Biotic and Abiotic Stress Responses in Crop Plants

Edited by
Thomas Dresselhaus and Ralph Hückelhoven

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Cover image courtesy of Kevin Begcy.
The image shows a semi-sterile maize cob fertilized with heat-stressed pollen.

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About the Special Issue Editors

Thomas Dresselhaus is chairman of the department of Cell Biology and Plant Biochemistry and speaker of the Graduate Research Academy RNA Biology, both at the University of Regensburg, Germany. He studied biology at the Universities of Münster and Tübingen and obtained a PhD degree in 1994 from the University of Hamburg. He qualified as a university lecturer after various research visits to other labs, group leader positions and working for a plant biotech company. Since 2006 he is full professor at the University of Regensburg, where he served the Faculty of Biology and Preclinical Medicine first as Dean of Research and thereafter as Dean. He served the Society of Plant Reproduction IASPRR from 2010 to 2014 as President. His main research interest is on plant reproduction with a focus on signaling and gene regulation during flowering time, male and female germline development, fertilization mechanisms, and patterning during early embryo and seed development. More recent work includes investigations on heat stress during reproduction in cereals. Arabidopsis, maize and wheat are used as model systems. His lab is known for their expertise in single cell handling, fluorescence microscopy, reverse genetic approaches to study gene functions, peptide signaling mechanisms and maize biotechnology.

Ralph Hückelhoven received his diploma in biology from the RWTH Aachen University and his doctoral degree from the University of Giessen. He continued his work in Giessen as a research associate until 2002, when the German research foundation awarded an independent junior research group grant to him for a total of six years. In 2005, he habilitated in the agricultural faculty in Giessen and achieved the *venia legendi* for Molecular Phytopathology and Cell Biology. Since 2006, he is full professor of Phytopathology at the Technical University of Munich School of Life Sciences Weihenstephan. His past and present main fields of research include (i) cellular and apoplastic defense reactions to directly invading fungal plant pathogens, (ii) regulation of programmed cell death in plants, (iii) production and function of reactive oxygen species in plant-microbe interactions, (iv) pattern-triggered immunity, (v) organization of the host cytoskeleton in the interaction with fungal parasites, (vi) regulation and function of ROP GTPases signaling in disease susceptibility, (vii) resistance to *Fusarium* spp. in barley and (viii) molecular mechanisms of fungal virulence effector function.

Preface to "Biotic and Abiotic Stress Responses in Crop Plants"

While the demand for crop products continues to increase strongly, agricultural productivity is threatened by various stress factors, often associated with global warming. To sustain and improve yield, it is necessary to understand how plants respond to various stresses, and to use the generated knowledge in modern breeding programs. Most knowledge regarding the molecular mechanisms associated with stress responses has been obtained from investigations using the model plant *Arabidopsis thaliana*. Stress hormones, such as abscisic acid, jasmonic acid, and salicylic acid, have been shown to play key roles in defense responses against abiotic and biotic stresses. More recently, evidence that growth-regulating plant hormones are also involved in stress responses has been accumulating. Epigenetic regulation at the DNA and histone level, and gene regulation by small non-coding RNAs appear to be important as well. Many approaches have used mutant screens and next generation sequencing approaches to identify key players and mechanisms how plants respond to their environment. However, it is often unclear to which extent the elucidated mechanisms also operate in crops.

This Special Issue Book, therefore, aims to close this gap and contains a number of contributions from labs that work both, on Arabidopsis and crops. The book includes contributions reporting how crop plant species respond to various abiotic stresses, such as drought, heat, cold, flooding, and salinity, as well as biotic stimuli during microbial infections. It contains reviews, opinions, perspectives, and original articles, and its focus is on our molecular understanding of biotic and abiotic stress responses in crops, highlighting, among other aspects, the role of stress hormones, secondary metabolites, signaling mechanisms, and changes in gene expression patterns and their regulation. Approaches and ideas to achieve stress tolerance and to maintain yield stability of agricultural crops during stress periods can be found in most chapters. These include also perspectives on how knowledge from model plants can be utilized to facilitate crop-plant breeding and biotechnology.

Thomas Dresselhaus, Ralph Hückelhoven
Special Issue Editors

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Editorial

Biotic and Abiotic Stress Responses in Crop Plants

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Abstract: Agricultural productivity depends on increasingly extreme weather phenomena, and the use of germplasm that has to be continuously improved by plant breeders to become tolerant to various biotic and abiotic stresses. Molecular plant biologists try to understand the mechanisms associated with stress responses and provide knowledge that could be used in breeding programs. To provide a partial overview about our current understanding about molecular and physiological stress responses, and how this knowledge can be used in agriculture, we have edited a special issue on "Biotic and Abiotic Stress Responses in Crop Plants". Contributions are from different fields including heat stress responses, stress responses during drought and salinity, as well as during flooding, and resistance and susceptibility to pathogenetic stresses and about the role of plant functional metabolites in biotic stress responses. Future research demand in particular areas of crop stress physiology is discussed, as well as the importance of translational research and investigations directly in elite crop plants and in the genetic resources available for breeding.

1. Introduction

While the demands for crop products continue to increase, agricultural productivity is threatened by various stress factors, often associated with global warming [1–3]. In Germany, for example, precipitation has increased by 11% during the past 100 years by up to 30% in the cold season, while summers are much dryer and the number of days with temperatures above 30 °C has doubled [4]. These conditions often favor pathogens and negatively affect plant productivity and fertility. Plants have to adapt in a physiologically costly way to these changing environmental conditions, resulting in reduced availability of resources to produce biomass, seeds, and thus yield. Moreover, the combination or alteration of diverse abiotic and biotic stress factors may further cause trade-off between plant responses that are appropriate for adaptation to one stress, but can enhance susceptibility to other stresses. Climate-driven migration of pathogens and pests further confronts locally adapted crop genotypes with new biotic stress factors. This clash of globally increasing yield demands with increasing yield-threatening environmental conditions asks for massive investments into plant stress resistance research and development. To sustain and improve yields, it is necessary to understand how plants respond to various stresses and to use the knowledge generated in modern plant breeding programs. Most knowledge regarding the molecular mechanisms associated with stress responses has been obtained from investigations using the model plant *Arabidopsis thaliana*. However, it is often unclear to which extent the elucidated mechanisms also operate in crops.

Financed by the German Research Foundation DFG and by the Bavarian State Ministry of the Environment and Consumer Protection, molecular plant researchers in Bavaria (in southeast Germany)

have now established two research consortia with the aim to elucidate the molecular mechanisms by which model and crop plants adapt to various biotic and abiotic stresses. The knowledge generated will then be used to develop crop plants better adapted to the consequences of global warming. Within the "BayKlimaFit" network (Strategies for the Adaptation of Crop Plants to Climate Change), seven groups study plant responses during flooding and cold as well as heat and drought stress, while two labs investigate symbionts and pathogens (for an overview, see http://www.bayklimafit.de) [5]. The Collaborative Research Centre (CRC) SFB924 aims to understand Molecular Mechanisms Regulating Yield and Yield Stability in Plants (http://sfb924.wzw.tum.de) [6] and to transfer knowledge from model plants to crop plants or from one crop plant to another crop plant. Within the center, 12 groups investigate molecular mechanisms regulating abiotic and biotic stress responses in *Arabidopsis* and crop plants such as maize, barley, tomato, and *Medicago*. As knowledge generated by molecular plant researchers often does not reach plant breeders and decision-makers, we discussed with our colleagues the idea of editing a Special Issue about *Biotic and Abiotic Stress Responses in Plants* in the journal *Agronomy*, with the goal of contributing to filling this gap and additionally highlighting this exciting research area.

This Special Issue, therefore, collected contributions from groups of both research consortia, complemented by papers from colleagues worldwide, reporting how crop plant species respond to various abiotic stresses, such as drought, heat, flooding, and salinity, as well as biotic stimuli during microbial infections. It contains reviews, opinions, perspectives, and original articles, and its focus is on our molecular understanding of biotic and abiotic stress responses in crops, highlighting, among other aspects, the role of stress hormones, secondary metabolites, signaling mechanisms, and their regulation, as well as state-of-the-art omics approaches. Strategies and ideas to achieve stress tolerance and to maintain yield stability of agricultural crops during stress periods are also discussed. These include perspectives on how knowledge from model plants can be utilized to facilitate crop-plant breeding and biotechnology.

2. Biotic Stress Responses and the Role of Secondary Metabolites

Plants function in complex environments and are increasingly often considered not as individual genotypes, but as holobionts that function in genetic, physiological, and evolutionary units with their associated microbiota [7]. The microbiota can be beneficial, detrimental, or neutral for plant fitness and their function can also change depending on environmental challenges. In their report, Aslam and Ali used the halotolerant plant species *Suaeda fruticosa* (L.) for the identification of a variety of plant-associated bacterial genera. They isolated bacteria and performed physiological characterization of auxin biosynthesis capacities, biofilm formation, and halotolerance, amongst others. Astonishingly, several bacterial strains showed an ability to support the growth of corn plants under salt stress conditions, and this was partially associated with higher antioxidant capacities. This finding shows the potential of plant-associated microbiota for application as biologicals in plant protection against abiotic stress and suggests that bacteria can prime plants for higher cell protection potential [8].

Interaction of plants with microbes or microbe-associated molecular patterns can also induce resistance to secondary infections by pathogens. This involves the production and systemic signaling of a complex of low-molecular-weight plant metabolites, which are well described for dicotyledonous plants, but poorly understood for monocotyledonous plants such as cereal crops. Lenk and coworkers used resistance-inducing compounds from the model *Arabidopsis thaliana* and tested their potential in barley. They discovered a challenging-pathogen-dependent effect of individual compounds in inducing either disease resistance or susceptibility in barley [9]. This highlights the conservation and diversification of plant responses to stress signaling compounds when comparing dicots and monocots.

Stress-related metabolic alterations occur in plants in a genotype-specific manner. Therefore, metabolic changes can be used in targeted breeding for specific agronomic traits such as stress resistance or food functions. In this context, Dawid and Hille provide a review on functional metabolomics and the analytical techniques used in this discipline [10]. They describe how metabolite profiles can be

translated into stress resistance or desired sensory traits of the crop plant, but also discuss the need for orchestrated biological and structural characterizations of unidentified marker metabolites.

Benzoxazinoids (BXs) are secondary plant metabolites from grasses with a high potential for the chemical defense against biotic stress factors from diverse kingdoms of life. Niculaes and coauthors provide a comprehensive overview of the biosynthesis, metabolism, and biological activities of BXs [11]. They report on the broad spectrum of biological functions of BXs, for example, from toxic to insect-health-promoting effects. Together, BXs belong to the best-characterized chemical defense compounds in maize and are of strong agronomic relevance. However, their exact biosynthesis pathways and functions in other *Poaceae* crops and their wild relatives appear to be not well understood. Future research demand is accordingly discussed.

Disease resistance and susceptibility are two sides of the same coin, aren't they? Two perspective articles in this Special Issue discuss how to make use of the recently expanding knowledge on how plants recognize and defend microbial intruders and how microbial pathogens trick the plant to overcome immunity and manipulate host functions for their own demand. Quantitative broad-spectrum immunity against microbial pathogens depends to a substantial extent on the efficient recognition of nonspecific microbe-associated molecular patterns and host-derived endogenous damage-associated molecular patterns. Ranf explains the mechanism of the sensitive detection of these molecules by cell-surface pattern recognition receptors (PRRs) [12]. She reports the great progress in the recent identification of novel PRRs from plants, with most of them being identified in *Arabidopsis thaliana*. However, she also shows the perspective for the translation of this knowledge into biotechnological approaches that will allow the establishing, enhancing, and even designing of pathogen pattern-sensing capacities in model and crop plants.

Engelhardt and coauthors discuss that disease susceptibility is more than just the failure of plant immunity, and involves so-called susceptibility (S) factors of the host plant [13]. They highlight physiological functions of S factors, give examples of how pathogen effectors manipulate S factors, and discuss whether and how we can use them by modern targeted breeding approaches, including guided endonuclease technology, to turn susceptibility into resistance. Furthermore, the authors discuss the natural occurrence, possible trade-offs, and durability of loss of susceptibility.

3. Abiotic Stress Responses and the Usage of RNA-seq Methodology

A number of papers consider the effect of heat and drought stress on crops. Nadeem and coworkers review the diverse effects of heat stress on crop yield [14]. They report that heat stress is especially deleterious to certain developmental stages, including the generation and function of reproductive organs. Physiological plant responses involve mechanisms to prevent membrane damage and to regulate photosynthesis, respiration, and transpiration. Molecular heat stress responses involve ROS, NO, and Ca^{2+} signaling as well as the induction of heat stress factor genes (*HSFs*) and other transcription factors, which is discussed to be used in future approaches to engineer tolerant crops. Authors conclude that is necessary to systematically assess wild species to increase the narrow gene pool of currently grown crops and to significantly intensify molecular studies, including omics approaches, to elucidate the molecular mechanisms underlying heat stress responses and tolerance. They suggest to use optimized transgenic plants to overcome yield losses associated with heat stress.

The effect of short-term moderate heat stress at a highly sensitive stage during reproduction in wheat is reported by Begcy et al. [15]. Authors imposed heat stress for two days at the pollen mitosis stage in four Australian and four European cultivars, and investigated the effects at the physiological and molecular level. In contrast to the Australian cultivars, photosynthetic and transpiration rates as well as pollen viability were strongly reduced in the European cultivars. Moreover, the *HSF* gene expression pattern showed little variation in the Australian cultivars, while *HSFs* were either down- or upregulated in the European cultivars. As a conclusion, it appears that European cultivars overreact during heat stress, while Australian cultivars are better adapted, showing smaller amplitudes of heat stress responses.

The performance and stability of commercial wheat cultivars under terminal heat stress was studied by Elbasyoni at two locations in Egypt, with the goal to identify the ideal cultivar for each condition [16]. It was shown that heat stress had a significant adverse impact on plant growth and contributed to overall yield losses of about 40%. Based on the findings, it was suggested that importing and evaluating heat-stress-tolerant wheat genotypes under late-sown conditions or heat-stressed conditions is required to boost heat-stress tolerance in adapted wheat cultivars.

Drought stress tolerance can be achieved by generating plants with improved water use efficiency (WUE). Plants with C_3 photosynthesis, such as *Arabidopsis*, are able to moderately increase WUE by restricting transpiration, but this also results in reduced CO_2 uptake and thus negatively influences photosynthesis, growth, and yield. In their opinion paper [17], Blankenagel and coworkers discuss recent data obtained for *Arabidopsis* and the potential to translate the findings to cereals, which are especially drought-sensitive. Their data on the C_4 crop maize subjected to progressive drought shows that there is potential for improvement in WUE at the whole plant level, but reduction in assimilation rate and growth trade-offs is even more pronounced compared with *Arabidopsis*. Authors assume that the CO_2 gradient established in C_4 metabolism limits the potential for further increases. In conclusion, it is demanded that many more studies are necessary in cereal crops to understand WUE mechanisms under water-deficit conditions.

During growth, plants are not only exposed to multiple abiotic and biotic stresses; access to water and minerals may also be limited simultaneously. Xiong et al. therefore investigated the influence of drought stress and nitrogen supply on the growth of pak choi. Drought was simulated by adding polyethylene glycol under hydroponic culture conditions, resulting in a significant decline in plant water content, transpiration rate, shoot biomass, and shoot nitrogen concentration [18]. Their results further indicated that pak choi supplied with higher nitrogen concentration showed a significant better growth performance under drought stress compared to the controls.

As pointed out already above, in addition to increasing heat and drought stress periods, the duration and frequency of precipitation has significantly increased, often resulting in soil flooding, especially early in the crop-growing season. The review by Mustroph [19] summarizes the progress and approaches to enhancing crop resistance to flooding. With a focus on maize, barley, and soybean, traits such as anatomical and metabolomic adaptations, including aerenchyma formation, adventitious roots, and the formation of a barrier against oxygen loss, but also optimized enzymes and transcriptional regulators, are discussed. Various quantitative trait loci (QTLs) have indicated the presence of tolerance genes. It will now be exciting to identify the corresponding genes and molecular mechanisms, and use the knowledge generated to engineer flooding-tolerant crops.

How natural stress-tolerant plants can be used to understand and exploit tolerance mechanisms is outlined in the opinion paper by Messerer and coworkers [20]. With a focus on salinity-tolerant quinoa, a crop of less economic importance, they highlight recent next-generation sequencing approaches using RNA-seq as a gold standard to identify novel and stress-associated genes. For example, in one study, 15 genes for putative transmembrane proteins were found that could potentially contribute to a higher salinity tolerance. Authors also point out that transcriptomic approaches should be combined with metabolomics to understand quinoa's salinity tolerance. Finally, they suggest applying similar approaches to understand tolerance in other naturally stress-tolerant plants.

4. Conclusions

In conclusion, plant sciences currently achieve good models of how model plants react to environmental factors by transcriptional and metabolic reprogramming. However, especially molecular research efforts in crops have to be strengthened considerably. Future biocomputational integration of multiple omics and meta-omics will help further understanding of the complexity of plant stress physiology. This needs further effort in developing innovative research tools and fundamental resources for crop plant research, such as reference genomes, proteomes, and metabolomes with comprehensive annotations and structure–function relationships, respectively. Even for the model

Arabidopsis, these resources are not fully available. Nevertheless, in several cases, *Arabidopsis* and other model plants have already been proved suitable for the translation of fundamental research into agronomically relevant crop traits. This is encouraging, but requires further and significant investment into translational research, as pointed out by many authors involved in this Special Issue. Besides this, it remains indispensable to investigate stress resistance mechanisms directly in elite crop plants and in the genetic resources available for breeding.

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Unraveling Field Crops Sensitivity to Heat Stress: Mechanisms, Approaches, and Future Prospects

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Abstract: The astonishing increase in temperature presents an alarming threat to crop production worldwide. As evident by huge yield decline in various crops, the escalating drastic impacts of heat stress (HS) are putting global food production as well as nutritional security at high risk. HS is a major abiotic stress that influences plant morphology, physiology, reproduction, and productivity worldwide. The physiological and molecular responses to HS are dynamic research areas, and molecular techniques are being adopted for producing heat tolerant crop plants. In this article, we reviewed recent findings, impacts, adoption, and tolerance at the cellular, organellar, and whole plant level and reported several approaches that are used to improve HS tolerance in crop plants. Omics approaches unravel various mechanisms underlying thermotolerance, which is imperative to understand the processes of molecular responses toward HS. Our review about physiological and molecular mechanisms may enlighten ways to develop thermo-tolerant cultivars and to produce crop plants that are agriculturally important in adverse climatic conditions.

Keywords: heat stress; thermotolerance; oxidative stress; heat shock proteins; QTLs; plant omics

1. Introduction

In recent years, temperature extremes and weather disasters have partially or completely damaged regional crop production [1–3]. The annual worldwide temperature has been increasing steadily, and is expected to be increased by 1.8–4.0 °C by the end of the 21st century [4]. This increasing trend in temperature creating curiosity among researchers, as temperature has an impact on life on earth, acting directly or indirectly. Regardless of these encounters, global food production will have to rise by 70% to meet the mandate of an expected rise in population growth to 9 billion by 2050 [5].

Plants as sessile organisms and cannot change their position or move to more suitable climatic conditions; therefore, plant activities are extensively affected by heat stress (HS), which often leads to mortally [6]. Specifically, HS has an impact on a number of various plant species [7]. HS significantly affect plant activities like seed germination, plant development, photosynthesis, and reproduction, which have a devastating impact on the overall yield of a crop [8]. It has been observed that HS leads to inhibition of pollen grain swelling leading to perturbed pollen dispersal and anther indehiscence during the reproductive process, which finally influence seed yield of rice [9]. Heat and drought are the key abiotic stresses to cereal crop production and resulted in the reduction of yield by 9% to 10% between 1964 and 2007 worldwide [2].

For survival in severe conditions, plants continuously struggle to modify their metabolic process in many ways in response to HS, specifically by generating key solutes that leads to establish proteins and osmotic adjustment and re-establish the redox balance of cell and homeostasis by modify the

antioxidant system [10,11]. A plant in defense from HS causes modifications at the molecular level in the expression of genes [12]. In HS conditions, change in biochemical and physiological activities by gene expression alter gradually, resulting in the development of thermotolerance [13]. In order to successfully produce HS-tolerant crop varieties in the light of global climate change, there is need of knowledge and investigations about HS-tolerance mechanisms at physiological, biochemical, and molecular levels.

At present, investigations into selection strategy and breeding for thermotolerant cultivars and understanding of heat tolerance mechanisms are more required today than ever before. Molecular and genetic mechanisms for avoiding HS-induced harmful changes play a crucial role in plant survival under such circumstances. In the present scenario of global warming, the major challenge for plant scientists is to develop new crop varieties tolerant to HS [14]. In the coming years, agricultural production will have to deal with growing crops under sub-optimal conditions accompanied by increased food demand, creating a gap between the current yield achievements and yield potential [15]. Developing genetically modified plants through target genes manipulation, QTLs, and omics techniques are widely studied molecular approaches in recent years. Our study about sensitivity, adaptations, mechanisms, and approaches may uncover ways to develop thermo-tolerant cultivars and to produce crop plants that are agriculturally important in adverse climatic conditions.

2. Plant Sensitivity to Heat Stress

Plant sensitivity to HS varies with duration, plant type, and the degree of temperature. Plant growth and development are greatly influenced by the series of morphological, physiological, and biochemical changes resulting from HS [16]. HS cause devastating impacts on crop plants by affecting vital physiological functions, including protein denaturation, increase in membrane fluidity, level of reactive oxygen species (ROS), decline in photosystem II (PSII)-mediated electron transport, as well as inactivation of chloroplast and mitochondrial enzymes activities [17–19]. HS due to the rising global temperature is becoming one of the main limiting factor to crop productivity and has an adverse impact on plants (Figure 1). This rising temperature may cause a change in the morphology, physiology, and growing periods of plants.
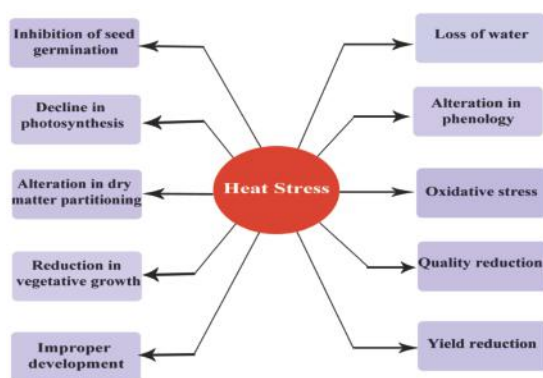
Figure 1. Impact of heat stress on plants.

