedom	Yield	Fruit number	Fruit weight	Plant weight
Experiment stratum	1	125.96	7.23	201.37	0.58
EXPERIMENT × LINE STRATUM					
Irrigation	1	0.66	0.00	1.88	0.63
Nitrogen	1	1.56	0.78	0.01	1.97
Irrigation × Nitrogen	1	0.41	0.09	1.89	0.08
Residual	11				
EXPERIMENT × LINE × BAG STRATUM					
AMF	2	3.43*	3.30*	1.41	1.89
AMF × Irrigation	2	0.27	0.10	0.50	0.81
AMF × Nitrogen	2	0.2	1.7	1.22	0.36
AMF × Irrigation × Nitrogen	2	4.59*	7.45**	1.52	0.09
Residual	24				

Plants were also subjected to two irrigation (high, low) and two nitrogen (high, low) treatments, delivered through automated fertigation pipe lines. A split-plot design was used in which the fertigation line and individual coir bags were the main and sub-plots, respectively.

*, **Significant at the level of 0.05 and 0.01, respectively.

substrates [$F_{(3, 43)} = 6.6$, $P < 0.001$]: for both coir and Terragreen, inoculation in sowing led to greater RLC than during transplanting, which was opposite to the situation for peat (Figure 4), and the difference for peat-free was very small.

When assessed 10 weeks after transplanting, the only significant difference in RLC was related to the four substrates [Table 2; $F_{(3, 42)} = 27.9$ and $F_{(3, 43)} = 91.7$ ($P < 0.001$)] for RLC near and further away from the inoculation site, respectively. For roots near the inoculation site, RLC was greatest for Terragreen (81.2%) and least for peat-free (16.2%; Figure 4B) and RLC did not differ significantly between Terragreen (81.2%) and peat (70.8%). The relative differences in RLC in roots further away from the inoculation site between the four substrates were similar as for the near-inoculation-site, except that the difference between Terragreen and peat was significant ($P < 0.05$). RLC differences between the two root positions also varied [$F_{(1, 42)}$

= 4.5, $P < 0.01$; Table 2] with substrates: for both coir and peat-free, RLC was less on the roots far from the inoculation site whereas no such differences were observed for Terragreen and peat (Figure 4B).

Effect of Coir Substrate on AMF Colonization

Overall AMF colonization was lower [$F_{(1, 23)} = 5.6$, $P < 0.05$] in coir (13.1%) than in Terragreen (29.3%); average colonization was greater [$F_{(1, 23)} = 10.5$, $P < 0.01$] in maize (36.8%) than in strawberry (13.4%). There were no significant interactions between hosts and substrates in affecting AMF colonization. Although, there were significant differences between treatments, the level of AMF colonization varied considerably within individual treatments.

The morphology of the mycorrhizal structures in coir was different from those in Terragreen (Figure 5), in which