2.1. Morphological Responses

2.1.1. Crop Growth and Development

During crop growth and development, temperature plays an important role in dry matter partitioning, transpiration [20,21], photosynthetic activity, respiration [22,23], and root and plant development [24]. The ideal conditions for plant growth and development generally occur within

a different range of temperature [25], with low or high temperature (HT) reducing growth and developmental rates [26,27]. In winter cereals, temperature acts as a signal stimulator in processes of vernalization to induce flowering in plants [28]. It has been investigated that the increase in temperature to optimum thresholds stimulates biochemical mechanisms, consequently affecting development rates and declining the lengths of growing seasons [29]. The shorter developmental phases could have an adverse impact on the formation of yield components [30]. In Germany, between 1959 and 2009, lengths of growing seasons of oats reduced by about two weeks, leading to an earlier occurrence of phonological phases due to by HT [31]. As HT trigger development of the crop, the phases of crop growth duration decline, producing a destructive impact on yield in field crops and final grain weight [30]. It is observed that temperature has negative effect on growth and phototropism of *Arabidopsis thaliana* (L.) seedlings [32]. Van Der Ploeg and Heuvelink [33] reviewed the Influence of sub-optimal temperature on growth and yield of tomato. In a recent study, Yang et al. [34] investigated the effects of different growth temperatures on growth, development, and plastid pigments metabolism of tobacco (*Nicotiana tabacum* L.) plants.

2.1.2. Reproductive Development

HS has the widest and most far-reaching effects on plant reproductive organ, seed weight, and number of seeds, but regulation of heat-shock responses in inflorescence is largely uncharacterized [32,33]. It has been reported that male and female organs are most sensitive to extreme temperature, especially ≥ 30 °C [34] (Figure 2a). HS damages both male and female gametophytes, resulting in decreased pollen viability, reduced pollen germination, pollen tube growth inhibition, stigma receptivity reduction and reduced ovule function, declined fertilization, limited embryogenesis, poor ovule viability, enhanced ovule abortion, and a decrease in yield [35,36] (Figure 2b).
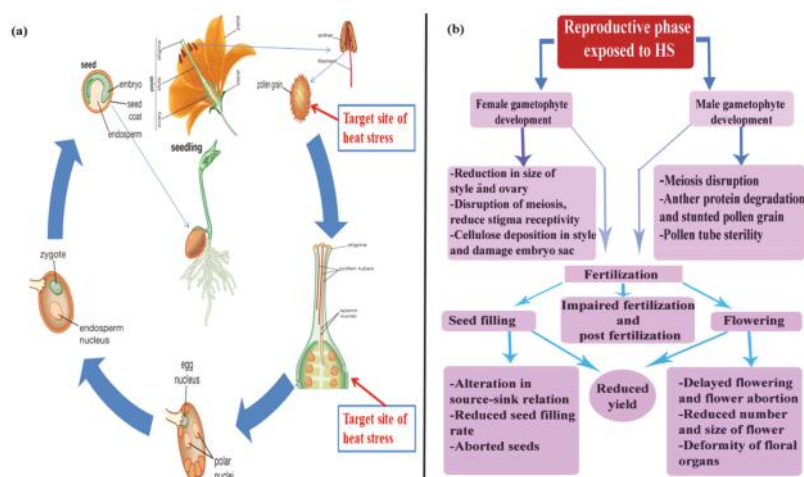
Figure 2. (**a**) Life cycle of an angiosperm representing target sites of heat stress (HS). Both male (pollen grains) and female (ovule) gametophytes are the main target sites of heat stress. (**b**) Sensitivity of reproductive phase to heat stress and consequences of heat stress on reproductive and grain-filling phases.

The number of seed year^{-1} increases with increasing air temperature from 16–28 °C, though it harshly declines with further increases in temperature [37]. During the period of grain filling, HS has a great impact on the quality and quantity of the final yield [38,39]. Enhancing temperatures from 25–31 °C increased the rates of grain filling, although the final yield steadily decreased due to shortening of the grain filling duration [40]. In wheat, at mean temperatures of 21/16 °C, the higher grain numbers are

obtained [35]. It has been found that the increase in temperatures to crop-specific thresholds (for wheat 10–21 °C) enhance the rate of grain filling by enhancing cell-division rates in the tissue of endosperm and increasing rates of metabolism [41]. Dry matter partitioning, which is the product of the movement of photosynthetic assimilate from source-sink organs, is enhanced between 10–30 °C in winter-season cereals [42]. The devastating decrease in growth and development, harvest index (HI), and seed yield were found for various crops. The decline of grain number resulted from the impact of HT on meiosis and transfer of pollen during anthesis and ovaries growth during pre-anthesis periods. It has been reported that in pepper plants, HS resulted in a drop in sucrose concentration in fruits/flowers, though in cereals, sucrose helps the plant avoid ovary abortion under water stress conditions [43]. The reproductive phase is more sensitive to HS, and high temperatures are likely to coincide with anther dehiscence and gametophyte development, which resulted in a final yield reduction.

2.1.3. Yield

HS has an adverse impact on various processes of crop growth and development and final yield [44]. It has been reported that HS leads to yield losses in various crops [45] (Table 1). With a 1 °C increase in global temperature, global wheat production is projected to decrease between 4.1% and 6.4% [46]. It is declared that the increase in temperature expected with environmental variability is likely to decrease wheat yields [47]. Additionally, in wheat, an annual worldwide yield loss of 19 million tons is observed, costing $2.6 billion, due to climate variability from 1981 to 2002 [6]. Globally, temperature trend analysis from 1980 to 2008 has revealed about a 5.5% decrease in the yield of wheat [1]. Similarly, it has been found that temperatures beyond 34 °C enhance the rates of senescence based on nine years of satellite data of wheat grown in northern India, thereby resulting in a significant reduction in yield [48].

Table 1. Yield reduction due to heat stress (HS) in some major crops.

Crop	Yield Reduction (%)	Reference
Wheat (*Triticum aestivum* L.)	31	[49]
Maize (*Zea mays* L.)	45	[50]
Rice (*Oryza sativa* L.)	50	[51]
Soybean (*Glycine max* L.)	46	[52]
Canola (*Brassica napus* L.)	50	[53,54]
Peanut (*Arachis hypogaea* L.)	31	[55]
Srghumm (*Sorghum bicolor* L.)	44	[56]
Sunflower (*Helianthus annuus* L.)	10	[57]

In addition to wheat, in maize, thermal warming form 1981 to 2002 has triggered a reduction in yield up to 12 million tons year^{-1}, comparable to a loss of $1.2 billion [6]. By 2100, a reduction of yield of about 30% in maize is recorded in the US by means of the nonlinear temperature and yield analysis [52]. Similarly, about a 3.8% yield loss in maize was observed by worldwide temperature trends analyses using past data from 1980 to 2008 [58]. These studies suggest that a rise in temperature beyond 30 °C have adverse effects on rainfed maize in Africa and the US [59]. It is reported that extreme HS at enthesis could reduce maize yield globally by 45% by 2080 as compared to the 1980s [50].

In the case of soybean, a decline in yield of about 46% in the US before the year 2100 is predicted by non-linear and asymmetric temperature and yield relationship analyses [52]. It is reported that during 1976 to 2006 in the US, a future yield reduction of about 16% has been observed in soybean due to a change in patterns of temperature [60]. In barley, thermal warming from 1981 to 2002 caused a yield reduction of 8 million tons year^{-1}, with loss of around $1 billion [6]. Cucumber (*Cucumis sativus* L.) is one of the most important horticultural crops and is highly sensitive to HS, particularly at the vegetative stage [61,62].

2.2. Physiological Responses

2.2.1. Membrane Damage

In a plant cell, the most sensitive component is the plasma membrane, as it is the primary sites of injury under HS [63]. HS severely affects the structure and functions of the membrane, thereby increasing membranes fluidity due to denaturation of proteins and increased level of unsaturated fatty acids, causing a transition from solid gel to flexible crystalline liquid structure [64]. HS damage can be assessed by loss of membrane integrity due to structural modifications of component proteins, which enhances the thermostability of the membrane and organic and inorganic ions leakage from the cells [65]. Therefore, an electrolyte leakage value acts as a pointer of membrane injury and reflects stress-induced alterations and has been used to evaluate the thermostability of membranes under HS [66]. The increased permeability and leakage of ions out of the cell has been used as a measure of cell membrane stability and as a screen test for HS tolerance [67]. The effects of HS on membranes have been reported in various crops. In cotton, sorghum, and soybean, HS-induced serious membrane injury and membrane lipid peroxidation have been observed [51,68]. The increased permeability of the membrane and electrolyte leakage is noticed under HS in soybeans, which declined the capacity of the plasma membrane to hold solutes and water [69]. Similarly, in chickpeas, injury of the membrane was noticed at 40/30 °C, which was intensified at 45/35 °C, especially in sensitive genotypes [70]. A recent study investigated cell membrane stability under drought and heat conditions in wheat [67]. Membrane fluidity in temperature tolerance has been delineated by mutation analysis and transgenic and physiological studies. For instance, a soybean mutant deficient in fatty acid unsaturation exhibited high tolerance to HS [71]. Also, the thylakoid membranes of two *Arabidopsis* mutants deficient in fatty acid unsaturation (fad5 and fad6) exhibited increased stability to HS and increased lipid saturation in tobacco caused by silencing a ω-3 desaturase gene, which also rendered the plants more tolerant to HS [72,73]. Wheat lines of high membrane thermal stability tended to yield higher than lines of low membrane thermostability when grain filling occurred under harsh climate [74]. It is investigated that *HIT1* functions in the membrane trafficking that is involved in the thermal adaptation of the plasma membrane for tolerance to HS in plants [75]. In transgenic tobacco, overexpression of the *PpEXP1* gene exhibited a less structural damage to cells, lower electrolyte leakage, and lower levels of membrane lipid peroxidation compared to wild-type plants [76]. Researchers identified tolerant genotypes that are proved to be more productive under extreme field stress conditions. The thermostability of the membrane has been successfully employed to evaluate HS tolerance in several crops worldwide.

2.2.2. Photosynthesis and Respiration

Due to ongoing climate change, it has been reported that severe climatic conditions with long light exposure and HT have increased dramatically. It is already proven that, for life on earth, photosynthesis is a vital process and is often restricted by various abiotic stresses like HS and high light conditions. The negative impact of HS on plant growth and crop yield were mainly caused by its negative impacts on the photosynthetic process, which is the most thermosensitive aspects of plant functions [77]. The relative water content (RWC), chlorophyll content, and PSII activity decreased under high light and heat co-stresses [78]. It has been found that the PSII reaction center is the vital site where damage is incurred by several abiotic stresses in the photosynthesis systems of plants [79]. It is found that photosystem II is thought to be more highly responsive to HS or high light than photosystem I [80]. The photosynthetic process is very sensitive under HS conditions, and reduction in chlorophyll contents might be one of the main reasons for the decline in photosynthesis, as an enzyme chlorophyllase helps in conversion of chlorophyll into phytol and chlorophyllide [81]. Photosynthetic acclimatization to various climatic conditions represents a modification in photosynthesis and structures at each level [80,82] (Figure 3). In thylakoids, the proton gradient and non-photochemical quenching (NPQ) are the vital photo-defense process in photosystem I and photosystem II, respectively [83]. In plants, climatic stress generally results in a decrease in chlorophyll concentrations and a reduction of the

photochemical reaction of thylakoid proteins [84–86]. It is reported that temperature is significantly affecting the photosynthetic activity of crops and photosynthetic pathways (C3 or C4 plants). In general, for cold season C3 crops, the temperature range for photosynthesis is between 0 °C to 30 °C, whereas the warm-adapted C4 plants that are grown in summer are photo-synthetically active between 7 °C to 40 °C temperature [22,81].

In plant species (C3), at current levels of CO_2 and light saturation, during the process of photo-phosphorylation, the photosynthetic response of plants to temperatures is measured by the availability of inorganic phosphates at a lower temperature, and it depends on the activity of Rubisco to fix atmospheric carbon in the optimum range of temperature. In plant species (C4) that are grown in a hot climate, the availability of Rubisco limits photosynthetic activity under low temperature, whereas at higher temperature in the thermal optimum level, it is unclear which mechanisms affect photosynthetic activity [87]. Photosynthetic rates decrease sharply as temperature increases past the optimum level [41]. This decreased rate of photosynthesis is related with declined light harvesting in photosystem II that results from cyclic electron flow [88], limitations in Rubisco, and thylakoid membrane instability [89]. In the process of photosynthesis, photoinhibition of PSII occur seven lower ranges of thermal stress [79]. However, some researchers reported slight or no harm to photosystem II due to moderate thermal stress [88].

HS decreases the rate of Photosystem II repair by the production of ROS across the thylakoid membrane [90], which is subject to influence by HS [91]. It has been found that the stability of the thylakoid membrane under HS, situated between 32–45 °C, is mainly determined by the stability of the double bonds of fatty acids of the membrane. Fatty acids double bonds decline due to excess generation of ROS under HS circumstances and increasing membrane electron leakage, thereby enhancing the denaturation of thylakoid membrane proteins [66]. It is described that maintenance respiration (turn-over of proteins complex) is high under HS, resulting in declining availability of assimilates for crop growth and development [92]. In maize, the increase in temperature from 18–33 °C raises the rates of maintenance respiration by greater than 80% [93]. Under elevated temperatures, the rate of respiration measurement could be an appropriate pointer for stimulation of plant response to HS, as the rate of respiration rises much more than the rate of photosynthesis initially decreases [25].
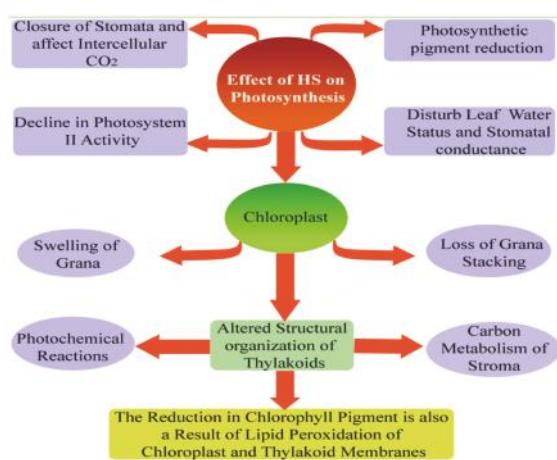
Figure 3. Decline in photosynthesis rate under heat stress. Heat stress leads to generation of reactive oxygen species, cell organelle and membrane damage, thylakoid membrane damage, thylakoid membrane lipid composition, swelling of grana, oxidative damage of cell organelle, and stomatal and non-stomatal limitations.

2.2.3. Water Relations

HS has the widest and most far-reaching effects on water relations leading to a severe decrease in yield potential in various crops. HS is often linked with rapid water loss from the surface of the plant, resulting in dehydration and ultimately leading to death [94]. The increase in transpiration and movement of water is an important tool for survival of the plant under extreme temperatures [8,95]. It is reported that HS influences plant–water relations due to the more rapid depletion of water from the soil, which affects the temperature of soil and transpiration [96]. HS directly and indirectly affects plant functions and leads to osmotic adjustments through impaired photosynthesis, enhanced respiration, a decline in leaf osmotic potentials, and decreased sugar concentration level [97]. Under HS, water loss during daytime was more common because of increased transpiration than night time, causing stress in snap bean (*Phaseolus vulgaris* L.) [98]. Under severe HS, high stomatal conductance boosts transpirational heat dissipation in tolerant genotypes of chickpea as long as soil water is available [99]. A rapid decrease in leaf tissue water contents was noted in sugarcane on exposure to extreme temperature despite the fact that an ample quantity of water was available in the soil [64]. In tobacco, stomatal conductance decreases markedly under severe HS, aggravating injury to leaves [68].

3. Oxidative Stress (OS)

Oxidative stress is a complex physiological and chemical phenomenon in plants and develops as a result of access production and accumulation of reactive oxygen species (ROS) under stress conditions. It has been reported that many metabolic pathways are subjected to depend upon different enzymes that are highly responsive to different ranges of HS. Like other abiotic stresses, HS might uncouple several enzymes and different metabolic pathways, which results in the accumulation of undesirable and dangerous ROS, generally hydrogen-peroxide (H_2O_2), hydroxyl-radical ($^\bullet OH$), superoxide-radical ($O_2^{\bullet-}$), and singlet-oxygen (O_2), which leads to OS [100]. The main sites of ROS production are reaction centers of photosystem I and photosystem II in chloroplasts, though ROS are also produced in other organelles, mitochondria, and peroxisomes [101] (Figure 4). A leaner relationship is present among accumulated ROS and highest efficiency of photosystem II. It is reported that because of heat injury to photosystem I and photosystem II under such HS condition, less absorption of photons occurs [102]. Among the ROS, photo-oxidation reactions lead to the generation of $O_2^{\bullet-}$ (flavoprotein, redox cycling), during mitochondrial ETCs reactions, in chloroplasts through Mehler reaction and glyoxisomal photorespiration, by xanthine oxidase, and NADPH oxidase in membrane polypeptides and plasmamembrane. A reaction between H_2O_2 and Fe^{2+} (Fenton reaction) and reaction between H_2O_2 and $O_2^{\bullet-}$ leads to the formation of hydroxyl radicals (Haber-Weiss reaction) and decomposition of O_3 in the apoplastic space region [103,104]. Photo-inhibition resulted in the formation of singlet oxygen and photosystem II electrons transfer reactions in chloroplast [104,105].

Due to HS, several physiological injuries occur in plants [102]. Hydroxyl radicals, which are produced during the process, can react with bio-molecules, pigments, lipids, proteins, DNA, and with all elements of the cell [104]. Protein denaturation as a result of thermal stress leads to OS through disruption of cell membrane stability and peroxidation of membrane lipids [106,107]. Photosynthetic activity of light reaction decreases under moderate HS is known to induce OS through the generation of ROS triggered by increased leakage of electrons from the membranes [108].

It is observed that the HT (33 °C) leads to OS in wheat, which can change the properties of membranes and cause the degradation of proteins and deactivation of enzymes that decrease the viability of cells. HS in wheat provokes OS and also amplifies the peroxidation of the membrane and decreases the thermal stability of the membrane by 28% and 54%, which enhances electrolytes leakage [109]. Premature leaf senescence in cotton was observed due to ROS generation by HS [54]. ROS accumulation at the outer surface of the plasma membrane due to continuous HS can cause membrane depolarization [110]. In such extreme cases, cell death can also occur because of ROS accumulation in cells [110]. ROS have a devastating impact on the metabolic processes of plants and triggered signaling behavior to activate the heat-shock responses toward the development of

thermotolerance in crops [100]. Research into plant oxidative stress (OS) has shown huge potential for developing HS-tolerant crops.
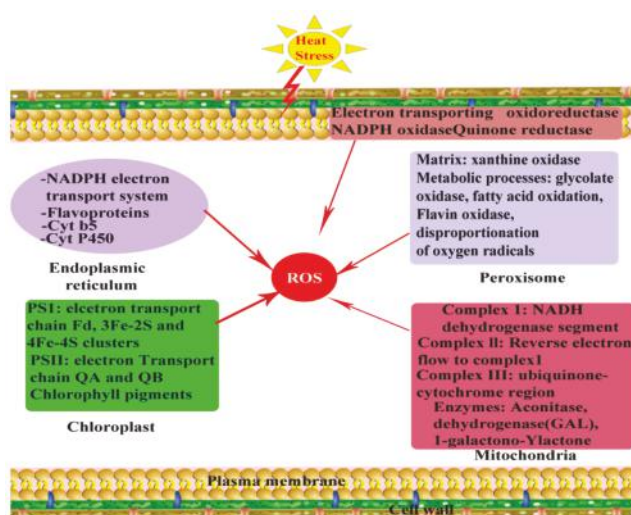
Figure 4. Reactive oxygen species (ROS) generate in plant cells as a consequence of heat stress. These reactive molecules are formed at different cellular sites, including chloroplasts, mitochondria, endoplasmic reticulum, peroxisomes, and at the extracellular side of the plasma membrane. The influence of ROS on cellular processes is mediated by both the perpetuation of their production and their amelioration by scavenging enzymes. The duration, location, and amplitude of production of ROS determine the specificity of the rapid responses under stress.

4. Avoidance and Tolerance Mechanism

Under HS conditions, plants show various survival mechanisms which include long-term morphological and phenological adaptations and short-term acclimation or avoidance mechanisms such as transpirational cooling, alteration of leaf orientation, or changing of membrane lipid compositions. Stomatal closure and water loss reduction, enhanced trichomatous and stomatal densities, and bigger xylem vessels are the main heat-induced features in crop plants [111]. Several plants growing in warm climatic conditions avoid HS by reducing the absorption of solar radiation. In order to avoid HS, some plants have small hairs on the leaf surface as well as cuticles (tomentose) and a waxy protective cover. The phenomenon called paraheliotropism occurs when plants often turn leaf blades away from sunlight, orient themselves parallel to solar rays, or roll their leaf blades. Under HS, early maturation is closely correlated with reduced yield losses in plants, which may be attributed to the engagement of an escape mechanism [107] (Figure 5). Such phenological and morphological adaptations are normally related with biochemical adaptations favoring net photosynthesis at HS (in particular CAM and C4 photosynthetic pathways), although C3 crop plants are also common in desert floras. HS can affect the degree of leaf rolling in many plants. The physiological role of leaf rolling is the maintenance of adaptation potential by enhancing the efficiency of water metabolism in the flag leaves of wheat under HS [112].
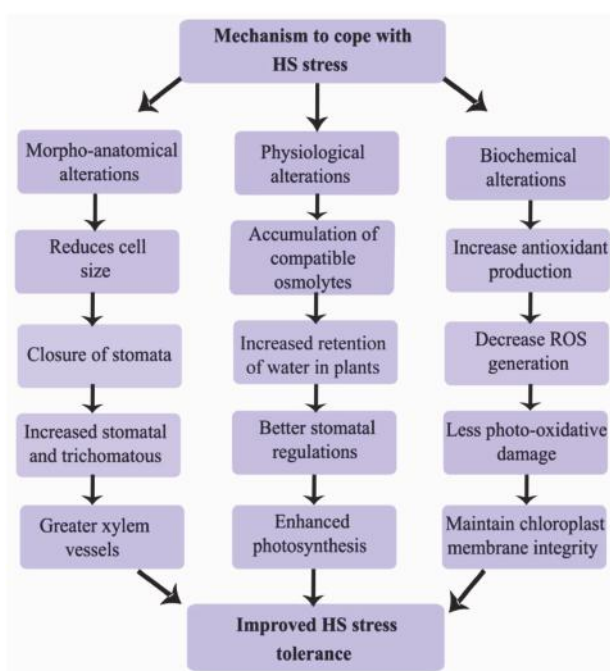
Figure 5. Flow chart of physiological, biochemical, and morphological adaptations of plants to deal with heat stress.

Thermotolerance is generally described as the capability of the plant to develop and grow to produce economic yield under HS. Plants have several survival mechanisms under HS conditions [113]. They included long-term adaptations or short-term acclimation/avoidance mechanisms. Some important mechanisms including ion transporter, late embryogenesis abundant proteins (LEA), antioxidant defense, osmoprotectants, and some factors linked with signaling cascade and transcriptional controls are fundamentally important to respond to HS [114] (Figure 5). It has been observed that several tissues in crop plants show differences in terms of developmental exposure, complexity, and responses toward the prevailing stress types [115]. These mechanisms help to regenerate homeostasis and to protect and repair damaged membranes and proteins [116].