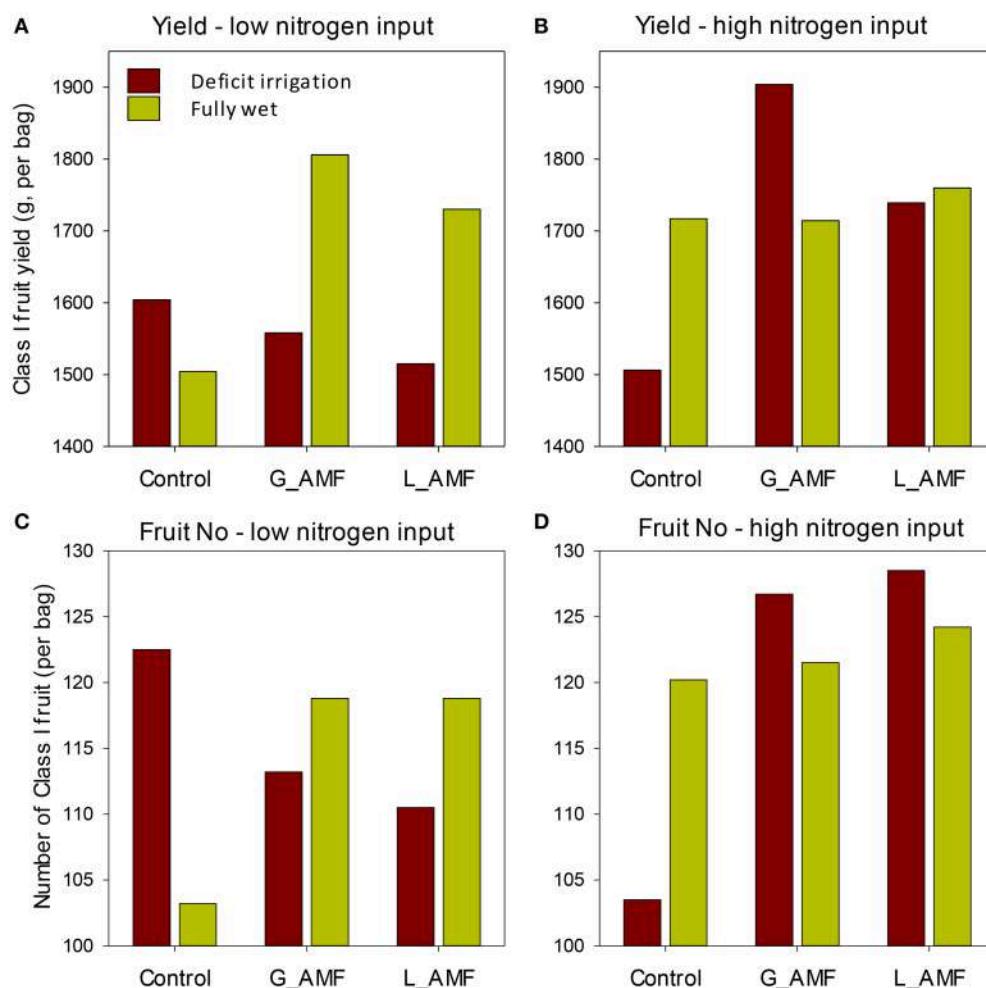


FIGURE 3 | Average class I fruit yield (A,B) and number of fruit (C,D) for each combination of AMF, nitrogen input and irrigation treatments for strawberry grown in coir bags in the replicate experiments. The standard error of differences [sed] for the main AMF treatments was 63.0 g and 3.6 for yield and fruit number, respectively; the corresponding value for irrigation and nitrogen was 83.2 g and 7.2. The sed for the means of each AMF, irrigation and nitrogen combination was 156.3 g [yield] and 11.7 [fruit number], except when comparing means with the same combination of irrigation or nitrogen—which were 126 g and 7.3, respectively.

normal colonization by AMF with fully-formed clear arbuscule structures was observed. Because of the changed structure in coir, a much larger root sample was assessed for colonization using a grid line technique. In strawberries growing in coir, the arbuscules and vesicles were small, underdeveloped, and their presence was inconsistent in the colonized roots—in many cases only hyphae were observed.

DISCUSSION

Inoculation with a commercial AMF product in coir increased yield and number of class I fruit of strawberry, particularly under stress conditions of deficient irrigation or low nitrogen input. The granular product of mixed AMF species resulted in a consistent limited benefit (though not statistically significant) to strawberry plants than the liquid inoculum, although the same number of infective propagules was added at planting in either formulation.

This difference could be because the liquid formulation has only a single species (*R. irregularis*), whereas the granular has four species of AMF. Thus, there could be synergistic interactions among AMF species in promoting plant growth (Wagg et al., 2011). However, recent work suggested that addition of two AMF species/strains did not result in improved performance of strawberry plants in compost or Terragreen relative to the use of individual species/strains (Robinson-Boyer et al., 2015). Another possible cause could be that with the liquid formulation there may be considerable losses of inoculum with irrigation water to the bottom of the bag that was not reachable by roots. The increase in fruit production was mainly associated with either of the two stress conditions singly, but not when combined. This suggests that AMF can alleviate the negative effects of either drought or low nitrogen. However, the positive effects of AMF on plant may be limited if plants are subjected extreme or combined stresses, which needs further research. Currently, we

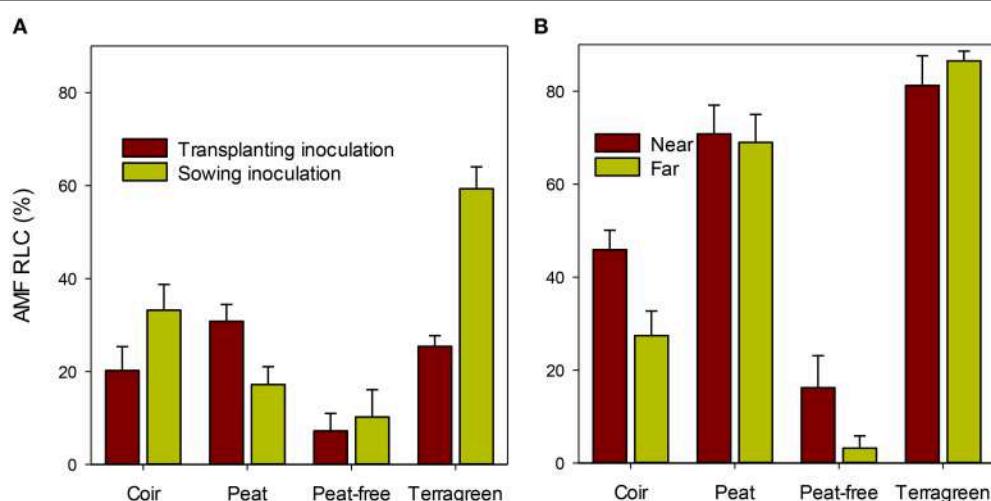
TABLE 2 | Summary of ANOVA (*F*-values) of AMF root length colonization (RLC) where granular AMF products were applied to maize plants grown in four different types of substrates at sowing time only (SO), or transplanting time only (TO), or both (ST).

Terms	Degree of freedom	RLC 4 weeks	RLC-near 10 weeks	RLC-away 10 weeks
Block	4	1.17	2.94	1.58
AMF inoculation time	2	2.80	0.95	1.51
SO vs. TO	1	0.10	0.95	2.37
ST vs. (SO + TO)	1	5.50*	0.94	0.64
Substrate	3	19.6***	27.9***	91.7***
AMF inoculation × Substrate	6	3.84**	1.24	1.79
(SO vs. TO) × Substrate	3	1.11	0.73	2.25
[ST vs. (SO + TO)] × Substrate	3	6.56***	1.76	1.33
Residual	43 ⁺			

AMF colonization was assessed at 4 and 10 weeks post-transplanting; for the 10 week assessment, two types of roots were sampled—those near or further away from the AMF inoculation site.

* , ** , ***Significant at the level of 0.05, 0.01, and 0.001, respectively.

⁺For RLC near the inoculation sites, there was only 42° of freedom for the residuals.

**FIGURE 4 |** Average root length colonization (RLC) by AMF of maize roots in four different substrates at two inoculation times (either during the sowing or during the transplanting) when assessed at 4 weeks (A) [sed for the substrate means = 7.75%]; average RLC at the two root positions relative to the inoculation site when assessed at 10 weeks (B) [sed is 6.7 and 4.9% for near to and away from inoculation sites, respectively].

are conducting transcriptomics research trying to shed light on this AMF-strawberry interaction in coir.