5. Signaling in HS Tolerance

Signaling molecules (calcium, nitric oxide) play vital roles in conferring tolerance during HS conditions. Calcium as a divalent cation (Ca^{2+}) the important part of the cell membrane and cell wall and is an important intercellular messenger in the cytosol [117]. Plant roots absorbed Ca^{2+} from soil and are transported to shoots via xylem. The plasma membrane of a plant cell comprises Ca^{2+}-permeable ion channels through which Ca^{2+} enters [118]. Higher concentrations of Ca^{2+} are cytotoxic, therefore, an optimum concentration is to be maintained within cells. This is done by H^+/Ca^{2+} antiporters and Ca^{2+} ATPase [119,120]. The function of calcium in plants during HS has been debatable among scientists for quite some time. Some scientists doubt that the calcium chloride pretreatment leads to enhance thermotolerance and that calcium inhibitors limits plants [121,122]. Calcium and calmodulin treatment was able to induce HS transcriptional factors (HSFs) and heat shock elements (HSEs) binding in vitro, while, when treated with their inhibitors, they prevented binding at extreme HS [123]. Nitric oxide (NO) is an essential signaling molecule that controls several physiological processes and HS responses in crop plants. NO is membrane-permeable and highly

reactive molecule that plays a vital role during critical growth and developmental stages such as germination, leaf expansion, decrease in dormancy, and plant maturation [124]. Recently, NO has gained courtesy due to its significant involvement in stress defense in several plants [8]. Application of 50 and 100 μM sodium nitroprusside at 33 °C on two wheat cultivars PBW550 (heat sensitive) and C306 (heat tolerant) showed improved activities of the antioxidative enzyme and increased heat tolerance and cellular viability [108]. In rice, pretreatment of seedlings with NO leads to less damage due to HS and enhanced rates of survival in wheat leaves and maize seedlings [125]. It is proposed that NO might be shielding plant by decreasing ROS levels, and NO was observed to activate antioxidant enzymes like APX, CAT, and SOD during HS [126]. Plants perceive internal and external signals under HS through many interlinked or independent mechanisms that are used to control several responses [127].

There are several signal transduction molecules linked to stress-responsive gene activation depending upon the type of stress and type of plant. These molecule complexes are interlinked with transcriptional factors for active stress-responsive genes. To understand the pathways and the signaling molecules involved in the development of thermotolerance, fundamental research is needed. The signaling complex mechanism under HS has been reported, which was typically found to comprise the basic bHLH (helix loop helix) transcription factors phytochromes interacting factor 4, whose ortholog has been recognized in various plant species [128].

6. Development of HS-Tolerant Plants using Molecular and Biotechnological Approaches

6.1. Quantitative Trait Loci (QTLs)

HS appears to be principally polygenic in nature, which might clarify why the genetic basis of HS tolerance in crop plants is inadequately understood [64,129,130]. In order to improve understanding about HS on a genetic basis, significant effort has been made to identify QTLs. Advances in DNA marker identification and genotyping assays have allowed the exact identification of the chromosomal position of the QTLs accountable for HS in crop plants [131–133]. A QTL study involving 90 introgression lines provided 5 QTLs enlightening PVs in the range of 6.83%–14.63% [134]. Likewise, Y106, an introgression line carrying 2 QTLs for HS tolerance (*qHTS1-1* and *qHTS3*), was identified while transferring genes from the wild rice (*O. rufipogon* Griff.) [134]. Additionally, three major QTLs were mapped on chromosomes 2B, 7B, and 7D in 148 RILs (NW1014 × HUW468) are associated with HS [135].

In wheat, nine QTLs were mapped on chromosomes (2A, 6A, 6B, 3A, 3B, and 7A) related to senescence [136]. In rice, a total of 14 QTLs related with a heat susceptibility index (HSI) were identified using parameters such as temperature depression (TD) of spike and spike yield [137]. Genome-wide association mapping as well as candidate-gene based strategies using SNP and diversity array technology (DArT) were helpful in chickpea to discover marker–trait associations for HS [138]. In tomato, six QTLs were identified for improving fruit set under HS [139]. In *Brassica campestris* L. ssp. *Pekinensis,* five QTLs related to HS has been identified [140]. In rice, a total of five QTLs (*qHTS1-1*, *qHTS1-2*, *qHTS2*, *qHTS3*, and *qHTS8*) were recognized on chromosomes 1, 2, 3, and 8 established on the heat response of the 90 inbred lines using 152 SSR polymorphic markers [134]. Composite interval mapping identified a total of nine HSI mapped on linkage groups 2A, 2B, and 6D in wheat [141]. Similarly, they reported that the major QTL HSI (*Qhsigfd.iiwbr-2B*) for grain filling duration was 28.01 cM away from marker gwm257. Major QTLs for HSI for grain filling duration had also been described on this chromosome [135]. Other QTLs identified on to this region were more obviously linked with heat tolerance for grain filling duration [141]. Furthermore, QTLs for HSI for grain filling duration were also stated on chromosomes 1D, 2A, 6D [142], and 2D (*Qlgfd.iiwbr-2D*) and 7A (*Qlgfd. iiwbr-7A*) [143].

In recent studies, three major QTLs encompassing *QHst.cph-3B.2*, *QHst.cph-3B.3*, and *QHst.cph-1D* exposed the presence of 12 potential genes having a direct role in heat tolerance in rice [144]. Similarly, five QTLs were recognized on chromosomes 3, 5, 9, and 12 against HS. Of these QTLs, two high-effect QTLs, one novel (*qSTIPSS9.1*) and one known (*qSTIY5.1*/*qSSIY5.2*), were mapped in

less than 400 Kbp genomic regions, encompassing of 65 and 54 genes, respectively [145]. Despite the major advances in QTL mapping, there is still huge room for improvement.

6.2. Heat Stress Proteins (HSPs) and Heat Shock Factors (HSFs)

Heat stress proteins (HSPs) and heat shock factors (HSFs) are master players for HS tolerance in plants. Except for different biochemical and physiological mechanisms, molecular techniques are helping to understand the concept of thermotolerance in crops. HS is responsible for the up-regulation of several heat-inducible genes, commonly referred as "heat shock genes" (HSGs), which encode HSPs, and these products play important functions under stress conditions. These HSPs protect cells from the harmful impact of HS [146]. Proteomic analysis revealed that HS could down-regulate proteins playing roles in photosynthesis, energy, and metabolism and up-regulating the resistance-related proteins [147]. These proteins are grouped into five classes in plants, according to their molecular weight: Hsp100, Hsp90, Hsp70, Hsp60, and small heat-shock proteins (sHSPs) [148] (Table 2). Successful transcription, translation and post-translational modification lead to produce functional HSPs to protect the plant cell and responsible for HS tolerance [8] (Figure 6). Tolerance against HS has been accomplished in plant species transferred with heat shock regulatory proteins.

HSPs with molecular weights of 100–104 kDa are categorized into the HSP100 family. In plants, HSP100 proteins are extensively studied for their functions in HS tolerance in plants [115,149,150]. Among the various *Arabidopsis* HSP100 proteins, the cytosolic form is important for HS tolerance but not normal growth [149]. HSP90 is the most abundant in the cytosolic HSP family in both prokaryotic and eukaryotic cells and is rapidly induced in response to HS conditions. Under physiological conditions, HSP90 interconnect with several other intracellular proteins, including calmodulin, actin, tubulin, kinases, and receptor proteins [151–153]. HSP90 has been reported as a key regulator of normal growth and development in *Arabidopsis* and *Nicotiana benthamiana* L. [154–157]. Recently, over-expression of five Hsp90 genes of *Glycine max* in *Arabidopsis* resulted in reduction in lipid peroxidation and loss of chlorophyll, higher biomass production and pod setting under HS [158].

Table 2. Demonstration of basic role of major heat shock proteins in plants under heat stress.

HSPs Major Classes	MW (KDa)	Subcellular Localization	Proposed Response	Exampled Plants	References
Small HSP or HSP20	15–30	Cytosol, ER, mitochondria, chloroplast	Require for the development of chloroplasts during HS, avoiding aggregation, co-chaperone, and formation of high weight molecular complex (oligo-meric), which act as a matrix for stabilization of unfolded protein. HSP40, HSP70 and HSP100 are required for its release.	*Arabidopsis*, Rice, Soybean, Wheat	[159–162]
HSP60	57–69	Cytoplasm, mitochondria	Specialized folding machinery, which depends upon ATP, role in embryo, and seedling development in some plants, function as a chaperon in the post-translational assembly of multi-meric proteins.	*Arabidopsis*, Maize, bentgrass, Barley, Rye, Wheat, Creeping grass	[113,163,164]
HSP70	68–75	Cytosol, ER, nucleus, mitochondria, chloroplast	Assisting refolding and proteolytic degradation of abnormal proteins, preventing aggregation, primary stabilization of proteins, metabolic detoxification, ATP dependent release and binding.	*Arabidopsis*, Rice, *Brachypodium distachyon* L., Tobacco, Soybean, Citrus	[159,165–173]
HSP90	82–90	Cytosol, ER, nucleus, mitochondria, chloroplast	Co-regulation of thermal stress associated with signal transduction and accomplishes protein folding. Genetic buffering, metabolic detoxification, regulation of receptors, protein translocation. ATP is required for its function.	*Arabidopsis*, Chickpea, Pigeonpea	[159,174–176]
HSP100	100–104	Cytosol, mitochondria, chloroplast	Sustain the functional integrity of convinced major polypeptides, assisting to degrade irreversibly damaged polypeptides, re-solubilizing protein aggregates via interactions with the sHSP chaperone system; ATP is required for its function.	Rice, Maize, *Arabidopsis*	[150,151,157,159, 177–182]

Note: HSPs, Heat shock proteins; ER, Endoplasmic reticulum; sHSP, Small heat shock proteins; MW, Molecular weight.
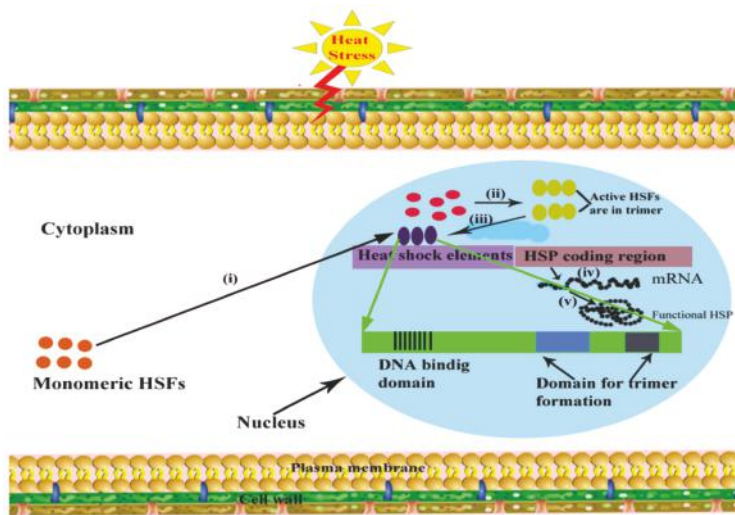
Figure 6. Illustration of the molecular regulatory mechanism of Heat Stress Proteins (HSPs) based on a hypothetical cellular model. Under heat stress, (**i**) monomeric Heat Shock Factors (HSFs) are entering into the nucleus from cytoplasm. (**ii**) HSF monomers are form active trimer in the nucleus, (**iii**) that will bind to the Heat Shock Element (HSE) of the respective Heat Shock Gene (HSG). Molecular dissection of the HSF binding region of HSE showing that it contains two domains for trimerization of HSFs and one DNA binding domain. (**iv**) Successful transcription, translation and post-translational modifications, (**v**) lead to produce functional HSPs (*Adapted and modified from* [8]).

HSP70 represents one of the most conserved classes of the heat shock proteins family. In plants and animals, HSP70 ensures proper protein folding during transfer to their final location and functions as a chaperone for newly synthesized proteins to prevent their accumulation as aggregates. HSP70 is the most highly conserved chaperones noted in plants, bacteria, and animals. These proteins associate with various other chaperones in a wide network and are implicated in diverse cellular activities [183]. Over-expression of HSP70 (HSP70-1) prevented degradation and fragmentation of nuclear DNA during HS conditions in tobacco [184]. Rice mitochondrial HSP70 over-expression resulted in the minor production of heat-induced ROS, suppressed programmed cell death, and higher mitochondrial membrane potential [184]. Chrysanthemum HSP70 expression in *A. thaliana* improved the tolerance against drought, salinity, and heat stresses [185].

Similar to other HSPs, sHSPs function as molecular chaperones, assisting refolding of denatured proteins and preventing undesired protein–protein interactions [151]. HSP20 or sHSPs are expressed in maximal amounts under HS conditions [160]. HSP20, a representative sHSP, maintains denatured proteins in a folding competent state and allows subsequent ATP-dependent disaggregation through the HSP70/90 chaperone system [166]. *Arabidopsis* plants over-expressing HSP17.5 of *Nelumbo nucifera* Gaertn., HSP17.8 of *Rosa chinesis* Jacq., HSP22 of *Zea mays* L., HSP26 of *Saccharomyces cerevisiae*, and HSP16.45 of *Lilium davidii* var. unicolor, showed heat tolerance to varying extents [184,186]. Tobacco plants over-expressing HSP16.9 of *Z. mays* resulted in increased early seed growth [184].

The research on plant HSFs regulation mainly emphasizes four levels, including transcriptional, post transcriptional, translational, and post-translation levels [187] (Figure 7). In transcription, the function of a gene can be regulated by binding of specific transcription factors (TFs) to the cis-acting elements located on the regulatory regions of its promoter [188]. Nishizawa-Yokoi et al. [188] reported that in *Arabidopsis* AtHSFA1d and A1e binding to the HSE in the 5′-flanking region of *AtHSFA2* gene is involved in high light (HL)-inducible *HSFA2* expression, triggering *AtHSFA2* transcription.

Dehydration-responsive element (DRE)-binding protein 2A (*DREB2A* gene) in the *Arabidopsis* directly regulates *AtHSFA3* transcription via binding the two DRE core elements in the *AtHSFA3* promoter under HS [189]. As *AtHSFA9* is mainly expressed in late stages of seed development, a TF may be involved in the regulation of *AtHSFA9* expression during the seed development stage.
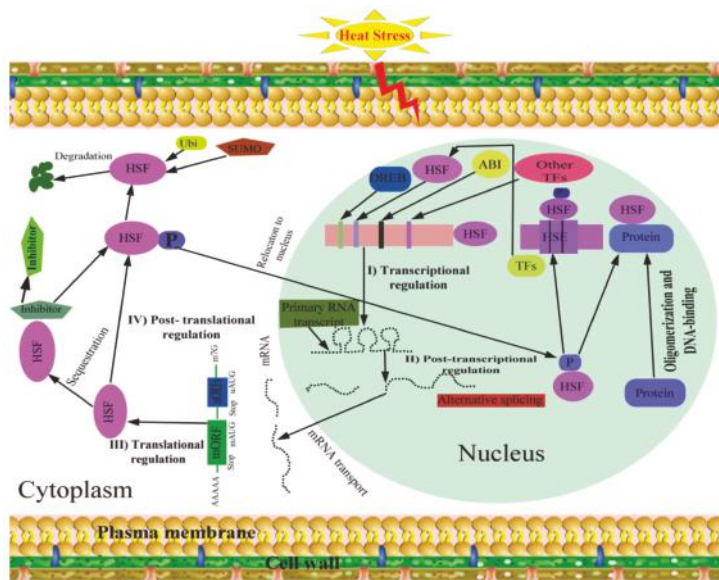
Figure 7. Illustration of the molecular regulatory mechanism of HSFs based on a hypothetical cellular model. The scheme illustrates the regulation of HSFs at (**I**) transcriptional, (**II**) post transcriptional, (**III**) translational, and (**IV**) post-translation level under heat stress. ABI, ABSCISIC ACID–INSENSITIVE protein; TFs, transcription factors; DREB, dehydration responsive element binding protein; AS, alternate splicing; m7G, cap of mRNA; mORF, major ORF; uORFs, upstream micro open reading frames; mAUG, AUG of mORF; uAUG, AUG of uORF; P, phosphate; mRNA, messenger RNA; Ubi, ubiquitination; SUMO, small ubiquitin-like modifier(*Adapted and modified from* [19]).

During several biological processes, HSFs post-transcriptional regulation involves alternative splicing in plants [187]. Keller et al. [190] examined HS-induced alternative splicing in the heat sensitive pollen tissue of two tomato cultivars. HS-induced alternative splicing is observed for *Arabidopsis AtHSFA2*, *A4c*, *A7b*, *B1* and *B2b* [191,192]. Alternative splicing induced by HS is also observed for rice *OsHSFA2d*, which encodes two main splice variant proteins, OsHSFA2dI located in the nucleus and OsHSFA2dII located in the nucleus and cytoplasm, respectively [193]. These studies suggest that the HSF regulation in the plant at posttranscriptional level is diversified. It is reported that the HSF regulation in the plant at the translational level is controlled by upstream micro open reading frames (uORFs) in their 5′ untranslated region [187,194,195]. However, the investigation on uORFs of plant *HSFs* is generally constrained to *Arabidopsis*.

HSFs also go through post-translational regulation included ubiquitination, phosphorylation, oligomerization, Small Ubiquitin-like MOdifier (SUMO)-mediated degradation, and interaction with other non-HSF proteins [196,197]. Mitogen-activated protein kinase MAPK6 targets the AtHSFA2, phosphorylates it on T249 and alters its intracellular localization under HS in *Arabidopsis* [198]. AtHSFA2 was regulated by the accumulation of polyubiquitinated proteins produced by the inhibition of AtHsp9026S and proteasome [199]. It is reported that AtSUMO1 interacts with AtHSFA2 at the core SUMOylation site Lys315, leading to the repression of its transcription and ultimately disrupting the

acquired thermotolerance in *Arabidopsis* [200]. Unfortunately, few active regulation factors involved in HSF regulation have been found to date.

6.3. Development of HS-Tolerant Plants Using Genetic Engineering and Transgenic Approaches

The devastating impacts of HS can be reduced by inducing thermotolerance in plants using different transgenic and genetic engineering techniques [107]. In addition to the investigations regarding manipulation expression of HSFs and sHSPs/chaperones, other genetically transformed plants with different degrees of thermotolerance have been developed (Table 3). Shockingly, such investigations have been relatively limited as compared to the investigations based on engineering cold, drought, or salt stress tolerance in plants. Lee et al. [201] successfully produced transgenic HS-tolerant *Arabidopsis* by changed HSP expression level by making a modification in the transcription factor (*AtHSF1*) that leads to the production of HSPs in *Arabidopsis*. It was reported that *AtHSF1* in *Arabidopsis* is constitutively expressed; in optimum temperature, its activity for DNA binding, trimer-formation, and transcriptional stimulation of genes (*HSPs*) are suppressed. When overexpression of the gene (*AtHSF1*) occurs, the transcription factor is not active for thermotolerance. A fusion protein was produced as a result of a fusion between the *AtHSF1* gene and N or C terminus of the *gusA* reporter gene (for β-synthesis of glucuronidase) that was able to trimerize itself and/or with the other HSFs in the absence of heat. That fusion protein transformation into *Arabidopsis* created a transgenic plant that expressed HSPs constitutive and showed increased heat tolerance without requiring prior thermal treatment [8]. It has been studied that in tomato, *MT-sHSP* (mitochondrial small HSP) has a molecular chaperone function in vitro [202], and it has already been reported that this gene is used to produce a thermotolerantly transformed tobacco plant [203]. Researchers were able to produce transgenic thermotolerant rice after the incorporation of HSP genes. In rice, thermotolerance was developed by successful overexpression of *Arabidopsis* gene *Hsp101* [204]. Additionally, in *E. coli* overexpression of *Oshsp26* (*sHSP*) gene in rice confer enhanced tolerance to HS and other related OS [205]. The *sHSP17.7* overexpression in rice plants confers thermotolerance [206].

Ono et al. [207] were able to successfully transfer a gene (*Dnak1*) in tobacco from the salt tolerant *Cyanobacterium aphanothece* halophytica and were able to successfully conferred HS tolerance. Yang et al. [208] reported that gene (*BADH*) transformation in the plant for the over-generation of GB osmolyte will improve thermotolerance. Heat tolerance is obtained when Rubisco activates gene transformation in tobacco for the Rubisco reversible decarboxylation; this defensive mechanism leads to safeguarding the plant photosynthetic apparatus and improvea thermotolerance [209]. The change in membrane fluidity may also alter the perception of the stress through lipid signaling, thus altering the response of defensive mechanism. Altering fatty acid composition in lipids to enhance HT stability of the photosynthetic membrane has also been shown to enhance thermotolerance and limits photo-oxidation due to the free radicals discharge. Murakami et al. [210] have been developing genetically modified *N. tabacum* L. with alteration in chloroplast membrane by silencing the gene encoding chloroplast omega-3 fatty acid desaturase. Such genetically transformed plants generate comparatively higher amounts of dienoic fatty acids and lower trienoic fatty acids in chloroplasts than the wild type. Remarkably, an *NPK1*-related transcript was significantly raised by heat in studies of [211]. A modest increase in thermotolerance of *Arabidopsis* plants constitutively expressing *APX1* gene of the barley has been reported [212]. Grover et al. [184] suggested many different means to use genetically modified plant in developing thermotolerance that may be attained by overexpressing genes (*HSPs*) or by changing level of HSFs that control expression of non-heat shock and heat shock genes by overexpressing of other trans-acting factors like bZIP28, WRKY, and DREB2A proteins.

Table 3. Transgenic crops, transgenes, source, and their responsible function for developing heat tolerance.

Transgenic Crop	Gene Transferred	Source	Function	Reference
Wheat	*TaMYB*	*Arabidopsis*	Response to various abiotic stresses	[213]
	TaFER-5B	*T. aestivum* L.	Transgenic plant exhibited enhanced thermotolerance	[214]
	TaGASR1	*T. aestivum* L.	*TaGASR1* overexpressing plant improved tolerance to HS and oxidative stress	[215]
	TaHsfC2a	*T. aestivum* L.	*TaHsfC2a* overexpressing wheat showed improved thermotolerance	[216]
Arabidopsis	*TaWRKY33*	*T. aestivum* L.	*TaWRKY33* transgenic lines showed enhanced tolerance to HS	[217]
	TaNAC2L	*T. aestivum* L.	Overexpression of *TaNAC2L* enhanced heat tolerance by activating expression of heat-related genes	[218]
	TaB2	*T. aestivum* L.	Overexpression of *TaB2* in Arabidopsis enhanced tolerance to HS	[219]
	TaGASR1	*T. aestivum* L.	*TaGASR1* overexpressing plant had improved tolerance to HS and oxidative stress	[215]
	TaLTP3	*T. aestivum* L.	*TaLTP3* overexpressing plant showed higher thermotolerance than control plants at the seedling stage	[220]
	TaOEP16-2-5B	*T. aestivum* L.	Transgenic plant overexpressing *theTaOEP16-2-5B* gene exhibited enhanced tolerance to HS	[221]
	Ot NOS	*O. tauri* L.	High accumulation of NO leading to thermotolerance and osmotic stress	[222]
Rice	*SBPase*	*Oryza. Sativa* L.	Transgenic more tolerant to HS during seed development	[223]
	HSP100, HSP101	*Arabidopsis*	Synthesis of HSPs for heat tolerance	[208]
	OsMYB48–1	*Oryza. sativa* L.	Plays a positive role in in stress tolerance	[224]
	OsHTAS	*Oryza. sativa* L.	Plays a positive role in heat tolerance at the seedling stage	[225]
	OsMYB55	*Oryza. sativa* L.	Improved high temperature tolerance	[226]
Tobacco	*BADH*	*S. oleracea* L.	Overproduction of GB osmolytes that will increase heat tolerance	[208]
	Fad7	*N. tabacum* L. and *O. sativa* L.	H_2O_2 responsive MAPK kinase kinase (MAPKKK) synthesis to protect against HS	[227]
Maize	*OsMYB55*	*Oryza. Sativa* L.	Improved plant growth and performance under high temperature and drought condition	[228]
	HSP100, HSP101	*Arabidopsis*	Synthesis of HSPs for heat tolerance	[115]
Wild carrot	*HSP 17.7*	*Daucus carota* L.	HSPs synthesis	[206]
Chili pepper	*CaATG8c*	*Capsicum annuum* L.	Plant tolerance to environmental stresses	[229]
Tomato	*LeAN2*	-	Conferred increased tolerance to HS by maintaining a low levels of reactive oxygen species and high non-enzymatic antioxidant activity	[230]

6.4. Development of HS-Tolerance Plants Using Omics Approaches

Keeping in view the importance of global warming as a potential threat, recent advances in "omics" approaches have offered new hopes and opportunities for the identification of post-translational, translational, and transcriptional mechanisms and signaling corridors that control the plants response to HS [8]. These "omics" approaches help to correlation and systematic analysis between alterations in microme, genome, proteome, metabolome, and transcriptome to the alteration in the responses of plant to HS and their application to enhance the probabilities of producing plants that are thermo-tolerant (Figure 8). In recent years, research has provided knowledge of the functions of proteins, metabolites, and several genes and molecular mechanisms involved in plant sensitivity to HS [96].

DNA is a basic unit of the entire molecular evidence related to thermotolerance in plant and comprises many different HS-responsive genes (genomics). Already, the identification of a huge number of genes with essential roles in HS responses has been done using genome-wide expression studies and genetic screens [231]. The transcriptory product like mRNA, from such genes in the genome, have made their transcriptomes (transcriptomics) and then proteomes (proteomics) when they translated into a functional protein (accountable for thermotolerance).

Many modern approaches such as RNA-sequencing have led to various deep expression investigations, ultimately unraveling various heat-responsive candidate genes in several

crops [36,232,233]. Transcriptomic investigations of HS effects on rice, wheat, tomato, grape, and tobacco have been reported [36,134,234]. Research in rice has unraveled that HS-responsive genes in panicles and flag leaves were mainly involved in transcriptional regulation, transport, protein binding, and anti-oxidants and stress responses [235]. Transcriptomic changes drive the physiological response to progressive drought stress and rehydration in tomato [236]. In a recent study, comparative transcriptome analysis revealed the transcriptional alterations in heat-resistant and heat-sensitive sweet maize (*Zea mays* L.) varieties under HS [237]. Chen and Li [238] found that DEGs were responsible for HS and protein folding in *Brachypodium distachyon*. It has been found that a huge number of genes were differentially expressed in leaves and roots in response to HS and/or desiccation, but only a few genes were identified as overlapping heat/drought responsive genes that are mainly involved in RNA regulation, transport, hormonal metabolism, and other stresses [239]. The transcriptome approaches are important to understand the molecular and cellular changes occurring in response to HS.
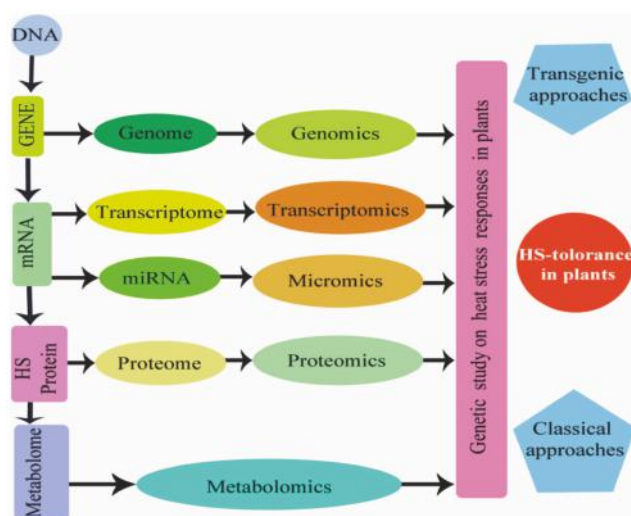
Figure 8. Illustration of integrated circuit of various "omics" techniques that correlated with each other at the molecular genetic level associated with thermotolerance in plants.

Micromics provide assistance for the better understanding of tolerance and miRNAs plays a significant role in such studies. Several thermo-tolerant responsive micro-RNAs have been recognized in plant, and their significant role in osmo-protection and nutrient deficiency response has been identified. In the onset of HS, up-regulated micro-RNAs may down-regulated their specific genes and act as negative regulators of thermotolerance, while stress down-regulated miRNAs may lead to accumulation of their target gene mRNAs, which may significantly regulate the thermotolerance. Overexpression of miRNA-resistant target genes plays an important role in plant post-transcriptional gene regulation and silencing and may result in the expression of improved traits in the genetically modified plant. Better understanding and knowledge about role of mRNAs in cellular tolerance, transcriptomehomeostasis, and the developmental and phenological plasticity of crop plants under HT and recovery will help with the genetic engineering of thermotolerance in plants.

Proteomics approaches provide important pieces of information, such as the heat-responsive proteins like HSPs and changes in proteomes under stress environments that associate to the analyses of transcriptomics and metabolomics, including the role of genes expressed in the genome's functionally translated regions linked to required traits [240]. The integration of proteomics with genetic information in legumes will give way to exciting opportunities to achieve crop improvement and

sustainable agriculture [241]. Proteomic analysis of wheat cultivar Jing411 revealed the expression of 256 different proteins under HS [215]. Further, proteomic analysis on leaves of wheat revealed different proteins that have roles in important photosynthesis, glycolysis, stress defense, heat shock, and ATP production [85]. The proteomics analyses provide a better understanding of the molecular basis of heat-stress responses in alfalfa [62]. Additionally, proteomic analysis on soybean leaves revealed the expression of 25 different proteins that have roles in important metabolic pathways, such as RuBisCo regulation, Calvin cycle, and electron transport under high temperature [242]. In an experiment concerning the proteome analysis of roots using heat stressed root hairs and normal root hairs, 30 commonly up and down-regulated proteins were identified [243]. In an investigation, three tomato (*Solanum lycopersicum* L.) cultivars (LA1310 (cherry tomato), Edkawi LA 2711 (unknown heat tolerance, salt tolerant), and Walter LA3465 (heat-tolerant)) were compared for changes in leaf proteomes after HS treatment [244]. In the reproductive phase, the response of pollen to HS is mainly regulated at the proteome level, whereby proteins related to degradation and synthesis of proteins are most HS-responsive and might play a vital role in the HS-response of pollen [245]. Exogenous spermidine-induced HT resistance by proteomic approaches in tomato [246]. Lin et al. [247] utilized a physiological and proteomic approach to discover the changes in protein expression profiles of tomatoes in response to heat and flood stresses.

Moreover, in several biochemical processes, proteomes are interlinked and will manufacture deferent metabolic product under metabolomics. Metabolomics comparison between HS and major types of abiotic stresses have recognized metabolites that are commonly essential in responses to stress [248,249]. In *Arabidopsis*, metabolites profiling demonstrated that HS reduced the toxicity of bio-active molecules like Pro, and these events reveal that during the more severe combination of stress treatments, sucrose replaces Pro in plant as the main osmoprotectant [250]. When comparing both cold and heat response, metabolomics reveals the nature of the overlapping of major metabolites in response to HS with those metabolites that are under cold stress responses in *Arabidopsis* [251]. Hence, it has been found that the metabolic complex of compatible solutes (galactinol-raffinose, fructose glucose, Pro, GB etc.) have a significant role in HS tolerance. A very important protein (ATGRP7) that acts as an RNA binding protein was found that increased in response to low temperature stress and reversed when under HS. Its excess was positively correlated with concentrations of Pro and glutamine. While concentrations of galactinol and raffinose were a significant marker for heat response, their responses were independent of the response of Pro, glutamine, and *ATGRP7* [248]. Such "omics" techniques are desired for the genetic molecular analyses on HS response in the plant in an integrated manner.

6.5. Epigenetics and Heat Tolerance

Epigenetics is defined as heritable modifications in expression and activity of a gene that occur without a change in DNA sequence and is linked with DNA methylation, histone modifications, and non-protein coding RNAs [252]. Knowledge and understanding about the Epigenetic regulation of HS responses has gained increasing interest [253,254]. A number of investigations have shown that these DNA and histone modifications play a key role in genes expression and plant development under stress conditions [255]. In Arabidopsis (*Arabidopsis thaliana* L.), a study investigated the malleability of the DNA methylome to stress within a generation and under repeated stress over five successive generations [256]. *Arabidopsis thaliana* L. imprinted gene SDC, which is silent during vegetative growth due to DNA methylation, is stimulated by heat and contributes to recovery from HS [257]. Recently, in wheat, a genome-wide survey revealed that elevated temperature had a dramatic effect on the expression of genes, but plants grown at 12 °C and 27 °C showed slight differences in methylation pattern. However, in only a few cases was methylation associated with small changes in gene expression [254]. It is observed that DNA methylation levels were different between a thermotolerant and heat-sensitive genotype under control environments [258]. Methylation enhanced more in the heat-sensitive genotype than in the heat tolerant genotype under heat treatments. It is

reported that more changes in the DNA methylation status of cultured microspores were observed under high temperature [259].

Histone variant deposition and histone modifications through SUMOylation and/or acetylation are considered to be involved in the HS response. Small ubiquitin-related modifier (SUMO) was recognized as a reversible post-translational modifier that contributed in the regulation of protein interactions in eukaryotes. Recent investigations have revealed that the occupancy of each histone variant of a core histone, in particular H2A and H3, play vital roles in not only genes expression but also in the assembly of chromosome centromeres and repair of DNA breaks in eukaryotes [260–262]. In *Arabidopsis*, it has been reported that H2A.Z deposition in gene bodies stimulates variability in the levels and gene expression patterns [261–263]. Additionally, H2A.Z plays an important role in the heat sensory response via its nucleosome occupancy. A screen of Arabidopsis (*Arabidopsis thaliana* L.) mutants deficient in temperature sensing under ambient temperatures (12–27 °C) identified actin-related protein 6 (*ARP6*) as a regulator of the coordinated alterations in gene expression in response to ambient temperature fluctuations [264]. *ARP6* encodes a subunit of the SWR1 complex [265] that is necessary for inserting the alternative histone H2A.Z into nucleosomes replacing the core histone H2A, and could be involved in heat sensing [264]. In *Arabidopsis*, it is reported that histone acetyltransferase GENERAL CONTROL OF NONREPRESSED PROTEIN5 (GCN5) plays a vital role in HS response by facilitating H3K9 and H3K14 acetylation of HSFA3 and UV-HYPERSENSITIVE6 (UVH6) under HS [266]. The histone acetyltransferase TaGCN5 gene in wheat is upregulated under HS and that it functions similarly to GCN5 in *Arabidopsis* [266]. Further studies are necessary to uncover what kinds of histone modification and histone deposition contribute to the HS response in crop plants.

Recent investigations suggest that a large part of the genome is transcribed, but among transcripts, only a minor portion encodes protein. The part of transcripts that do not encode proteins are generally termed as non-protein coding RNAs (npcRNA). These npcRNAs are subdivided as regulatory npcRNAs and housekeeping npcRNAs, with the latter being further divided into long regulatory npcRNAs (greater than 300 bp in length) and short regulatory npcRNAs (less than 300 bp in length, such as microRNA, siRNA, piwi-RNA) [267–271]. Xin et al. [269] recognized 66 HS-responsive long npcRNAs that performed their roles in the form of long molecules. To check whether miRNAs have any functions in regulating response to HS in *T. aestivum* L., Xin et al. [270] cloned small RNA exposed to HS and found that 12 of the 153 miRNAs identified were responsive to HS. Kumar et al. [267] identified 37 novel miRNAs in wheat and validated six of the identified novel miRNA as HS-responsive. Interestingly, TamiR159 was down-regulated after two hours of HS treatment in *T. aestivum* L., but TamiR159 overexpressing *Oryza sativa* L. lines were more sensitive to HS relative to the wild type, showing that down-regulation of TamiR159 in *T. aestivum* L. after HS might participate in a HS-related signaling pathway, in turn contributing to HS tolerance [272].

6.6. CRISPR/Cas9 Power in Genome Editing

CRISPR/Cas9 is the most powerful gene editing tool ever seen to date. Developing more crop plants able to sustainably produce higher yield when grown under HS is an important goal if food security and crop production are to be guaranteed in the face of increasing human population and unpredictable climatic conditions. However, conventional crop improvement through random mutagenesis or genetic recombination is laborious and cannot keep pace with increasing food demands. Targeted genome editing (GE) technologies, especially CRISPR/Cas9, have great potential to produce high-yielding crops under HS. This is due to their low risk, high accuracy, and efficiency of off-target effects compared with conventional random mutagenesis approaches. [273] reviewed recent applications of the CRISPR/Cas9-mediated GE as a means to produce plants with greater resilience to the stressors they encounter when grown under harsh environments. CRISPR/Cas9-mediated GE will allow the fast development of new crop cultivars with a very low risk of off-target effects, especially

for the crop plants that have complexity in their genomes and are not easily bred using conventional breeding approaches [274,275].

CRISPR/Cas9-mediated GE enabling the suppression or activation of target genes is also an essential tool for understanding the functioning of genes involved in plant abiotic stress resistance [276]. CRISPR/Cas9 targeted mutation of the *TMS5* gene in rice cultivars led to the rapid development of temperature-sensitive lines for use in hybrid rice production [277]. Klap et al. [278] carried out CRISPR/Cas9-induced knockdown of the tomato *slagamous-like 6 (SlAGL6)* gene, making tomato mutant plants able to produce parthenocarpic fruits under HS. Genome editing using the CRISPR/Cas9 system can be used to modify plant genomes. However, improvements in specificity and applicability are still needed in order for the editing technique to be useful in various plant species.

7. Conclusion and Future Prospects

From the forgone discussion, it can be concluded that HS has significantly affected crop yield in the past several years and currently has become a key concern for crop production because it significantly affects all stages of plant growth and development. One of the reasons of the enigmatic nature of heat tolerance mechanism in plants is the dissection of a very narrow genetic pool that does not provide ample information to explore it in worldwide commercially important crop plants such wheat, cotton, maize, rice, and soybean. There is an immense need to systematically assess wild species and accessions tolerating extreme degrees of higher temperatures. In that regard, searching for novel donors with high heat tolerance or escape mechanisms is of key importance. Crop responses to HS are for the most part grouped under heat tolerance categories without having explored heat avoidance or escape phenomena, which are equally viable under field conditions. Phenotyping techniques classifying HS response into the appropriate tolerance, escape, or avoidance category is a vital first step toward developing stress-resilient crops for the future hotter climate. A complete understanding and knowledge of the nature of heat shock signaling and specific gene expression under HS will be vital for developing HS-tolerant crop plants. Despite the major advances in genetic strategies like transgenic approaches and QTL mapping, there is still huge room for improvement. For example, genetic and environmental interactions are poorly understood. However, a brief mechanism of thermotolerance remains indefinable and needs appropriate research directions. In our opinion, the genetic base for heat tolerant mechanisms in crop plants can be expanded significantly through advances in the phenotyping approaches using biochemical means including lipidomics or metabolomics which that off an impression of being promising strategies in identifying robust biochemical markers to supplement breeding efforts. Furthermore, advances in ground-based or aerial (unmanned aerial vehicles) sensor technology may also help field-based high-throughput phenotyping, which may greatly facilitate marked expansion of the genetic base incorporated into abiotic stress breeding programs. On the field scale, multiple stresses interact, and dealing with the entire complexity could be challenging, and hence addressing subcomponents (such as HS or drought stress) independently and using advanced techniques for needs based traits/genes stacking based on the target environment would be a handy mechanistic research strategy for producing crop plants that will stand out firmly in the hottest season of the year.

In the future, integration of advanced high0throughput approaches, such as microarray, genomics, and proteomics, in various developmental stages and stress conditions will provide us with transgenic plants developed for combating with HS.

Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

APX	ascorbate peroxidase
Ca	calcium
CAT	catalase
GB	glycine betaine
HS	heat stress
HT	high temperature
HSPs	heat shock proteins
HSFs	heat shock transcriptional factors
HSEs	heat shock elements
LEA	late embryogenesis abundant proteins
MDA	malondialdehyde
NPQ	non-photochemical quenching
NO	nitric oxide
OS	oxidative Stress
PS	photosystem
QTLs	quantitative trait loci
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase
RuBP	ribulose bisphosphate
ROS	reactive oxygen species
SOD	superoxide dismutase

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Compared to Australian Cultivars, European Summer Wheat (*Triticum aestivum*) Overreacts When Moderate Heat Stress Is Applied at the Pollen Development Stage

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Abstract: Heat stress frequently imposes a strong negative impact on vegetative and reproductive development of plants leading to severe yield losses. Wheat, a major temperate crop, is more prone to suffer from increased temperatures than most other major crops. With heat waves becoming more intense and frequent, as a consequence of global warming, a decrease in wheat yield is highly expected. Here, we examined the impact of a short-term (48 h) heat stress on wheat imposed during reproduction at the pollen mitosis stage both, at the physiological and molecular level. We analyzed two sets of summer wheat germplasms from Australia (Kukri, Drysdale, Gladius, and RAC875) and Europe (Epos, Cornetto, Granny, and Chamsin). Heat stress strongly affected gas exchange parameters leading to reduced photosynthetic and transpiration rates in the European cultivars. These effects were less pronounced in Australian cultivars. Pollen viability was also reduced in all European cultivars. At the transcriptional level, the largest group of heat shock factor genes (type A *HSFs*), which trigger molecular responses as a result of environmental stimuli, showed small variations in gene expression levels in Australian wheat cultivars. In contrast, *HSFs* in European cultivars, including Epos and Granny, were strongly downregulated and partly even silenced, while the high-yielding variety Chamsin displayed a strong upregulation of type A *HSFs*. In conclusion, Australian cultivars are well adapted to moderate heat stress compared to European summer wheat. The latter strongly react after heat stress application by downregulating photosynthesis and transpiration rates as well as differentially regulating *HSFs* gene expression pattern.

Keywords: wheat; heat stress; heat shock factors (*HSF*); pollen mitosis; pollen viability; gas exchange parameters; photosynthesis; transpiration

1. Introduction

Wheat (*Triticum aestivum*) is one of the three major cereal crops, which contributes to more than 20% of the total human caloric and protein intake worldwide [1]. The first evidence of wheat domestication dated of about 12,000 years ago in the Middle East contributing to the transition from hunting and gathering of food to settled agriculture during human civilization [2,3]. Since then, wheat plants have been cultivated in a wide range of climatic conditions and in many geographic regions. Currently, Australia and Europe are two main hubs for wheat production. A large degree of heat stress resilience has been identified within Australian cultivars, with a significant number of cultivars exhibiting high levels of tolerance [4]. Australian wheat production has increased largely as a result of improved cultivars and well established crop management practices. In Europe, together with

maize, barley, and rye, wheat is one of the main agricultural crops. European wheat production represents around 20% of the total production worldwide [5]. However, largely attributed to climate conditions, a decrease in total cereal production has been observed in European countries over the past two decades [6].

Wheat adaptation to the Australian environment started more than 200 years ago, when the First Fleet arrived to Sydney around 1788 [7]. The first evidence of a successful cross-breeding employing a European cultivar (Italian wheat Tuscan) in Australia dated back to 1860 [7]. Since then, two major events have defined the evolution of the Australian wheat germplasm. First, the introduction of earlier flowering material and second the use of semi-dwarf germplasm during the early 70s of the last century [7]. In Europe, wheat breeding has a longer history, since it was spread from the Middle East to the entire European continent, first via Greece and then through the Danube river and the Balkans to Northern Europe [2]. Different breeding strategies have been employed in Europe depending on specific interests and market requirements. For instance, highly productive British wheat varieties with low tolerance to climate conditions have been crossed with high yielding German lines to develop varieties better adapted to increased temperatures with higher productivity [8]. However, even though some progress has been made with European varieties, long-term climate adaptation of Australian wheat varieties during breeding have generated genetic pools more resilient to environmental stresses compared to their European counterparts.

Gas exchange parameters, especially photosynthetic and transpiration rates, are particularly sensitive to heat stress [9]. Studies in wheat, rice, and tomato have shown that high temperatures lead, for example, to deactivation of the key CO_2-fixating enzyme RUBISCO [10,11], which correlates with a decline in photosynthesis observed at higher temperatures [12–14]. Therefore, maintenance of photosynthetic activity as well as high transpiration rates are considered indicators of heat tolerance. Furthermore, at the molecular level, heat shock factors (HSFs) are important regulators during plant response to increased temperatures. HSFs have been described as inducible transcriptional regulators of numerous genes encoding—for instance, molecular chaperones, ion transporters, aquaporins, and other stress proteins—in order to regulate stress responses. Moreover, HSFs have been shown to be master regulators for triggering acquired thermotolerance and heat stress responses [15,16]. In wheat, HSFs have been categorized into three groups [17], of which type A represents the largest group containing some gene members that are specifically expressed during reproductive development.

At the reproductive stage, heat stress results in severe yield losses in several crop species including wheat [18–21]. Particularly, male reproductive development is sensitive to environmental stresses [18,22]. Elevated temperatures during pollen development are detrimental to the formation of functional pollen. Anthers and pollen itself represent important photosynthetic sink tissues as high accumulation of photoassimilates, including starch and monomers of carbohydrates, are required during their development [18,22]. Starch and other reserve substances are accumulated in pollen grains and required during pollen tube growth to deliver the sperm cells towards the female gametes (egg and central cell) to ensure proper fertilization. Thus, disruption of pollen development or decrease in nutrient supply is expected to lead to sterility and failure of seed set, which potentially results in yield decrease.

Our current understanding of pollen development is mainly derived from model species such as maize, rice, and Arabidopsis [23–25]. Male gametophyte development (pollen) in wheat is poorly characterized, and even less is known how environmental stresses impact pollen development at the physiological and molecular level in this species. In wheat, studies associated with pollen susceptibility to heat stress are scarce [26–29] and were not performed under highly controlled moderate environmental stress conditions, allowing to separate heat stress from other stresses. Most studies have been focused on grain yield and quality [30,31]. Physiological and molecular parameters were usually not investigated, thus the mechanisms underlying reduced pollen viability, an important yield component, remained unexplored.

Understanding the effect of heat stress in two contrasting germplasms will help to mitigate the impact of increased temperature on wheat performance. With the goal to understand the adaptation of Australian summer wheat cultivars and the susceptibility of European cultivars to moderate heat stress, we selected four cultivars of each germplasm and compared them by measuring transpiration and photosynthetic rates, viability of male reproductive structures, and the response in gene expression levels of type A *HSF* genes. In the long term, this study may contribute to the development of an improved European germplasm with enhanced resilience to increased temperatures.