Surprisingly, with such an appreciable increase in plant growth/yield, there were very low levels of root colonization by AMF under any of the conditions in coir. This is remarkable given that the original native colonization found in the planting material (runners lifted from field-grown mother plants) had ca. 20% RLC by AMF, which did not appear to spread and establish into the roots produced post-planting in the control plants. The detection of AMF was by root staining and thus does not give an indication of the viability of the AMF colonization present. This may be important considering these runner plants would have overwintered at -2°C prior to planting. Root colonization by AMF in the treated plants was also mostly found in roots that had developed in close proximity to the inoculation site. Reduced root colonization by AMF in coir was also shown for

maize, normally recognized for high RLC levels of AMF. Here we clearly show that the level of root colonization in maize was affected by the substrate in which the plant was grown and was significantly reduced when grown in coir. Again the colonization detected in maize grown in coir was largely close to the site of inoculation.

Such low colonization of roots in coir could indicate that (1) coir is a harsh environment for mycorrhiza to colonize roots, (2) movement of inoculum in coir is limited (as irrigation is well controlled to prevent run-off), (3) spore production from colonized roots is limited and (4) changes to plant root physiology in substrate. In addition to the low level of colonization by AMF in coir, the AMF structures appear to be more compact and immature in coir than in soil and Terragreen on both maize and strawberry. This compact AMF structure in coir was also observed in clover (data not shown).

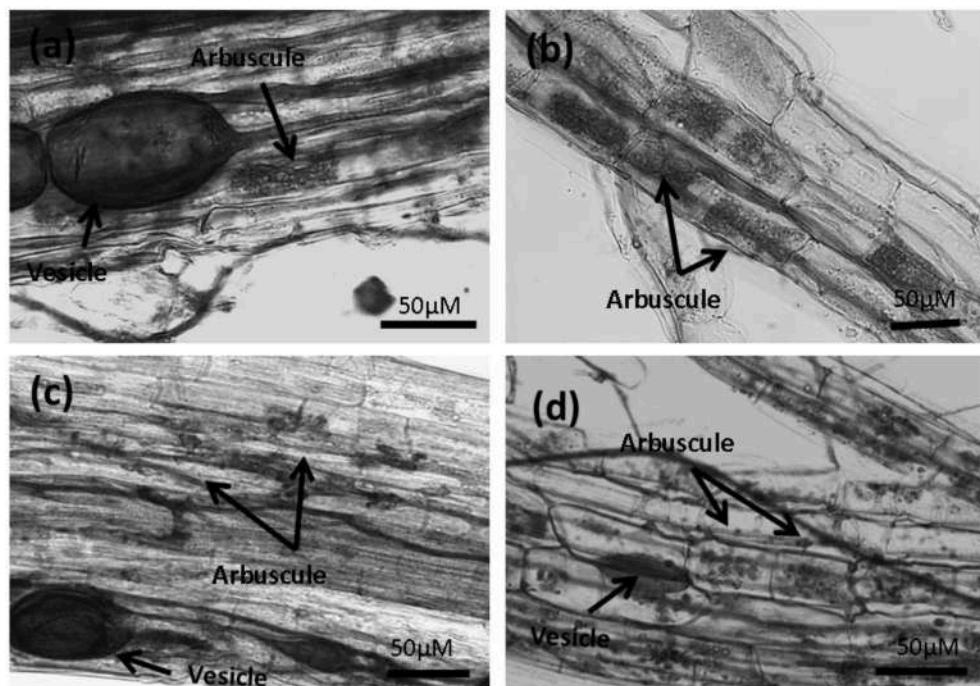


FIGURE 5 | Micrographs of strawberry roots stained using trypan blue showing mycorrhizal structures. (A,B) Plants grown in Terragreen substrate, and **(C,D)** plants grown in coir substrate. Differences can be seen between the arbuscule formation and development in the coir substrate.

Studies (Isayenkov et al., 2004; Hart et al., 2013) have shown that the level of colonization of a root is not necessarily an indicator of mycorrhizal benefit; however it is notable to record such low levels providing large and consistent plant growth promoting effects. It is possible that in a substrate environment, which is highly artificial for plant growth containing no background level of beneficial micro-organisms, colonization by AMF, even at a low level, may be highly beneficial for plant production.