2. Materials and Methods

2.1. Plant Growth Conditions and Heat Stress Treatment

Wheat cultivars from Australia (*Triticum aestivum* L. cv. Kurki, Drysdale, Gladius, and RAC875) and Europe (*Triticum aestivum* L. cv. Epos, Cornetto, Granny, Chamsin) (Table 1) were germinated in an incubator and then transferred to pots (10 cm diameter, two seedlings per pot) containing a mixture of 15% sand, 25% Liapor (swelling clay), and 60% substrate (Einheitserde). Ten seedlings of each cultivar in each of three independent experiments were then transferred to 10 L pots to the greenhouse under controlled conditions of 14 h L/10 h dark photoperiod, with 21 °C ± 2 °C daytime temperature and 18 °C ± 2 °C night temperature, and a constant air humidity of 60–65%. An automated temperature—water based irrigation system was used to supply water according to plant consumption in a time-based pre-programmed schedule. Plants were fertilized twice a week with 2% fertilizer (Hakaphos) and monitored throughout their entire vegetative and reproductive developmental stages. After spikes developed, the auricle distance (the distance between the auricles of the flag leaf and the second last leaf) of each spike was measured. Spikes with auricle distance between 13–15 cm were marked and used for heat stress experiments; at this stage wheat plants enter pollen mitosis [32]. Spikes with an auricle distance of different lengths were not considered. Plants at pollen mitosis were then transferred to walking growth chambers. Non-heat-stressed control plants were maintained in chambers with identical conditions as described above. For heat stress, temperatures during the light/dark photoperiod were increased to 35 °C/25 °C, respectively, but otherwise conditions remained unchanged. Plants were kept for 48 h under heat stress conditions and then directly analyzed.

Table 1. List of summer wheat varieties (all bread wheat) used in this study. Their respective country of origin and flowering time are indicated.

Variety Name	Country of Origin	Flowering Time
Kukri	Australia	40 ± 1 day
Drysdale	Australia	48 ± 1.5 days
Gladius	Australia	49 ± 1.8 days
RAC875	Australia	45 ± 2.3 days
Epos	Czech Republic	52 ± 2.4 days
Cornetto	Germany	57 ± 1.1 days
Granny	Czech Republic	50 ± 1.6 days
Chamsin	Germany	53 ± 2 days

2.2. Physiological Measurements

Wheat plants of Australian and European cultivars were continuously monitored during the time course of the experiments. Gas exchange parameters were recorded at 0, 24, and 48 h in control and heat stressed plants. Fully expanded flag leaves were used to estimate net photosynthetic rate (A) and transpiration rate (E). Daily measurements were taken with an Infrared Gas Analyzer (IRGA, LCpro+; ADC Bioscientific, Hoddesdon, UK) at a CO_2 concentration of 360 μL L^{-1}, a saturating light intensity of 1000 μmol m^{-2} s^{-1} and a gas flow rate of 200 mL min^{-1} as described before [33–35]. For each time point, 15 to 20 plants per treatment were examined and measurements completed between 10:30 a.m. and 2:00 p.m. Data were recorded when gas exchange and chlorophyll fluorescence parameters became stable during measurements.

2.3. Histological Analysis

Anthers of non- and heat-stressed plants were fixed in 3.7% (*w/v*) formaldehyde, 5% (*v/v*) acetic acid and 50% (*v/v*) ethanol, vacuum infiltrated, and stored at 4 °C overnight. Samples were then embedded in 10% (*w/v*) low melting agarose dissolved in distilled water. Samples were kept in gelatine blocks and post-fixed with 10% formaldehyde and 0.1 M standard PBS buffer at 4 °C overnight. Embedded blocks (1.5 × 1.5 × 1.0 cm) were stored at 4 °C in PBS until sectioning. Cross-sections (60 μm thick) were prepared using a vibratome Hyrax V50 (Carl Zeiss MicroImaging, Thornwood, NY, USA). For quality comparisons, at least 10 cross sections were prepared per sample. Three independent experiments were each performed to collect anthers for sectioning. Images of anther sections shown in Figure S1 are representatives of observations across replicates.

2.4. Microscopy and Pollen Viability Assay

Mature pollen grains of non- and heat-stressed wheat of all eight cultivars were isolated and mounted on glass slides containing 3.33 g/L iodine and 6.66 g/L potassium iodide, covered with a cover slip and observed in a Zeiss Axio Imager Z1 microscope equipped with an apotome module and an AxioCam MRM monochromatic camera. We tested on average three thousand grains per cultivar/condition.

2.5. RNA Isolation and RT-qPCR

Total RNA from pollen was isolated using the RNA Plant Mini kit (Ambion, Waltham, MA, USA) following the manufacturer's instructions. cDNA synthesis was performed using reverse transcriptase (Invitrogen SuperScript II, Carlsbad, CA, USA) and oligo(dT) primers. Real time PCR reactions were performed using KAPA SYBR Fast qPCR master mix (Peqlab Biotechnology, Erlangen, Germany) as described [25,35]. Expression levels of a wheat tubulin gene (GenBank accession No. U76558) were used as internal standards for normalization of cDNA template quantity using tubulin-specific primers (Table S1). PCRs were performed using a MasterCycler® RealPlex² system (Eppendorf, Hamburg, Germany) in a 96-well reaction plate according to the manufacturer's recommendations. Primers used in this study are listed in Table S1. Cycling parameters consisted of 5 min at 95 °C, and 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 70 °C for 30 s as described previously [24]. qPCR reactions were performed in triplicate for each RNA sample on at least three biological replicates. Specificity of the amplifications was verified by a melting curve analysis to test product specificity. Results from the MasterCycler® RealPlex2 detection system were further analyzed using Microsoft Excel. Relative amounts of mRNA were calculated from threshold points (Ct values) located in the log-linear range of real time PCR amplification plots using the $2^{-\Delta Ct}$ method [36].

2.6. Statistical Analysis

Statistical analyses were performed using the R software/environment. A one-way ANOVA was used to compare flowering time between Australian and European cultivars. Data from at least three independent experiments, where each experiment had at least *n* = 6 plants per cultivar/condition were used. Data were expressed as means with standard deviation, and *p*-value of 0.05 was used as the significance level.

3. Results

Eight Australian and European summer wheat cultivars (Table 1) were exposed to a moderate heat stress (35 °C during the day and 25 °C during the night) for 48 h after flowering induction when anthers contained pollen at the mitosis stages of development. Flowering time was significantly shorter in all-Australian cultivars compared to the European varieties. Our experimental design aimed to reflect short heat temperature episodes, which are very frequent in Europe as a consequence of global warming.

3.1. Physiological Responses of Australian and European Summer Wheat Cultivars under Heat Stress

3.1.1. Transpiration Rate Was Strongly Reduced in European Cultivars

Since heat stress events lead to damaging the photosynthetic apparatus and withering of plants [37], we first compared gas exchange parameters between Australian and European wheat cultivars. We measured the transpiration rate at the beginning of the experiment (0 h) as well as 24 and 48 h after heat stress (HS). A control group of non-stressed plants was measured in a neighboring growth chamber at the same time-points. Apart from the temperature, all other conditions were kept constant. Transpiration rates in the Australian varieties Kukri and Gladius, remained comparable in both HS and control conditions (Figure 1A,G). Phenotypically, these cultivars appeared unchanged even 48 h after HS treatment (Figure 1B,C,H,I). Plants looked healthy, showed almost no sign of stress with only a few curled leaves. In the varieties Drysdale and RAC875, a decrease in transpiration rates was observed 24 h after HS (Figure 1D,E) and a further reduction was noticed after 48 h of HS. However, transpiration rates were still significantly above a 50% level of the non-stressed control plants. These cultivars also looked healthy, though RAC875 showed partly yellowish leaves and a larger number of curly leaves compared with the other Australian varieties.
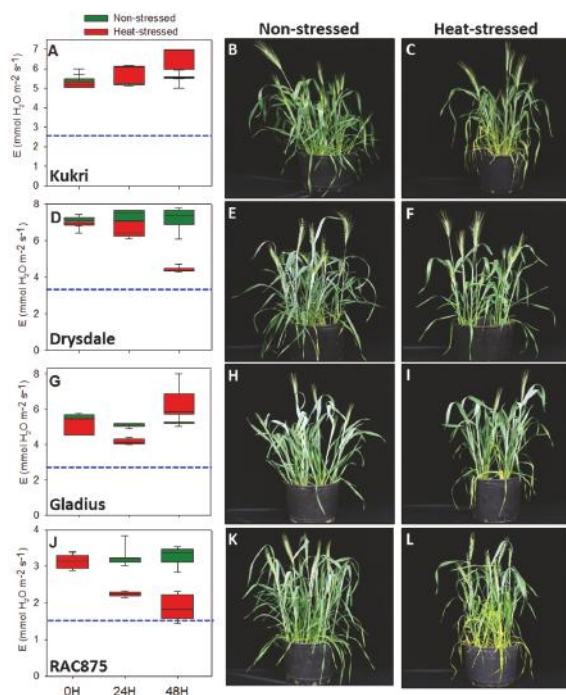
Figure 1. Transpiration rate is maintained under moderate heat stress by Australian summer wheat varieties. Physiological and phenotypical effects on varieties Kukri (**A–C**), Drysdale (**D–F**), Gladius (**G–I**), and RAC875 (**J–L**) are shown. Box plots show the central tendency and dispersion of the transpiration rate among all Australian cultivars. Green and red box plots represent non- and heat-stressed plants, respectively. Blue bars show 50% transpiration rate of non-stressed plants. 15–20 plants of each line were examined.

In contrast, the European summer wheat cultivars showed a pronounced decline in transpiration rate after HS imposition (Figure 2). With the exception of the cultivar Chamsin, elevated temperatures

resulted on average in a 25% reduction of transpiration rates in European cultivars after 24 h of HS. After 48 h of HS, all cultivars showed a reduction in transpiration rates close to 75% and in some instances even higher as observed in Epos (Figure 2A,D,G,J). This effect was confirmed phenotypically, as heat-stressed plants wilted and showed yellowish leaves (Figure 2). In summary, Australian cultivars showed a higher tolerance to moderate heat stress indicated by their appearance and sustained high levels of transpiration rates. European lines were less tolerant and showed a marked reduction of transpiration rate.
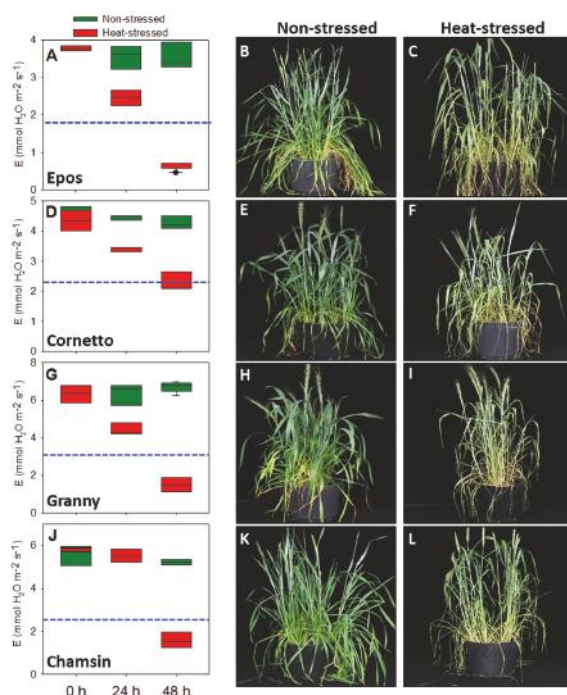
Figure 2. Moderate heat stress strongly alters transpiration rate of European summer wheat varieties. Physiological and phenotypical effects on varieties Epos (**A–C**), Cornetto (**D–F**), Granny (**G–I**), and Chamsin (**J–L**) are shown. Box plots show the central tendency and dispersion of the transpiration rate among all European cultivars. Green and red box plots represent non- and heat-stressed plants, respectively. Blue bars show 50% transpiration rate of non-stressed plants. 15–20 plants of each line were examined.

3.1.2. Photosynthesis Rate Was Strongly Reduced in European Cultivars

Another physiological parameter highly affected by increased temperatures is the net photosynthetic rate. During stress treatment, a significant decline in gas exchange parameters occurred in all cultivars tested. However, a less accentuated drop was observed in the Australian cultivars (Figure 3A–D) compared with the European cultivars (Figure 3E,F). At 24 h after HS, photosynthesis remained close to the non-stress conditions in Kukri and Gladius as well as in Epos (Figure 3A,C,E). In Drysdale and Granny a decline of 22% and 25% on net photosynthesis were observed, respectively (Figure 3B,G). Notably, RAC875, Cornetto, and Chamsin showed a more pronounced decline, with a reduction of net photosynthetic levels of 55, 60, and 77%, respectively, relative to non-stressed control plants (Figure 3D,F,H). After 48 h of HS the Australian cultivars Kukri, Drysdale, and Gladius showed a higher tolerance to heat stress (Figure 3A–C) as indicated by a higher photosynthetic activity. None of

these cultivars showed a decrease below 50% of the photosynthetic rate under non-stress conditions. The photosynthetic rate of the fourth Australian cultivar tested (RAC875), declined close to 50% already after 24 h of HS and remained at this value during continuation of HS treatment (Figure 3D). Unlike the Australian cultivars, European summer wheat cultivars with the exception of cultivar Epos were strongly affected already after 24 h of HS showing a sharp decline in photosynthesis rate (Figure 3E–H). In Cornetto and Chamsin (Figure 3F,H) a drop below 50% of the values observed under non-stress conditions was observed and in Granny (Figure 3G) to about 60% levels of control plants. After 48 h of HS exposure all European cultivars showed a net photosynthetic reduction of more than 75% reaching almost zero in Chamsin (Figure 3H). In conclusion, two days after HS treatment, the net photosynthetic rate decreased on average over 80% in European cultivars, while none of the Australian cultivars showed a drop below 50% compared to control plants. Two Australian cultivars (Kukri and Gladius) were especially well-adapted to HS and showed a drop of 25% in photosynthesis rate.
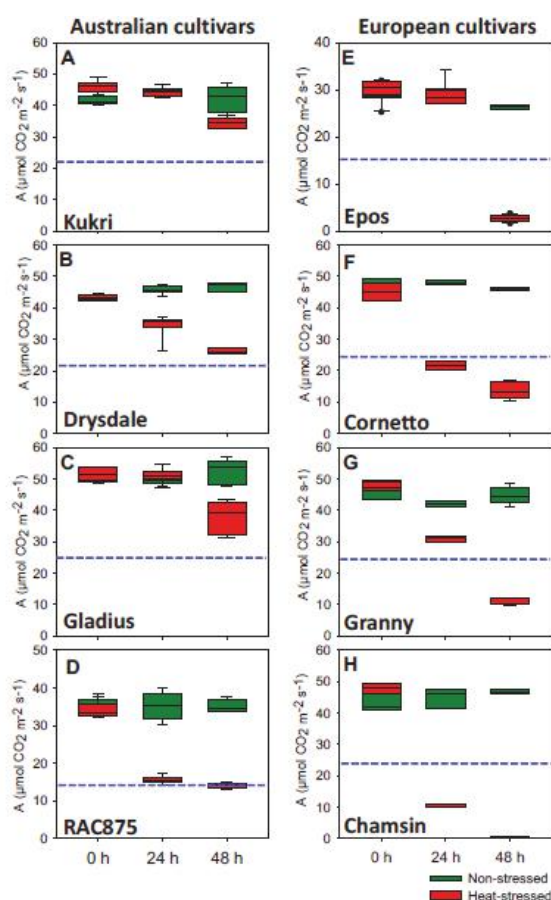
Figure 3. Effect of moderate heat stress on net photosynthesis rates of Australian (**A**–**D**) and European (**E**–**H**) summer wheat varieties during the pollen division stage. Box plots show the central tendency and dispersion of the net photosynthetic rate among all Australian and European wheat cultivars. Green and red box plots represent non- and heat-stressed plants, respectively. Blue bars show 50% net photosynthesis rates of non-stressed plants for comparison. 15–20 plants of each line were examined.

3.2. Analysis of Male Reproductive Structures after a Short-Term Moderate Heat Stress

3.2.1. Anther Morphology Was Not Significantly Modified after Moderate Heat Stress

One of the most susceptible developmental stages to environmental stresses is male gametophyte development (pollen formation), which occurs in anthers. We collected anthers of all wheat cultivars under non-stress and heat-stress (HS) conditions (Figure S1). To illustrate how our histological analysis were carried out, a scheme indicating a wheat anther and its cross section is shown in Figure S1A. A schematic overview of the internal part of an anther is illustrated in Figure S1B and a detailed view explaining the various anther cell types in Figure S1C.

Under non-stress conditions, spikes containing anthers of both Australian and European cultivars were at the mitosis stage (Figure S1D–K). Identification of developmental stages was conducted based on the auricle distance, which is the distance between the auricles of the flag leaf and the second last leaf [33]. Spikes with an auricle distance between 13 and 15 centimeters were tagged and used for heat stress experiments; at this auricle distance, developing pollen grains completed meiosis and were at the microspore stage shortly before pollen mitosis. After two days of HS, Australian cultivars Kukri, Drysdale, Gladius, and RAC875 displayed normal anther and pollen development (Figure S1D–G). Notably, European wheat varieties also displayed normal pollen development both under HS and control conditions (Figure S1H,K). Pollen grains attached to the anthers were clearly noticeable. Overall, even though European cultivars were strongly affected at the physiological level, the morphology of male reproductive structures (anthers and pollen) did not appear to be severely affected.

3.2.2. Pollen Viability Was Only Affected in European Cultivars after Moderate Heat Stress

Decreased pollen viability has been reported as a major effect of heat stress during reproductive development. Even though anthers of Australian and European varieties did not show a significant damage after 48 h of heat stress (HS), we investigated to which extent pollen of both sets of plants were affected by HS. Pollen viability was tested using iodine-potassium (I2-IK) staining. As shown in Figure 4, almost all pollen grains from Australian varieties were viable (Figure 4A–D) as indicated by strong dyed starch coloration. On average 96–99% of pollen tested were viable (Figure 4I). In contrast, European wheat varieties exposed to HS had a significant amount of non-viable pollen grains, which either did not stain at all or were partially stained (Figure 4E–H). The most severe effect of HS was observed on pollen grains of the Epos cultivar (Figure 4E). Approximately 40% of pollen grains were non-viable (Figure 4I). Similarly, around 25% of pollen grains from other European cultivars were non-viable. We could not test to which extent pollen germination and growth of viable pollen were affected, because wheat pollen generally germinate very poorly in vitro [38]. Nonetheless, our findings suggest that high temperatures during pollen development significantly impair pollen grain development of European cultivars, but not of Australian ones.
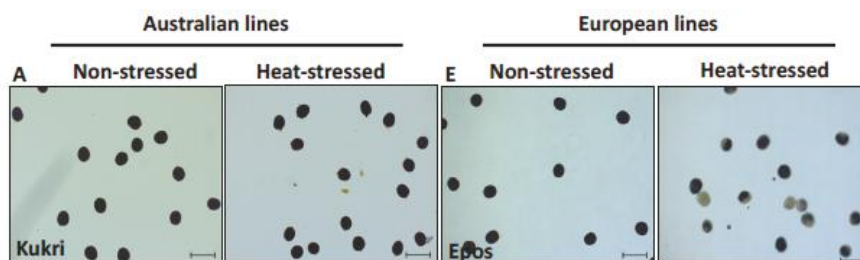
Figure 4. *Cont.*
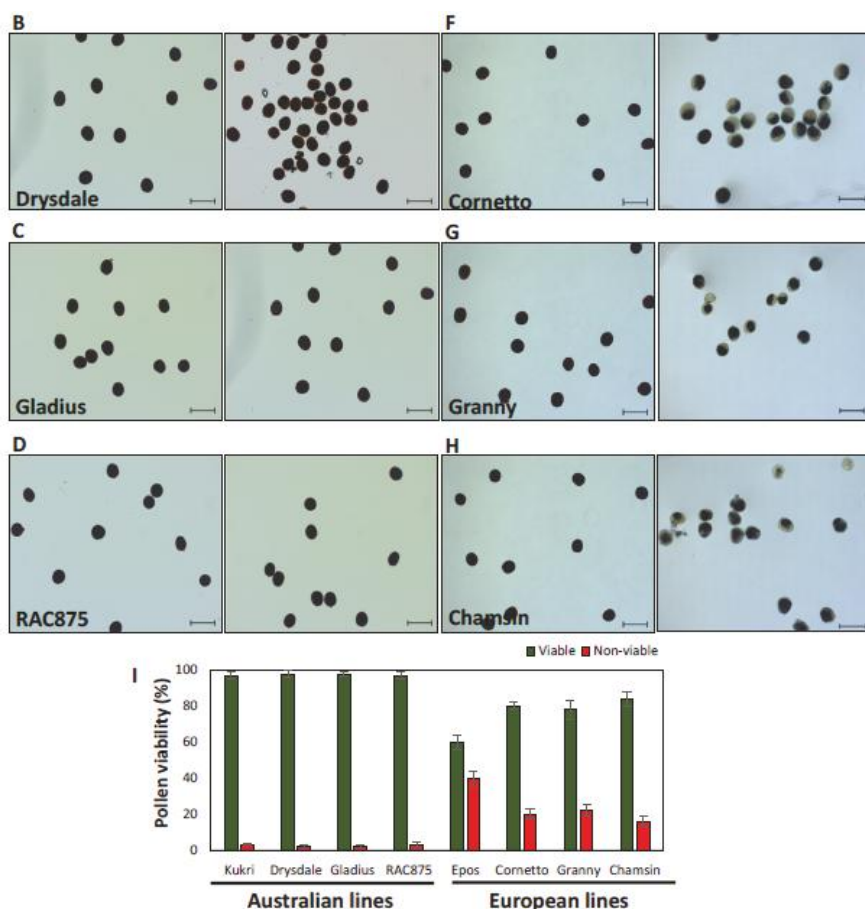
Figure 4. Pollen viability determined in Australian (**A–D**) and European (**E–H**) summer wheat varieties under non-stress and moderate heat-stress conditions. Pollen released from anthers were stained with lugol-iodine (I2-IK). (**I**) Percentage of viable pollen compared between Australian and Europeans summer wheat varieties after exposure to moderate heat stress during pollen mitosis.

3.3. Heat Shock Factors Genes Were Strongly Regulated in European, but Not in Australian Cultivars

In cereals and other plants, heat shock factors (*HSFs*) play an important regulatory role in response to heat stress (HS) and acquired thermotolerance. To understand the molecular basis of the HS response in Australian and European wheat cultivars, we analyzed and compared the gene expression level of several members of the *HSF* gene family. In wheat, 56 *HSF* genes have been identified, which were categorized into classes A, B, and C [17]. Many genes of the largest *TaHSF* class, A class, were previously shown to be predominately expressed in reproductive tissues under non-stress conditions [17]. Therefore, we selected this class to analyze their expression using anthers containing pollen at the mitosis stage under control and HS conditions.

TaHSF class A contains 25 genes divided into seven subclasses. As shown in Figure 5, all Australian cultivars responded to HS as indicated by significant up- and downregulation of some selected genes, while the relative expression levels of most genes were not dramatically altered. In Kukri only genes A3a, A3b, A4e, and A7e were strongly upregulated, while A6c, A6d, and A6e were strongly

downregulated (Figure 5A). In Drysdale, the expression level of all other class A members remained low and invariable upon heat stress treatment (Figure 5B). Similarly, in Gladius, only five genes of subclass A6 were strongly downregulated with A6c and A6d even being completely switched off (Figure 5C). Interestingly, RAC875 showed a different response; subclasses A1 and A2 genes were upregulated after HS, while genes of the other subclasses were lowly expressed and did not alter their expression significantly after HS (Figure 5D). In general, class A *HSF* genes in the Australian wheat cultivars were similarly expressed both under HS and control conditions.
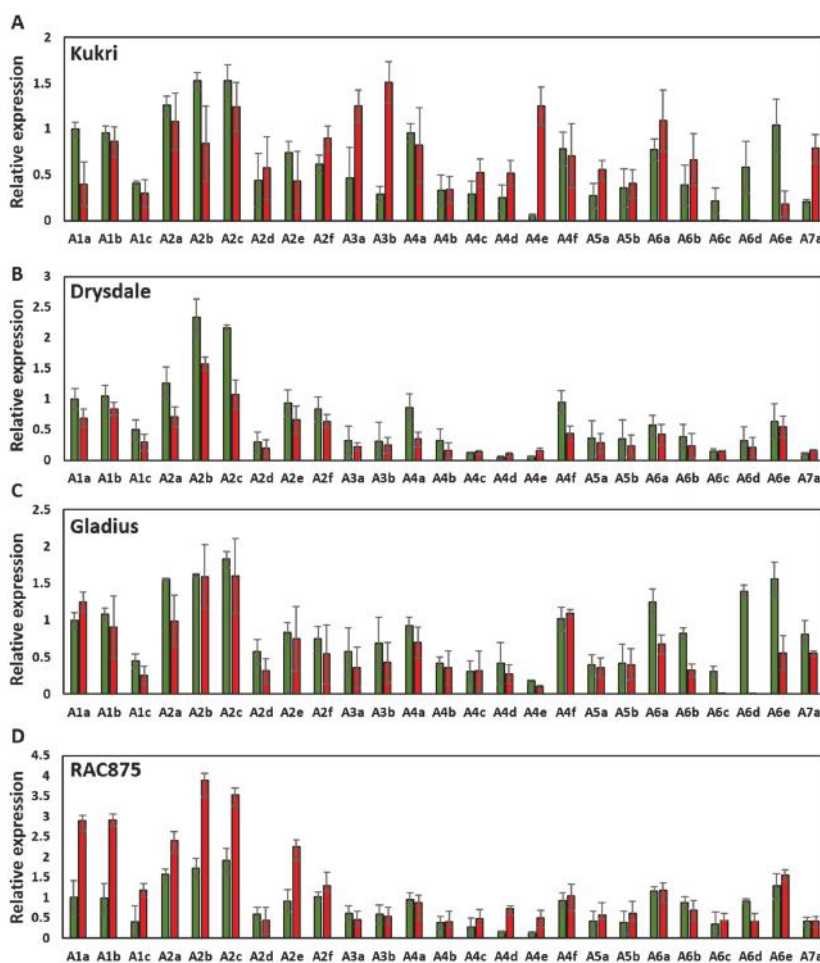
Figure 5. Relative mRNA expression levels of class A heat shock factor genes (*TaHSFs*) in anthers of Australian summer wheat varieties comparing heat stressed and control plants. A tubulin gene was used as a housekeeping gene for normalization. Expression values were set in relation to the average expression level of A1a HSF. Kukri (**A**), Drysdale (**B**), Gladius (**C**), and RAC875 (**D**). Green and red bars represent non- and heat-stressed plants, respectively. Values are means ± SD of three biological replicates.

The European wheat cultivars showed a much stronger response to HS. In Epos, only *HSF* genes A1a, A1b, A2a, A2c, A2e, and A6a were expressed under non-stress conditions. With the exception

of A6a, these genes were completely silenced after HS (Figure 6A). Similarly, Cornetto showed downregulation of almost all class A genes, except for A4e, which was upregulated, while A2a, A3a and A4f expression levels did not change significantly (Figure 6B). In Granny, low expression levels of *HSFs* were observed through the entire class A genes (Figure 6C). Under HS, with the exception of A2a, which showed comparable expression under non-stress conditions, all gene members of the subclasses A1 and A2 had low expression levels. Finally, Chamsin was the only European cultivar that showed a strong upregulation of *HSF* genes in response to HS. In general, class A *HSF* genes are expressed at low levels in this cultivar under non-stress conditions and a strong induction, especially of subclass A3 and A4 as well as of A2a was detected (Figure 6D). Taken together, our results indicate that the relatively stable transcriptional expression of *HSF* genes in the Australian cultivars is associated with their high level of acquired HS tolerance. Contrastingly, at the transcriptional level, the European cultivars showed a strong response in gene expression after HS exposure.
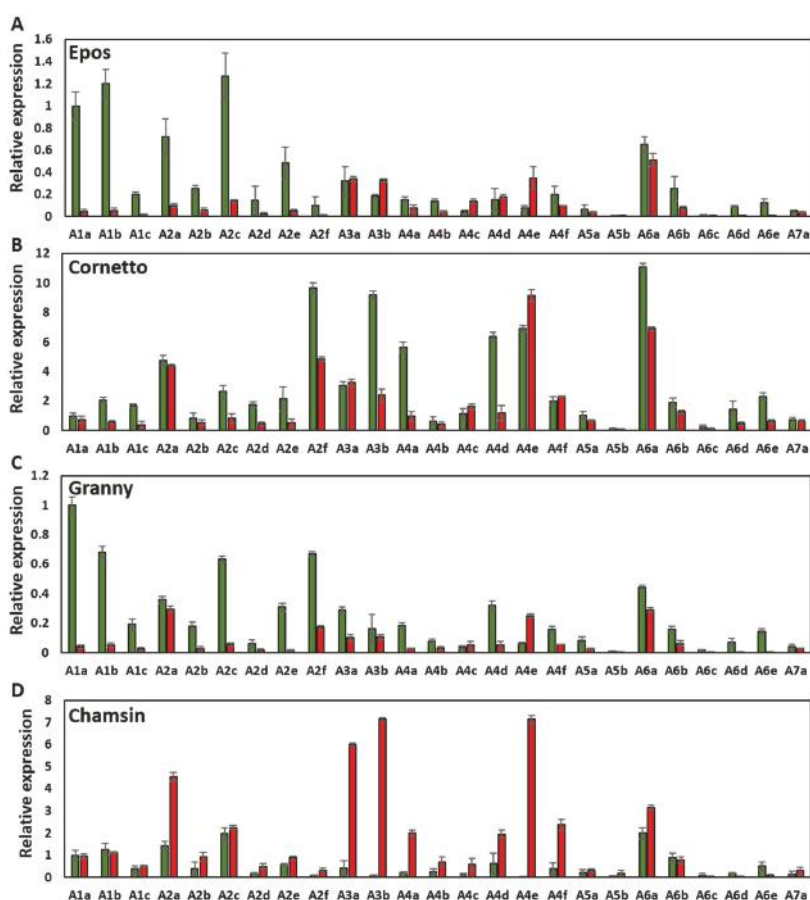
Figure 6. Relative mRNA expression levels of class A heat shock factor genes (*TaHSFs*) in anthers of European summer wheat varieties comparing heat stressed and control plants. A tubulin gene was used as housekeeping gene for normalization. Expression values were set in relation to the average expression level of A1a HSF. Epos (**A**), Cornetto (**B**), Granny (**C**), and Chamsin (**D**). Green and red bars represent non- and heat-stressed plants, respectively. Values are means ± SD of three biological replicates.

4. Discussion

The continuous improvement of wheat cultivars better adapted to environmental stresses, including increased temperatures, is an important goal of modern breeding and agriculture. To achieve this aim, it is necessary to comprehensively explore available germplasms to obtain a deeper understanding of the physiological and molecular adaptive mechanisms that differ between genotypes under changing environmental stress conditions. In this study, we analyzed two sets of summer wheat cultivars from different germplasms exposed to elevated temperatures. Our study confirms previous findings that long-term adaptation and breeding strategies have played fundamental roles in Australian wheat cultivars, which possess an enhanced tolerance to heat stress (HS) compared to European cultivars.

In general, plants use different strategies to overcome heat stress conditions. One of these strategies involves the adjustment between photosynthetic and transpiration rates through the regulation of stomata opening and closure. Stomata closure avoids the loss of water, but leads also to a reduction in CO_2 assimilation and thus photosynthetic rate, while their opening increases gas exchange, including the uptake of CO_2 and simultaneously to the cooling of the leaf surface at elevated temperatures. This avoids, for instance, membrane and thylakoid damage. Thus, a tight regulation is required to balance the various effects caused by increased temperatures [9]. Compared with HS susceptible European cultivars, we observed that Australian cultivars maintained higher photosynthetic and transpiration rates under moderate HS conditions. Similar observations have been made in tomato [12,13] and rice [14], where tolerant varieties displayed higher photosynthetic and transpiration rates compared to susceptible lines confirming our observations. The decline in these two gas exchange parameters as the result of HS has been suggested to be cultivar- and stage-dependent. Exposure of winter wheat cultivars to elevated temperatures resulted, for example, in a reduction of photosynthesis during the grain-filling phase compared to the vegetative stage [21,39]. Similarly, we found that all cultivars tested displayed an individual physiological and molecular HS response. Among the Australian summer wheat cultivars tested, Kukri and Gladius showed a relatively constant photosynthetic rate at increased temperatures, while Drysdale and RAC875 showed a significant decline. Natural variation in photosynthetic and transpiration rates are known to exist within crop species [40–43]. These variations have not shown a consistent correlation between photosynthetic rate during anthesis and grain yield or biomass when wheat cultivars were compared [40–43]. Particularly, wheat cultivars with the highest photosynthetic rates did not necessarily generate highest yields. Therefore, the large variation observed in Australian wheat cultivars could be attributed to unintentional selection traits that result in high photosynthetic rate [41,42]. Notably, Kukri, a drought-sensitive cultivar, and RAC875, a drought-tolerant one [44], displayed opposite responses to heat stress, indicating that these drought tolerant/susceptible Australian wheat cultivars display unrelated responses to heat stress. Similarly, a recent study showed large differences in gene expression responses to drought and heat stress between two barley cultivars [45] supporting this observation.

Unlike vegetative development, where stresses can be tolerated to a certain extent, reproductive development is highly sensitive to environmental stresses [18–20,34,45]. Male gametophyte or pollen development is especially susceptible to environmental stimuli, resulting in morphological, metabolic, and epigenetic alterations [23]. With the exception of a decrease in pollen viability in all European cultivars, we did not observe obvious alterations in anther and pollen morphology after HS. A moderate HS for two days is probably too short to cause more severe effects, which ultimately lead to complete sterility. Moreover, measuring the 100-grain weight and grain number per plant did not show significant differences when the heat stress was applied for only two days during the pollen mitosis stages (data not shown) and plants were allowed to quickly recover and grow at optimal conditions afterwards during the fertilization and seed filling stages. Thus the observed 20–40% loss in pollen viability in European cultivars could be compensated by viable pollen. A major future direction thus will be focused on investigating how longer HS periods affect sterility, seed set, and grain yield. Since starch, sugars, and other photoassimilates are generated during photosynthesis, a reduction in

pollen viability detected in European cultivars could be linked to reduced photosynthetic activity and a reduction of photoassimilate transport to major sink organs, including anthers and pollen [22,46–48].

The ability of plants to respond to environmental stresses, especially to heat stress, is strongly associated with the integration of heat shock factors (*HSFs*), which are gene regulators of more elaborated responses [49]. Class A *HSFs* are the most abundant class of regulators and have been shown to be the main heat stress regulators in Arabidopsis [49] and tomato [50,51]. They also play a fundamental role in the regulation of abiotic stress responses in wheat [17]. Subclasses A1 and A2 genes that were predominantly expressed in all cultivars tested in our experiments, both under HS and control conditions, were also expressed in endosperm samples of spring wheat cultivar Bobwhite [17]. This indicates a preferential expression of these genes during reproductive development. In heat-stressed tomato anthers, A2 *HSF* members were highly induced under moderate and severe heat stress conditions. Their high expression levels were maintained even after several days of the stress treatment [51]. Expression levels of European summer wheat cultivars were generally lower and responses more variable compared to the Australian cultivars. For instance, in Epos, one of the most susceptible cultivars to HS, almost all *HSF* class A genes were strongly downregulated by heat stress, indicating a low sustained response to increased temperatures. Cultivars like Chamsin strongly induce *HSF* genes ultimately leading to a complete switch off of net photosynthesis.

5. Conclusions

In conclusion, *HSF* genes appear to be already expressed at substantial levels in HS tolerant wheat cultivars such as the Australian ones used in this study. In contrast, European cultivars showed lower expression levels under non-stress conditions and in most cultivars even a further reduced expression after exposure to moderate HS. By increasing the basal expression level of *HSF* genes, either by genetic engineering or by selecting corresponding wheat lines during breeding programs, we assume that better wheat germplasm can be generated to ameliorate adaptation to increased temperatures. This will also be relevant for short and transient heat waves occurring during reproductive development, as especially male flower organs as well as pollen development are very sensitive to elevated temperatures. A key approach will be a systematic analysis of the regulation of *HSF* genes, but how does HS lead to the complete silencing of *HSF* genes in some cultivars, while others show a strong upregulation or even de novo induction of gene expression? The European cultivars Epos and Chamsin are very suitable to address these interesting questions in future studies.

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Performance and Stability of Commercial Wheat Cultivars under Terminal Heat Stress

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Abstract: Egypt, the fifteenth most populated country and the largest wheat importer worldwide, is vulnerable to global warming. Ten of the commercial and widely grown wheat cultivars were planted in two locations, i.e., Elbostan and Elkhazan for three successive seasons 2014/2015, 2015/2016, and 2016/2017 under two sowing dates (recommended and late). Elbostan and Elkhazan are the two locations used in this study because they represent newly reclaimed sandy soil and the Nile delta soil (clay), respectively. A split-plot, with main plots arranged as a randomized complete block design and three replicates, was used. The overall objective of this study was to identify the ideal cultivar for recommended conditions and heat stressed conditions. The results revealed that heat stress had a significant adverse impact on all traits while it raised the prevalence and severity of leaf and stem rust which contributed to overall yield losses of about 40%. Stability measurements, the additive main effects and multiplicative interaction model (AMMI) and genotype main effect plus genotype × environment interaction (GGE), were useful to determine the ideal genotypes for recommended and late sowing conditions (heat stressed). However, inconsistency was observed among some of these measurements. Cultivar "Sids12" was stable and outperformed other tested cultivars under combined sowing dates across environments. However, cultivar "Gemmeiza9" was more stable and outperformed other cultivars across environments under the recommended sowing date. Moreover, cultivar "Gemmeiza12" was the ideal cultivar for the late sown condition. Based on our findings, importing and evaluating heat stress tolerant wheat genotypes under late sown conditions or heat stressed conditions in Egypt is required to boost heat stress tolerance in the adapted wheat cultivars.

Keywords: wheat; heat stress; stability

1. Introduction

Wheat (*Triticum aestivum* L.) accounts for 30% of the cereal grains production while providing 55% of the carbohydrates and 20% of the food calories consumed globally [1]. Thus, wheat is considered a strategic cereal crop for several countries around the globe including Egypt, in which wheat production became one of the crucial elements of food security [2]. Furthermore, wheat is cultivated and grown in a wide range of environmental and climatic conditions [3]. Therefore, the impact of climate change is expected to affect wheat production in several regions around the globe. The Mediterranean basin is one of the regions (hot spots) that is expected to have an annual mean temperature increase of 3 to 4 °C, which might lead to total grain yield reduction of about 18% to 24% [4]. Heat stress was defined as the rise in temperature for a period and beyond the point that causes irreversible damage to the plant growth and development [5].

The impact of heat stress on several aspects of wheat phenology and physiology during the reproductive stage was studied by several researchers, in which they reported that heat stress trigger senescence-related metabolic changes in wheat [6]. Heat stress decreases photosynthesis as a result of

photosystem II (PSII) inhibition [7–10]. Moreover, during the reproductive stage, heat stress decreased the grain-filling duration significantly [11], increased floral abortion, and decreased the number of seeds [12–14]. Nevertheless, heat stress significantly increased protein concentration, but with lower end-use quality because the functionality of protein was reduced by the high temperature [15]. The increased protein concentration under terminal heat stress could be due to upregulation of heat shock proteins (HSPs) which is a plant mechanism to alleviate the effect of heat stress [16]. In wheat, 6560 probe sets for HSPs displayed expression upregulation under heat stress treatment of 34 °C and 40 °C [16].

Conceptually, the phenotype (P) of any plant is a result of the genotype (G), the environment (E), and the genotype–environment interaction (G × E). Based on this concept, one can cope with the negative impact of the heat stress either by altering the environment or using heat stress tolerant genotypes. Exploring highly adapted local genotypes to identify heat stress tolerant wheat genotypes is the first step to start a wheat breeding program for heat stress [5,17,18]. Whereas, if the breeder was able to find high yielding and stress tolerant genotype, then years of evaluation and crosses to develop such a cultivar can be saved. Plant breeders always look for genotypes that perform better across environments with minimal G × E interaction, but that seldom occurs, especially under the dynamic weather conditions and the fluctuations in the environmental conditions from year to year and location to location. Thus, measuring the stability of a given cultivar is an essential criterion before releasing new cultivars. Selecting superior genotypes using stability measurements instead of average performance is highly recommended because genotypes selected using stability measures are more reliable across environments with a minimized G × E interaction, or the provide a predictable response across environments. Studies have shown that stability analyses according to various measures can result in better identification of stable genotypes, even when there were no interactions among the measures [19]. Stability and G × E measurements can be classified into parametric and non-parametric measurements [20]. The most frequently utilized two parametric stability methods are partitioning of G × E interaction [21] and the regression model [22]. The additive main effects and multiplicative interaction model (AMMI) [23] and the genotype main effect plus G × E interaction (GGE) [24] are the most frequently utilized non-parametric methods. Both AMMI and GGE biplot analyses are based on the principal component analysis (PCA). However, GGE biplot is based on environment-centered principal component (PCA), whereas AMMI analysis is a double centered PCA method [25].

In the current study, ten wheat cultivars that represent around 60% of the commercially grown wheat cultivars in Egypt (based on 2017/2018 grown wheat cultivars) were evaluated under recommended and late sowing conditions (heat stressed). These cultivars were used to study the effect of heat stress (late sown) on several physiological and morphological traits. Egypt, the fifteenth most populated country worldwide, is one of the Mediterranean basin countries that is vulnerable to the effects of global warming [26]. In addition to the impact of the global warming, the Egyptian population is projected to be 125,870,736 inhabitants in 2030, which will require the production of more wheat grain. The recommended sowing date for wheat in Egypt is around mid-November. However, due to limited availability of land and water, several growers in Egypt tend to grow wheat after sugar beet (*Beta vulgaris* L.), carrot *(Daucus carota* L.), or pea (*Pisum sativum* L.), which results in sowing wheat around Mid-January. These later sowing dates expose wheat plants to terminal heat stress during the reproductive stage.

Therefore, evaluating wheat cultivars for heat tolerance has environmental and socioeconomic importance in this region of the world. Although screening for heat stress tolerance in wheat has been done in the past, limited research was conducted to study the effect of heat stress on the grain yield stability or wheat resistance to leaf and stem rust using commercial newly developed wheat cultivars under clay and sandy soils across three successive growing seasons. Thus, the primary objectives of this study were to; 1—evaluate ten of the commercially and widely distributed wheat cultivars under heat stressed and recommended (control) conditions across several environments.

2—Study the relationships among relevant phenological and physiological traits under normal and heat stress conditions.

2. Materials and Methods

2.1. Plant Materials and Field Conditions

Ten of the commercially widely distributed Egyptian wheat cultivars were used in this study (Table 1). The studied materials were planted in three successive growing seasons, i.e., 2014/2015, 2015/2016, and 2016/2017, in two locations; Elkhazan (31°05′35.2″ N, 30°30′10.4″ E) and Elbostan (30°45′19.4″ N, 30°29′04.8″ E), Behera governorate, Egypt. In each growing season for both locations, two sowing dates were used, i.e., recommended and late sown dates. The recommended sowing dates were 19, 12, 14 of November for 2014/2015, 2015/2016, and 2016/2017, respectively. While the late-sown dates were 7, 9, 13 of January, for 2014/2015, 2015/2016, and 2016/2017, respectively. A Split-plot, with main plots arranged as a randomized complete block design and three blocks, was used. The two sowing dates were randomly assigned to the main plots within each of the three blocks. The ten wheat cultivars were assigned randomly to the subplots within each main plot (sowing date). The experimental units (plots) were two meters long and four rows wide by 25 cm between rows. The surrounding border of the experimental areas of one meter wide, planted with the wheat cultivar "Morocco", a "spreader" cultivar so named because it is susceptible to currently prevalent races of leaf and stem rust. Standard agronomic practices including recommended fertilization and irrigation schedules were followed.

Table 1. Name, pedigree, and year of release of the ten wheat cultivars used in this study.

Cultivar	Pedigree	Year of Release
Giza168	MRL/BUC//SERI	1995
Gemmeiza7	7CMH74A-630/SX//SERI82/AGEN	1999
Gemmeiza9	ALD"s"/HUAC//CMH74A-630/SX	1999
Gemmeiza10	Maya74"S"/ON/1160-147/3/Bb/G11/4/chat"S"/5/crow"S"CGM5820-3GM-1GM-2GM-0GM	2004
Gemmeiza11	BOW "S"/KVZ "S"//7C/SERI82/3/GIZA168/SKHA61	2011
Gemmeiza12	OTUS/3/SARA/THB//VEECMSS97Y00227S-5Y-	2011
Sakha94	OPATA/RAYON//KAUZ	2004
Sids12	BUC//7C/ALD/5/MAYA74/ON//1160-147/3/BB/GLL/4/CHAT"S"/6/MAYA/VUL-4SD-1SD-1SD-0SD.	2007
Sids13	KAUZ "S"//TSI/SNB"S". ICW94-0375-4AP-2AP-030AP-0APS-3AP-0APS-050AP-0AP-0SD.	2010
Misr2	SKAUZ/BAV92. CMSS96M0361S-1M-010SY-010M-010SY-8M-0Y-0S.	2011

2.2. Phenotypic Measurements

The number of days to flowering date was recorded visually as the number of days to anther exertion from 50% of the main spikes (days). Leaf area (LA) was estimated on three samples according to the following equation [25]:

$$\text{Leaf area (LA)} = L \times W \times 0.75 \tag{1}$$

where L and W are the length and width, respectively, of the flag leaf.