Another consideration in applying AMF in commercial agriculture is to what extent there is a specific interaction of plant growth environment with AMF species or strain genotype. Multiple variants of sequences have been shown to occur within individual spores and isolates, as well as within and between species of the Glomeromycota (Rodriguez et al., 2001). It is important to determine how different growing environments and host plants could influence AMF genomic changes (Krüger et al., 2015) and consequently their beneficial effects on plant growth, enabling specific AMF products under specific conditions to maximize their beneficial effects. Further research is needed to investigate the inter-relationship of AMF effect, colonization structure and colonization levels in different types of substrates.

It is known that in other crop plants, e.g., wheat, genotypes and cultivars can differ in the extent to which they form an association with AMF (Al-Karaki and Al-Raddad, 1997). Further work is needed to assess to what extent the benefit associated with AMF inoculation in coir is dependent on strawberry genotypes (cultivars). To fully exploit the positive

effect of AMF on strawberry in coir substrate, further work is needed to clarify to what extent the ability of specific strawberry genotypes being colonized by AMF in coir is heritable. If this trait is controlled genetically, this could be exploited to breed strawberry plants that can be easily colonized by AMF in substrate to increase their cropping potential and tolerance to pathogens, e.g., powdery mildew and *Phytophthora* diseases.

In conclusion this work demonstrates that there is a role for AMF in the commercial production of strawberry when grown in substrate and they could be a valuable tool for sustainable cropping of this important fruit crop especially under low-input production systems. Current levels of high intensity agriculture are no longer sustainable primarily due to energy costs of N fertilizers and the decreasing supplies of P (Cordell et al., 2009), along with a decreasing armory of pesticides (due to legislation) and water limitation. Further studies such as this are needed to improve our knowledge of how best to apply and use these beneficial organisms to successfully incorporate them into sustainable commercial cropping systems for soft fruit and other commercial crops. With a greater understanding of the application and benefits of these beneficial microbes there is a real possibility for their use in aiding sustainable crop production.

AUTHOR CONTRIBUTIONS

LR, is the lead author, and undertook the bulk of project management, practical work and writing. WF contributed largely to the metagenomics studies undertaking practical work and

writing. NG, KH worked on inoculum preparation and the strawberry experiments with practical work, data collection, analysis, and writing. RH contributed to the metagenomics analysis and writing. PJ was a PI on the metagenomics work, contributed to analysis, interpretation and writing. XX was PI on all the work, contributing to analysis, metagenomics analysis, interpretation, and writing.