Plant height was measured on a random sample of five plants in each plot as the distance from the soil surface to the tip of the spike awns excluded at harvest time (cm). Grain yield was measured by harvesting the four rows of each plot (tons/ha). Total leaf chlorophyll content (SPAD index) estimated using spad-502 chlorophyll meter (spad-502 plus, Konica Minolta, Kearney, NE, USA), during the flowering stage. Canopy temperatures (Tc) were measured using a handheld infrared thermometer (KM 843, Comark Ltd., Hertfordshire, UK) with a field view of 100 mm to 1000 mm. Canopy temperatures (Tc) data were taken from the same side of each plot at 1m distance from the edge and approximately 50 cm above the canopy at an angle of 30° to the horizontal. Readings were made between 1300 and 1500 h on sunny days. Grain filling duration (GFD) was measured from flowering to physiological maturity (when the peduncle changed color). Leaf and stem rust screening under recommended and late sown conditions were conducted using the modified Cobb's scale described by Peterson et al. [26]. The infection type was expressed in the following types, i.e., Immune = 0,

R = resistant, small uredinia surrounded by necrosis; MR = Moderately resistant, medium to large uredinia surrounded by necrosis; MS = moderately susceptible, medium to large uredinia surrounded by chlorosis; S = susceptible, large uredinia without necrosis or chlorosis [27]. The statistical analysis was conducted on the infection type after replacing the infection types with 0, 0.2, 0.4, 0.80, and 1 scores for immune, resistant, moderately resistant, moderately susceptible, and susceptible, respectively.

2.3. Statistical Analysis

Analysis of variance was carried out using SAS 9.2 (SAS v9.2; SAS Institute Inc., Cary, NC, USA), by fitting the following linear model [27]:

$$Y_{ijlm} = \mu + E_i + EB_{(i)j} + T_l + ET_{il} + TBE_{(i)jl} + G_m + EG_{im} + ETG_{ilm} + \varepsilon_{ijlm} \tag{2}$$

where Y_{ijlm} is the response measured on the $_{ijlm}$ plot, μ is the overall mean, E_i is the effect of ith environment (three seasons and two locations which compose six environments), $EB_{(i)j}$ is j^{th} block nested within i^{th} environment, T_l is the effect of the lth sowing date, ET_{il} is the interaction between i^{th} environment and l^{th} sowing date, $TBE_{(i)jl}$ is interaction between l^{th} sowing date and j^{th} replicates within i^{th} environment as an error term for environment, sowing date, and environment × sowing date. G_m is the effect of mth cultivar, ETG_{ilm} is the interaction effect among i^{th} environment, l^{th} sowing date, and G^{th} cultivar, and ε_{ijlm} is the experimental error.

Means were compared using the least significance difference (*LSD)* test at *p*-value < 0.05, according to Gomez and Gomez [28]. Homogeneity of the variance in different environments was tested following Bartlett's Test [29]. Combined analyses of variance were performed among the different environments with homogeneous variance, as outlined by Cochran and Cox [30]. Correlation coefficients were conducted using Pearson correlation coefficient.

2.4. Stability Analysis and Genotype × Interaction (G × E)

The following stability measurements were performed on grain yield under the six environments (three seasons and two locations which compose six environments), i.e., coefficient of variability (CV$_i$) [31], regression coefficient (b$_i$) [22], Wricke's ecovalance (W$_i$) [32], superiority measure (P$_i$) [21], Perkins and Jinks (D$_i$) [33], and average absolute rank difference of genotype on the environment (S$_i$(1)) [24] . Moreover, the additive main effects and multiplicative interaction model (AMMI) [23] was applied on the grain yield variable for each sowing date separately, and after combing them. Then the genotype main effect plus G × E interaction (GGE biplot) [24] was used to visualize the G × E interaction. The stability and G × E analysis was conducted using R (software) package GEA-R (Version 4.0, 2017, CIMMYT, El Batán, Mexico) [34].

3. Results

3.1. Analysis of Variance

The analysis of variance for total chlorophyll content, canopy temperature, leaf area, grain filling duration, plant height, grain yield, leaf rust scores, stem rust scores, and the number of days to flowering are presented in Table 2. The results indicated highly significant effect *(p*-value < 0.01) for the six environments (three growing seasons and two locations which compose six environments) on all traits except for leaf rust scores and stem rust scores, in which the environmental effect was not found to be statistically significant. Furthermore, sowing dates (Sd) had a highly significant effect on all traits. More importantly, the analysis of variance revealed highly significant variance among the studied cultivars. Our results suggested that the magnitude of differences among cultivars was sufficient to provide a scope to characterize the effect of terminal heat stress (late sown condition). All traits except leaf and stem rust scores, had significant two-way and three-way interactions (environments × cultivars, environments × sowing dates, sowing dates × cultivars and environments × sowing dates × cultivars) effects. As for leaf and stem rust scores, the interaction effect of environments ×

cultivars and sowing dates × cultivars found to be highly significant, while the interaction effect of environments × sowing dates × cultivars was not statistically significant.

Table 2. Analysis of variance for total chlorophyll content (CHLOR), canopy temperature (CANO), leaf area (LA), grain filling duration (GFD), plant height (PH), grain yield (YIELD), leaf rust (LR), stem rust (SR), and number of days to flowering (NDF) under two sowing date (SD) and different environments (ENV).

SOURCE	DF	Mean Squares								
		CHLOR	CANO	LA	GFD	PH	YIELD	LR	SR	NDF
ENV	5	72.6 **	1316.1 **	212.7 **	492.5 **	785.5 **	34.02 **	0.025	0.016	2263.5 **
Replication(ENV)	12	0.60	2.27	1.44	3.36	37.73	0.10	0.017	0.017	67.00 **
SD	1	1055.4 **	61871.3 **	11969.29 **	6002.5 **	63374.9 **	836.1 **	14.88 **	16.72 **	85069.9 **
ENV * SD	5	119.0 **	463.2 **	115.33 **	16.3 *	393.3 **	13.0 **	0.02	0.036	360.9 **
Main plot Error	12	0.59	1.36	2.10	6.83	37.55	0.12	0.042	0.011	68.9
CULTIVAR	9	15.1 **	182.5 **	142.8 **	116.7 **	1141.2 **	6.6 **	1.46 **	1.77 **	3500.5 **
SD * CULTIVAR	9	6.4 **	84.3 **	20.2 **	43.8 **	761.1 **	2.9 **	1.47 **	0.89 **	2003.76 **
ENV * CULTIVAR	45	9.08 **	109.7 **	53.4 **	38.04 **	371.1 **	2.2 **	0.02	0.035 *	600.3 **
ENV * SD * CULTIVAR	45	6.8 **	193.18 **	28.59 **	44.9 **	423.2 **	2.8 **	0.019	0.031	682.9 **
ERROR	216	0.14	2.71	0.69	1.4	14.95	0.1	0.019	0.02	22.2

*, **: Significant at the 0.05 and 0.01 probability levels, respectively. SD: Sowing date; ENV: Environment.

The late sown condition had a significant adverse effect on the total chlorophyll content, leaf area, grain filling duration, plant height, and grain yield. The average of the total chlorophyll content measured for all cultivars under recommended and late sown condition was 31.9 and 28.5, respectively. Moreover, the late sown condition decreased leaf area from 35.15 to 23.5 cm^2. In the same manner, the late sown condition shortened the grain filling duration from 32.5 days to 25.5 days. The late sown condition had an adverse effect on plant height in which mean plant height across cultivars was dropped from 83.00 cm to 56.77 cm. Moreover, the late sown condition increased canopy temperature from 32.76 °C to 56.16 °C. Furthermore, the late sown condition decreased overall resistance to leaf rust (leaf rust scores increased from 0.34 to 0.73) and stem rust (stem rust scores increased from 0.36 to 0.79).

Results in Table 3 illustrate the effect of sowing date on the response of the studied cultivars for the total chlorophyll content, canopy temperature, leaf area, grain filling duration, plant height, grain yield, and leaf and stem rust scores across environments. Cultivar "Giza168" exhibited the tallest plants (95.8 and 64.9 cm), while the shortest plants were for cultivar "Gemmeiza10" (65.7 and 52.2 cm) obtained from recommended and late sown conditions, respectively. Furthermore, cultivar "Gemmeiza12" produced the highest grain yield (8.8 ton/hectare) under the recommended sowing date. While cultivar "Gemmeiza9" produced the highest grain yield (4.87 ton/hectare) under the late sown condition. Results in Table 3 indicated that cultivars Gemmeiza10 and Gemmeiza12 flowered earlier than other cultivars under the recommended and late sown conditions. Recommended sowing date extended the number of days to flowering for all cultivars across environments. Results of the adult plant resistance to leaf and stem rust indicated a negative impact of late sowing date on wheat resistance to both stem and leaf rust. Among the ten wheat cultivars tested based on the infection type (IT) and under the recommended sowing date, five cultivars were resistant to leaf rust, i.e., Gemmeiza9, Gemmeiza10, Gemmeiza12, Giza168, and Sids13. Furthermore, Sids12, Gemmeiza7, and Gemmeiza11 were moderately resistant to leaf rust. Whereas, Sakha94 was susceptible to leaf rust under the recommended sowing date. Under late sowing date, Gemmeiza9, Gemmeiza11, and Sids12 showed moderate resistance (MR) to leaf rust across all environments, while the rest of the cultivars were moderately susceptible or susceptible to leaf rust. Moreover, the stem rust results under recommended sowing date indicated that five wheat cultivars were stem rust resistant, i.e., Sids12, Sakha94, Gemmeiza10, Gemmeiza11, and Gemmeiza12, across all environments. Furthermore, Gemmeiza7, Gemmeiza9, and Misr2 were moderately resistant to stem rust, but Giza168 and Sids13 were susceptible. Nevertheless, stem rust results obtained from the late sowing date indicated that Sids12, Gemmeiza9, and Gemmeiza11 were moderately resistant, but Gemmeiza7, Gemmeiza10, Gemmeiza 12, Misr2, Sakha94, Giza168, and Sids13 were susceptible.

Table 3. Response of the ten wheat cultivars to recommended (R) and late sowing (L) dates for total chlorophyll content (CHLOR), canopy temperature (CANO), leaf area (LA), grain filling duration (GFD), plant height(PH), grain yield (GY), leaf rust scores (LR), stem rust scores(SR), and number of days to flowering (NDF).

Sowing date	PH		GY		NDF		LR		SR		CHLOR		CANO		LA		GFD	
	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L	R	L
Sids12	83.5	62.5	8.08	4.27	139.5	112.1	0.40 (40.7)	0.36 (49.9)	0.09 (37.52)	0.46 (53.2)	32.6	29.0	33.9	46.9	36.1	24.6	31.32	28.9
Gemmeiza10	65.7	52.2	7.65	3.12	116.2	103.4	0.06 (40.5)	0.85 (57.1)	0.14 (32.6)	0.90 (46.8)	31.3	28.0	32.2	44.4	33.8	22.4	32.2	27.9
Gemmeiza7	88.3	58.3	4.67	4.15	132.5	124.4	0.41 (30.4)	0.94 (54.1)	0.40 (39.2)	0.95 (43.2)	32.6	28.6	36.6	42.6	38.36	25.2	34.8	25.25
Gemmeiza9	89.3	58.3	7.6	4.87	133.7	119.8	0.11 (38.8)	0.41 (68.1)	0.40 (32.8)	0.46 (55.5)	32.4	28.5	37.1	44.1	38.5	25.1	34.5	29.3
Gemmeiza11	88.4	52.3	5.0	2.9	126	109.6	0.32 (20.6)	0.36 (43.9)	0.16 (26.3)	0.41 (54.6)	30.7	28.4	34.3	41.9	34.4	24.6	30.2	24.2
Gemmeiza12	76.3	60.1	8.8	3.12	117.5	95.2	0.05 (36.6)	0.84 (47.7)	0.16 (26.3)	0.93 (54.6)	30.8	28.3	33.1	47.6	33.4	23.1	33.3	22.3
Misr2	90.6	61.5	5.0	3.4	128.4	110.7	0.78 (38.4)	0.83 (56.7)	0.35 (38.0)	0.91 (55.1)	31.3	28.7	32.4	60.4	36.25	24.2	34.9	25.3
Sakha94	75.5	63.9	5.0	3.3	127.0	121.4	0.94 (46.5)	0.82 (44.3)	0.08 (39.4)	0.95 (56.8)	30.8	27.8	31.1	47.5	29.9	20.1	28.3	21.8
Giza168	95.8	64.9	5.4	4.0	121.8	114.0	0.06 (39.4)	0.93 (59.8)	0.91 (32.9)	0.97 (45.5)	33.5	28.5	29.6	42.5	34.5	22.9	32.5	25.9
Sids13	79.9	58.3	5.0	3.7	126.7	110.3	0.11 (29.7)	0.92 (52.2)	0.88 (45.6)	0.85 (52.5)	32.7	29.5	33.5	48.7	36.5	23.2	32.7	24.2
Lsd	6.0		0.5		1.8		0.071		0.065		0.6		2.6		1.3		1.8	
Sowing date mean	83.3	59.2	6.2	3.7	126.9	112.1	0.32	0.73	0.36	0.78	31.9	28.5	33.4	46.7	35.2	23.5	32.5	25.5

Lsd: least significant difference for the interaction between sowing dates and genotypes at α = 0.05. Numbers in brackets refer to the severity of leaf and stem rust diseases at the adult plant stage.

Even though wheat cultivars showed highly significant variance across environments for total chlorophyll content, the range of difference among values was rather narrow (Table 3). Cultivar Giza168 exhibited the highest values for total chlorophyll content (33.5 SPAD units) while the lowest values were for cultivar "Gemmeiza11" (30.7 SPAD) obtained from the recommended sowing date. Furthermore, under the late sown condition, cultivar "Sids13" has the highest total chlorophyll content value (29.5 SPAD). Besides, canopy temperature measurement indicated higher values on all cultivars under the recommended sowing date compared to the late sown condition. As shown in Table 3, cultivar "Gemmeiza9" had the highest canopy temperature (37.1) under the recommended sowing date. Cultivar "Misr2" had the highest canopy temperature (60.4) under the late sown condition. Significant reduction in leaf area due to the late sown condition was also detected. Leaf areas under the recommended sowing date ranged from 38.6 to 29.9 cm^2 for cultivars Gemmeiza7 and Sakha94, respectively. However, leaf area ranges from 25.2 to 20.1 cm^2 for the same cultivars under late sown condition. Under the recommended sowing date, grain filling duration ranged from 34.8 to 28.3 days for cultivars Gemmeiza7 and Sakha94, respectively. Furthermore, under the recommended sown date, cultivar "Misr2" had the most extended grain filling duration (34.9 days). Nevertheless, under the late sown condition cultivar, Gemmeiza9 had the most extended grain filling duration (29.3 days).

3.2. Interrelationships among the Studied Traits under Recommended Sown Condition

Pearson correlation coefficients among the studied traits under the recommended sowing date (normal) are presented in Table 4 (above diagonal). Results in Table 4 indicated significant positive correlation (*p*-value < 0.05) among total chlorophyll content, leaf area, and plant height. Significant negative correlation was detected for the relationship among total chlorophyll content, the number of days to flowering and leaf rust. However, non-significant correlation (*p*-value > 0.05) of total chlorophyll content with canopy temperature, grain filling duration, grain yield, and stem rust was detected (Table 4, above diagonal). There was a significant positive correlation between canopy temperature and leaf area. Furthermore, canopy temperature was negatively correlated with the number of days to flowering. A non-significant correlation was detected for the canopy temperature with grain filling duration, plant height, grain yield, and leaf and stem rust scores. Correlations of leaf area with grain filling duration, plant height, and grain yield were significant and positive. Whereas, the correlation of leaf area with stem rust and number of days to flowering were significant but negative. Grain filling duration was positive and significantly correlated with grain yield, but it was negative and significantly correlated with stem rust and the number of days to flowering. No significance was detected for the correlation among grain filling duration, plant height, and leaf rust. Plant height was significantly and positively correlated with grain yield but significantly and negatively correlated with both stem rust and number of days to flowering. Additionally, non-significant correlation was detected for plant height with stem and leaf rust scores. Grain yield was significantly and negatively correlated with the number of days to flowering and stem rust, but it was not significantly correlated with leaf rust scores. The correlation between leaf rust and number of days to flowering was positive and significant. The correlation between stem and leaf rust was significant. Finally, the correlation between stem rust and number of days to flowering was not significant.

3.3. Interrelationships among the Studied Traits under the Late Sown Condition

The Pearson correlations coefficients among the studied traits under the late sown condition are presented in Table 4 (below diagonal). The correlation of total chlorophyll content with leaf area, grain filling duration, plant height, and grain yield were significant and positive, but it was not significantly correlated with canopy temperature, leaf rust, stem rust, and the number of days to flowering. Canopy temperature was significantly and positively correlated with leaf and stem rust scores but negatively correlated with grain filling duration, grain yield, and the number of days to flowering. Non-significant correlation for canopy temperature with plant height and leaf area was detected. Leaf area was significantly and negatively correlated with leaf and stem rust, but it was significantly and positively

correlated with grain filling duration, plant height, and grain yield. Furthermore, Leaf area was not significantly correlated with the number of days to flowering. Grain filling duration was significant and positively correlated with plant height and grain yield, but significant and negatively correlated with leaf and stem rust scores. However, non-significant correlation for grain filling duration with the number of days to flowering was detected. The correlation of plant height with grain yield was significant and positive. Plant height was significantly and negatively correlated with leaf rust scores, stem rust scores, and the number of days to flowering. Grain yield was significantly and negatively correlated with stem and leaf rust, but non-significant correlation was detected between grain yield and number of days to flowering. The correlation between leaf rust and stem rust scores was significant and positive. Furthermore, leaf rust was significant and negatively correlated with the number of days to flowering. Nevertheless, non-significant correlation between stem rust and the number of days to flowering was detected.

Table 4. Pearson correlation coefficients among total chlorophyll content (CHLOR), canopy temperature (CANO), leaf area (LA), grain filling duration (GFD), plant height (PH), grain yield (YIELD), leaf rust (LR), stem rust (SR) scores, and the number of days to flowering (NDF) under recommended sowing date (above diagonal), and correlation coefficients among same traits under late sown condition (below diagonal).

	CHLOR	CANO	LA	GFD	PH	YIELD	LR	SR	NDF
CHLOR		−0.17	0.55 **	0.27	0.43 *	0.07	−0.33 *	0.07	−0.47 **
CANO	0.00		0.44 **	−0.11	0.10	−0.20	−0.05	−0.25	−0.31 *
LA	0.42 *	−0.21		0.61 **	0.50 **	0.43 *	0.06	−0.63 **	−0.79 **
GFD	0.40 *	−0.57 **	0.56 **		0.31	0.60 **	0.17	−0.69 **	−0.56 **
PH	0.55 **	−0.08	0.60 **	0.39 *		0.45 *	0.01	−0.90 **	
YIELD	0.39 *	−0.55 **	0.55 **	0.77 **	0.43 *		0.01	−0.67 **	−0.59 **
LR	−0.33	0.37 *	−0.71 **	−0.81 **	−0.53 **	−0.82 **		−0.38	0.42 *
SR	−0.28	0.41 *	−0.63 **	−0.84 **	−0.42 *	−0.85 **	0.97 **		−0.06
NDF	−0.30	−0.80 **	−0.30	0.25	−0.46 **	0.23	−0.53 **	−0.1	

*, **: Significant at the 0.05, 0.01 probability levels, respectively.

3.4. Genotype × Environment Interaction (G × E) for Grain Yield

Grain yield is a quantitative and complex trait that was found to be responsive to genotype by environment interaction (G × E). Additionally, grain yield is the most critical parameter that determines a cultivar's acceptance by growers. Thus, in this part of the study, we performed stability analysis on the grain yield. The results of the stability parameters used in this study are presented in Table 5. Under the combined sowing dates (12 environments) model, i.e., two sowing dates, two locations and three years, Gemmeiza9 was the most stable cultivar in several measurements such as coefficient of variation (C.V%), Superiority measure (P_i) and Wrike's ecovalence (W_i).Gemmeiza10 was the most stable cultivar for other measurements such as Regression coefficient (b_i), and Perkins and Jinks (D_i). However, Gemmeiza12 was the most stable genotype for the average absolute rank difference of genotype on the environment ($S_i(1)$). Moreover, under the recommended sowing date, i.e., two locations and three years (six environments), coefficient of variation (C.V%), and Superiority measure (P_i) identified Sids12 to be the most stable cultivar. Wrike's ecovalence (W_i) and average absolute rank difference of genotype on environment ($S_i(1)$) identified Gemmeiza12 to be the most stable cultivar. Moreover, regression coefficient (b_i) and Perkins and Jinks (D_i) identified Gemmeiza10 to be the most stable cultivar. Furthermore, under the late sown condition, coefficient of variation (C.V%), Wrike's ecovalence (W_i), Superiority measure (P_i), and the average absolute rank difference of genotype on the environment ($S_i(1)$) identified Sids12 to be the most stable genotype. Furthermore, Giza168 was the most stable genotype under regression coefficient (b_i) and Perkins and Jinks (D_i).

Table 5. Stability parameters estimates for grain yield and ten cultivars, under combined, early, and late sown conditions.

Sowing Date	Genotypes	Coefficient of Variation C.V%	Regression Coefficient (b_i)	Perkins and Jinks (D_i)	Wrike's Ecovalence (W_i)	Superiority Measure (P_i)	Average Absolute Rank ($S_i(1)$)
Recommended & late combined	Sids12	49.03	1.60	0.60	28.42	0.78	0.82
	Gemmeiza10	32.43	0.60	−0.40	12.53	6.35	0.32
	Gemmeiza7	31.70	0.88	−0.12	10.38	2.29	0.61
	Giza168	40.77	1.10	0.10	8.82	2.66	0.41
	Gemmeiza11	47.59	1.46	0.46	13.67	1.34	0.68
	Gemmeiza12	32.28	0.61	−0.39	12.83	5.75	0.21
	Misr2	33.89	0.75	−0.25	8.11	4.58	0.45
	Sakha94	47.22	1.11	0.11	18.29	3.56	0.52
	Gemmeiza9	30.03	1.06	0.06	3.91	0.54	0.24
	Sids13	35.91	0.83	−0.17	7.49	4.39	0.48
Recommended	Sids12	6.14	0.32	−0.68	4.15	0.16	0.53
	Misr2	31.73	1.47	0.47	2.52	7.75	0.53
	Gemmeiza7	24.62	0.62	−0.38	5.79	11.01	0.47
	Gemmeiza9	31.65	1.73	0.73	6.18	4.50	1.00
	Gemmeiza11	30.89	1.54	0.54	7.61	4.44	1.20
	Gemmeiza12	13.53	0.86	−0.14	1.09	0.91	0.33
	Gemmeiza10	19.67	0.18	−0.82	9.98	9.74	0.60
	Sakha94	23.90	0.83	−0.17	4.52	7.84	1.00
	Giza168	34.97	1.47	0.47	13.11	5.37	1.47
	Sids13	17.07	0.98	−0.02	3.31	1.08	0.60
late	Sids13	24.03	2.32	1.32	1.30	1.40	0.67
	Gemmeiza9	22.99	2.48	1.48	1.35	1.03	0.73
	Gemmeiza7	25.24	2.26	1.26	1.36	1.69	0.67
	Gemmeiza10	6.74	−0.33	−1.33	1.47	0.09	0.60
	Gemmeiza11	8.37	−0.12	−1.12	1.21	0.88	0.87
	Gemmeiza12	30.57	2.56	1.56	2.74	1.77	0.73
	Misr2	11.30	0.50	−0.51	0.63	1.77	0.80
	Sakha94	17.77	0.68	−0.32	1.65	1.32	0.83
	Giza168	24.80	−0.84	−1.84	5.09	1.75	0.97
	Sids12	5.27	0.49	−0.51	0.36	0.00	0.07

The results of stability measures used in the current study indicated inconsistency among some of the stability measures used. Thus, to complement the results of the previous stability measures, Genotype by environment (G × E) was further investigated using the additive main effect and multiplicative interaction (AMMI) analysis (Table 6) and genotype main effect plus genotype × environment interaction (GGE). Additionally, two models were fitted in the AMMI and GGE biplot; the first was by considering sowing dates as part of the environments, i.e., 12 environments, while the second was by running the AMMI analysis across years and locations within each sowing date, i.e., six environments. In the first model (12 environments), the analysis of variance for AMMI model indicated significant effect of the environments, genotypes, and genotype × environment interaction. Whereas the variance of the environment was 63.2%, while the variance due to genotypes was 14.6% and that for genotype × environment interaction was 22.2%. In the second model (6 environments within each sowing date), the variance of the AMMI model for the recommended sowing date was 29.52%, 46%, and 24.48% for the environment, genotypes, and genotypes–environment interaction, respectively. Moreover, the variance of the AMMI model for the late sown condition was 12.99%, 48.57%, and 38.44% for the environment, genotypes, and genotypes–environment interaction, respectively. In both models, the genotype–environment interaction was highly significant (*p*-value < 0.01) implying differential response of genotypes to environments. Substantial variance for the environment in the first model compare to the second model was detected, which indicates an amplification effect of sowing dates on the environmental effect.