REFERENCES

- Al-Karaki, G. N., and Al-Raddad, A. (1997). Effects of arbuscular mycorrhizal fungi and drought stress on growth and nutrient uptake of two wheat genotypes differing in drought resistance. *Mycorrhiza* 7, 83–88.
- Borkowska, B. (2002). Growth and photosynthetic activity of micropropagated strawberry plants inoculated with endomycorrhizal fungi (AMF) and growing under drought stress. *Acta Physiol. Plant* 24, 365–370. doi: 10.1007/s11738-002-0031-7
- Borowicz, V. A. (2001). Do arbuscular mycorrhizal fungi alter plant-pathogen relations? *Ecology* 82, 3057–3068. doi: 10.1890/0012-9658(2001)082[3057:damfap]2.0.co;2
- Castellanos-Morales, V., Villegas, J., Wendelin, S., Vierheilig, H., Eder, R., and Cárdenas-Navarro, R. (2010). Root colonization by the arbuscular mycorrhizal fungus *Glomus intraradices* alters the quality of strawberry fruits (*Fragaria x ananassa* Duch.) at different nitrogen levels. *J. Sci. Food Agric.* 90, 1774–1782. doi: 10.1002/jsfa.3998
- Cochran, W. G. (1950). Estimation of bacterial densities by means of the “most probable number”. *Biometrics* 6, 105–116. doi: 10.2307/3001491
- Cordell, D., Drangert, J.-O., and White, S. (2009). The story of phosphorus: global food security and food for thought. *Glob. Environ. Change Hum. Policy Dimens.* 19, 292–305. doi: 10.1016/j.gloenvcha.2008.10.009
- DEFRA (2015). *Basic Horticultural Statistics 2014*. Department for Environment, Food and Rural Affairs. Available online at: <https://www.gov.uk/government/statistics/basic-horticultural-statistics>
- Hart, M., Forsythe, J., Oshowski, B., Bücking, H., Jansa, J., and Kiers, E. T. (2013). Hiding in a crowd—does diversity facilitate persistence of a low-quality fungal partner in the mycorrhizal symbiosis? *Symbiosis* 59, 47–56. doi: 10.1007/s13199-012-0197-8
- Isayenkov, S., Fester, T., and Hause, B. (2004). Rapid determination of fungal colonization and arbuscule formation in roots of *Medicago truncatula* using real-time (RT) PCR. *J. Plant Physiol.* 161, 1379–1383. doi: 10.1016/j.jplph.2004.04.012
- Ismail, Y., and Hijri, M. (2012). Arbuscular mycorrhisation with *Glomus irregularare* induces expression of potato PR homologues genes in response to infection by *Fusarium sambucinum*. *Funct. Plant Biol.* 39, 236–245. doi: 10.1071/FP11218
- Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., and Barea, H. M. (2003). The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. *Biol. Fertil. Soils* 37, 1–16. doi: 10.1007/s00374-002-0546-5
- Krüger, M., Teste, F. P., Laliberté, E., Lambers, H., Coghlann, M., Zemunik, G., et al. (2015). The rise and fall of arbuscular mycorrhizal fungal diversity during ecosystem retrogression. *Mol. Ecol.* 24, 4912–4930. doi: 10.1111/mec.13363
- Martin, F. N. (2003). Development of alternative strategies for management of soilborne pathogens currently controlled with methyl bromide. *Annu. Rev. Phytopathol.* 41, 325–350. doi: 10.1146/annurev.phyto.41.052002.095514
- McGonigle, T. P., Miller, M. H., Evans, D. G., Fairchild, G. L., and Swan, J. A. (1990). A new method which gives an objective measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. *New Phytol.* 115, 495–501. doi: 10.1111/j.1469-8137.1990.tb00476.x
- Ren, L., Lou, Y., Zhang, N., Zhu, X., Hao, W., Sun, S., et al. (2013). Role of arbuscular mycorrhizal network in carbon and phosphorus transfer between plants. *Biol. Fertil. Soils* 49, 3–11. doi: 10.1007/s00374-012-0689-y
- Robinson-Boyer, L. R., Brain, P., Xu, X.-M., and Jeffries, P. (2015). Inoculation of drought-stressed strawberry with a mixed inoculum of two arbuscular mycorrhizal fungi: effects on population dynamics of fungal species in roots and consequential plant tolerance to water deficiency. *Mycorrhiza* 25, 215–227. doi: 10.1007/s00572-014-0603-6
- Rodriguez, A., Dougall, T., Dodd, J. C., and Clapp, J. P. (2001). The large subunit ribosomal RNA genes of *Entrophospora infrequens* comprise sequences related to two different glomalean families. *New Phytol.* 152, 159–167. doi: 10.1046/j.0028-646X.2001.00237.x
- Smith, S., Facelli, E., Pope, S., and Andrew Smith, F. (2010). Plant performance in stressful environments: interpreting new and established knowledge of the roles of arbuscular mycorrhizas. *Plant Soil* 326, 3–20. doi: 10.1007/s11104-009-981-5
- Wagg, C., Jansa, J., Schmid, B., and van der Heijden, M. G. A. (2011). Belowground biodiversity effects of plant symbionts support aboveground productivity. *Ecol. Lett.* 14, 1001–1009. doi: 10.1111/j.1461-0248.2011.01666.x
- Xu, X.-M., Robinson, J., and Else, M. A. (2013). Effects of nitrogen input and deficit irrigation within the commercial acceptable range on susceptibility of strawberry leaves to powdery mildew. *Eur. J. Plant Pathol.* 135, 695–701. doi: 10.1007/s10658-012-0106-2

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