Table 6. Summary of the analysis of variance and partitioning of the G × E interaction by , the additive main effects and multiplicative interaction model (AMMI) for grain yield.

| Sowing Date	Source	DF	MS	% of Variance Explained
Recommend and late combined	Environments (E)	11	96.80 **	63.20
	Genotypes (G)	9	27.40 **	14.60
	G × E	99	3.80 **	22.20
	PC1	19	8.90 **	45.30
	PC2	17	6.00 **	27.10
	PC3	15	3.70 **	14.80
	Residuals	240	0.10	
Recommend	Environments (E)	5	42.15 **	29.52
	Genotypes (G)	9	36.50 **	46.00
	G × E	45	3.88 **	24.48
	PC1	13	6.74 **	50.13
	PC2	11	4.82 **	30.36
	PC3	9	2.25 **	11.59
	Residuals	120	0.19	
late	Environments (E)	5	3.48 **	12.99
	Genotypes (G)	9	7.23 **	48.57
	G × E	45	1.14 **	38.44
	PC1	13	2.42 **	61.16
	PC2	11	1.20 **	25.59
	PC3	9	0.54 **	9.41
	Residuals	120	0.03	

**: Significant at 0.01 probability level.

Based on AMMI analysis, the genotype–environments interaction was divided into three main principal components that explain 87.2% of the total variance under the first model (combined sowing dates). Furthermore, the first three principal components explained 92.08% and 96.16% of the interaction between genotype and environment under recommended and late sown conditions, respectively. A graphical representation of the relationship between cultivars and sowing dates across environments regarding grain yield is shown in a GGE biplot (Figure 1A,B). The previous biplot and the AMMI analysis of variance indicated a variable response of the genotypes under the two sowing dates. Therefore, in addition to running the biplot and stability analysis on the combined sowing dates, it was refitted within each sowing date. Thus, three biplots were generated; for combined, early, and late sown conditions (Figures 1–3). For the three biplots, a polygon was formed by connecting the genotypes that were further away from the biplot origin, such that all other genotypes were contained in the polygon. Genotypes located on the vertices of the polygon performed either the best or the poorest in one or more locations since they had the longest distance from the origin of biplot. The vertex cultivars in the first GGE biplot (combined sowing dates) were Giza168, G7 (Gemmeiza7), Misr2, Sids13, Sakha94, G9 (Gemmeiza9), Sids12, and G10 (Gemmeiza10) (Figure 1B). Under the recommended sowing date (Figure 2B) the vertex genotypes were Giza168, G7 (Gemmeiza7), Sids13, Sakha94, and G10 (Gemmeiza10). Moreover, the vertex genotypes for the late sown condition were G9 (Gemmeiza9), Sids12, G12 (Gemmeiza12), Giza168, and Sakha94 (Figure 3B). The best genotype for combined sowing dates was Sids12 (Figure 4A). However, the best genotype for recommended sowing date was G12 (Gemmeiza12 (Figure 4B). Furthermore, G9 (Gemmeiza9) followed by Sids12 were the better genotypes for the late sown condition (Figure 4C).
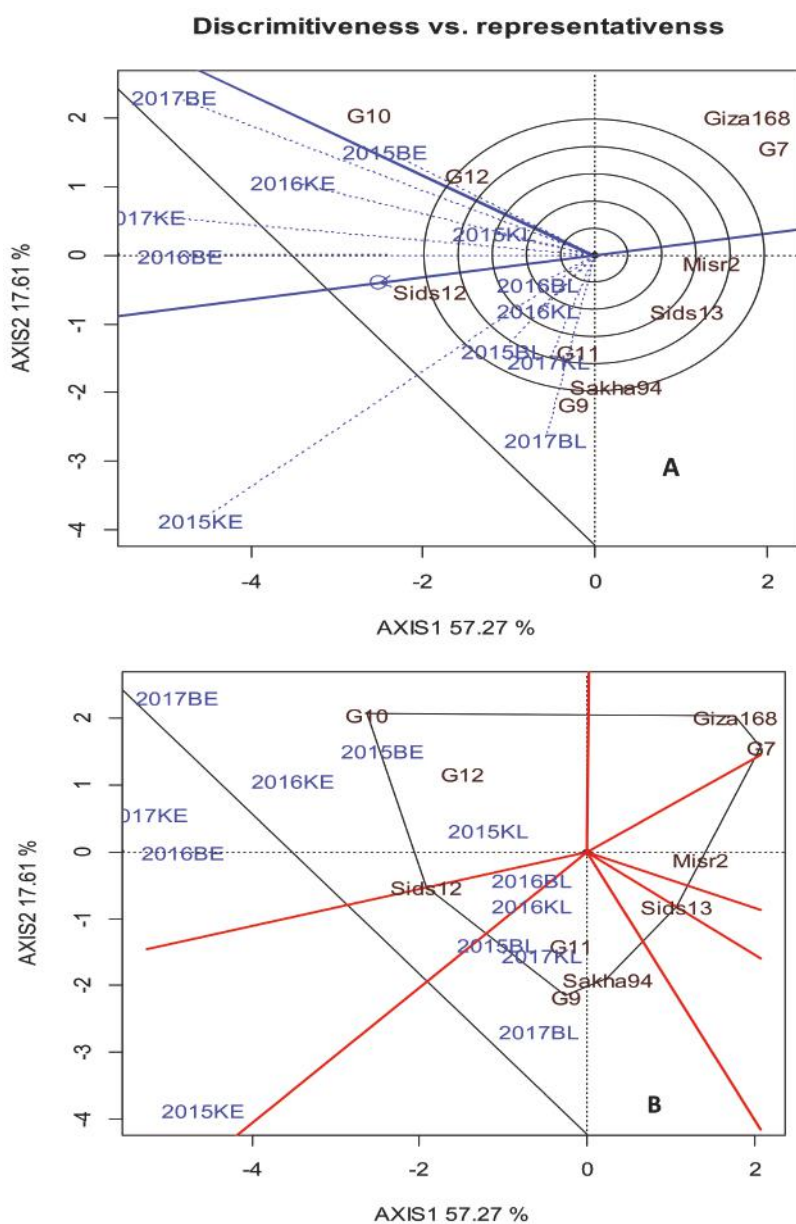
Figure 1. Vector view of GGE biplot for relationships among environments (**A**), and GGE biplot identification of winning cultivars across environments (two years i.e., 2015 and 2016, and two locations i.e., Elbostan (B), and Elkhazan (K)) under recommended (E) and late (L) sowing dates (**B**). AXIS1 and 2 refer to Principal component 1 (PC1) and Principal component 2 (PC2), respectively.

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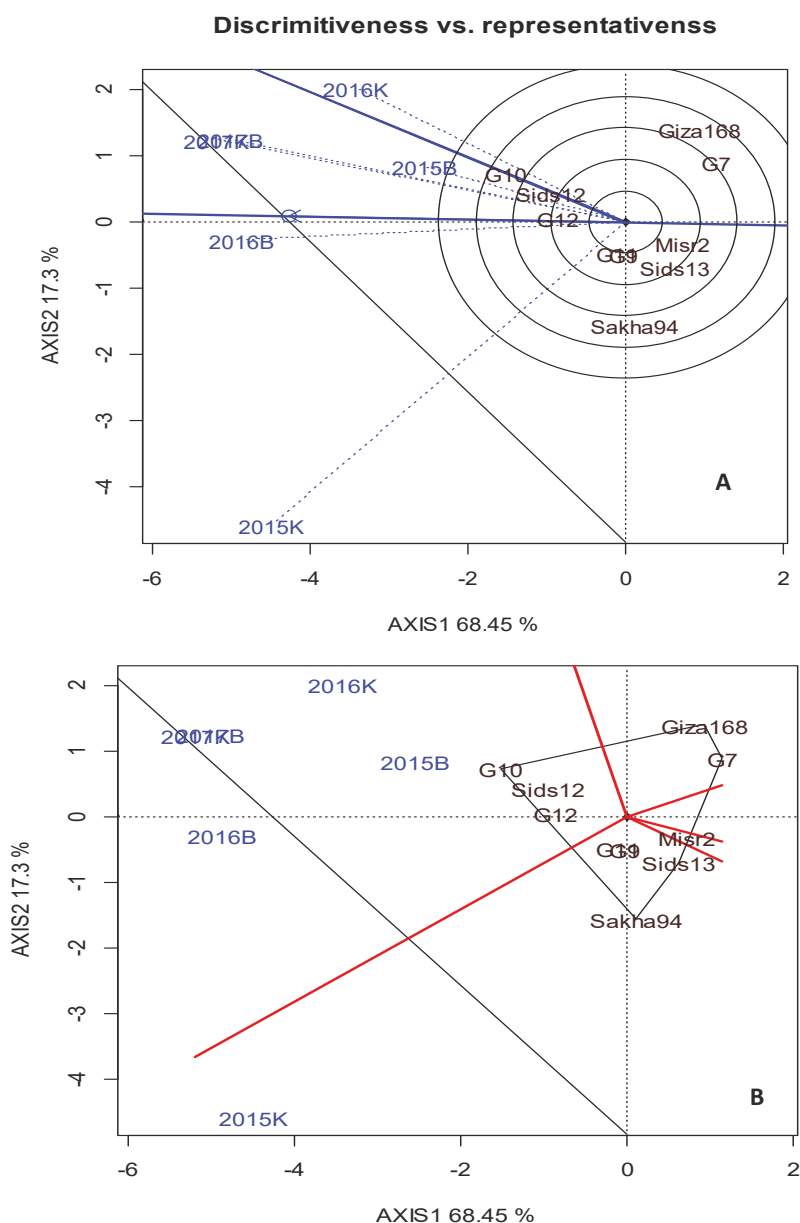
Figure 2. Vector view of GGE biplot for relationships among environments (**A**), and GGE biplot identification of winning cultivars across environments (two years, i.e., 2015 and 2016, and two locations, i.e., Elbostan (B), and Elkhazan (K)) under early sowing date (**B**). AXIS1 and 2 refer to Principal component 1 (PC1) and Principal component 2 (PC2), respectively.
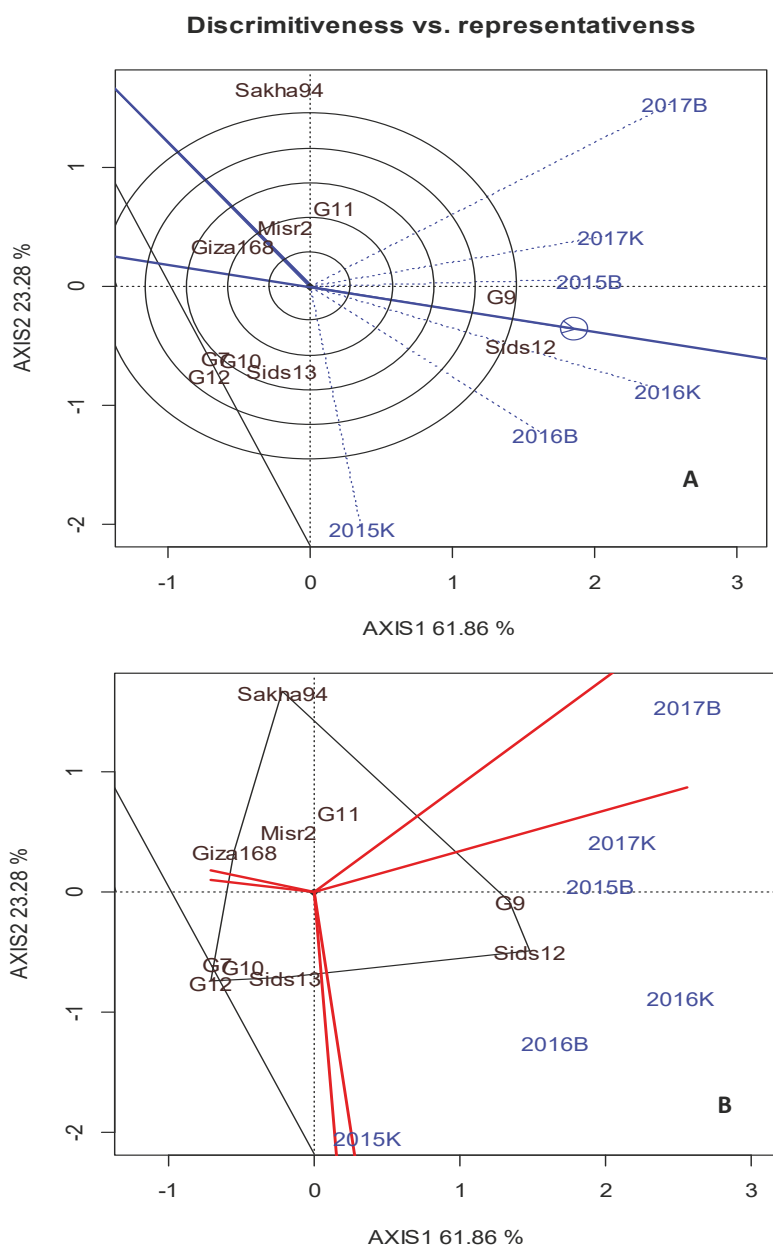
Figure 3. Vector view of GGE biplot for relationships among environments (**A**), and GGE biplot identification of winning cultivars across environments (two years, i.e., 2015 and 2016, and two locations, i.e., Elbostan (**B**), and Elkhazan (**K**)) under late sown condition (**B**). AXIS1 and 2 refer to Principal component 1 (PC1) and Principal component 2 (PC2), respectively.
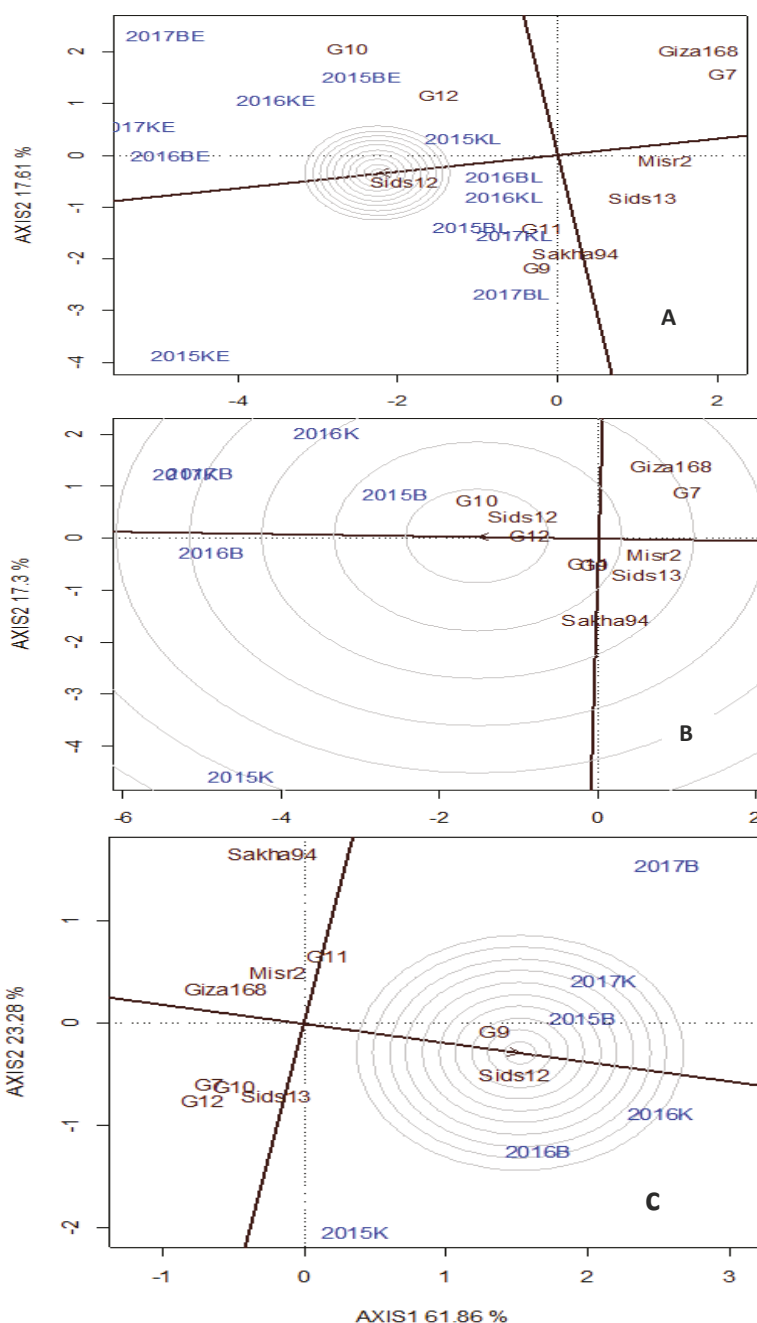
Figure 4. GGE biplot for the ideal genotype under both sowing dates (**A**), recommended sowing date (**B**) and late sown condition (**C**), across environments (two years, i.e., 2015 and 2016, and two locations, i.e., Elbostan (B), and Elkhazan (K)). AXIS1 and 2 refer to Principal component 1 (PC1) and Principal component 2 (PC2), respectively.

4. Discussion

The world bank identified Egypt as one of the potentially vulnerable countries in the Mediterranean basin that might be affected by global warming [35]. Wheat is the backbone of food security in Egypt, where it provides more than 30% of the population's calorie intake [36]. Wheat is a cool season crop that found to be sensitive to heat stress during the reproductive stage [37]. Heat stress tolerance is a complicated process controlled by several small effect genes or QTLs and is often confounded by differences in plant morphology and physiology under different environments [38–40]. Thus, to understand the complexity of plant responses to heat stress, it is vital to account for the morphological, physiological, and genetic basis of this response under the field conditions.

The current study intended to investigate the effect of heat stress on several morphological traits in addition to the grain yield using commercial wheat cultivars that are widely grown in Egypt [41]. Two locations in Egypt were used; Elbostan and Elkhazan, the first represents newly reclaimed sandy soil while the former represents the Nile delta soil (clay). In Elbostan the mean temperature during flowering under the late sown condition was higher than the recommended sowing conditions by 3.8, 2.3, and 3.2 °C for the 2015, 2016, 2017 growing seasons, respectively. Moreover, the mean temperature during flowering for Elkhazan was higher than the recommended sowing conditions by 3.8, 2.2, and 3.4 °C for the 2015, 2016, 2017 growing seasons, respectively.

The increased temperature during the late sown condition decreased total chlorophyll content, leaf area, grain filling duration, plant height, and grain yield. Moreover, the increased terminal temperature during flowering increased canopy temperature and leaf and stem rust susceptibility. Total chlorophyll content declined significantly under the late sown condition, suggesting structural damage to the chloroplast due to heat stress [7–9,42]. The decrease in the total chlorophyll content in response to induced heat stress has also been reported previously in several crops [43–47]. However, in the current study, total chlorophyll content was not significantly correlated with grain yield in either the timely-sown or the late-sown plots. The reasons for the weak correlation between total chlorophyll content and yield might be due to the growth stage in which we measured the total chlorophyll content. Several researchers indicated that plants start losing chlorophyll when the grain filling stage starts [48].

Heat stress (late sown condition) increased average canopy temperature across all cultivars. The correlation between canopy temperature and grain yield was negative and significant which agrees with previously reported results [49–51]. The prevalence and severity of stem and leaf rust were increased due to the late sown condition. A possible explanation is that the late sown condition creates favorable environmental conditions for both stem and leaf rust [52–58].

Overall, the late sown condition decreased the period of grain filling duration, while the sudden rise in the temperature during the reproductive stage decreased the amount of the assimilates [33]. High temperature increases the rate of seed filling, but the increase in the seed filling did not compensate for the loss in the grain filling duration [39]. Our results indicated variable response of the studied cultivars to heat stress across all traits under different environmental conditions which agrees with previous studies [40,59]. Moreover, the high temperature increased stem and leaf rust prevalence and severity during flowering stage under the late sown condition. Failure to understand the mechanisms of grain yield stability might impact both traditional breeding and the use of modern genetics in improving yield production [60]. Heat stress tolerance and maintaining high grain yield under heat stress is considered as one of the most critical aspects of wheat improvement [61–64]. Genotype–environment (G × E) interaction makes genotypic evaluation a complicated process because of the different response of genotypes under different locations or years. Obviously, grain yield is the most important trait that might solely determine the success of a plant breeder. At the same time, grain yield is a complex and quantitative trait that has strong G × E interactions. Thus, measuring the stability of a giving cultivar became one of the plant breeding routines before releasing the cultivar. The advantage of selecting superior genotypes using stability analysis instead of average performance is that stable genotypes are reliable across environments which reduce G × E interaction. Studies have shown that stability analyses according to various measures can result in better identification of stable genotypes, even

when there were no interactions among the measures. In the current study, seven stability parameters were used, which were inconsistent in identifying ideal genotypes for environments or sowing dates; this result agrees with previous results [65–67]. The inconsistency could be attributed to the difference in statistical and mathematical methods that the stability parameters rely on [68]. AMMI and GGE biplot were also applied to identify the most stable genotypes. In our study, the ideal genotype was defined agronomically as the genotype that performs well, as "High yielding" and stable across a wide range of environments. Furthermore, in our study, we defined the ideal genotype statistically as the genotype that was stable in at least one of the seven traditional stability measures in addition to AMMI.

In the current study, some genotypes performed well under recommended sowing date but not under the late sown condition (heat stressed conditions) and vice-versa, while some of the studied materials performed well under both sowing dates. Previous results imply that the genotypes used in this study contain different combinations of genes governing their response to sowing dates and tolerance to heat stress, and this result agrees with previous findings [59,69–73]. The ultimate goal of this study was to estimate the effect of global warming and heat stress on grain yield using a representative sample of commercially distributed and widely grown wheat cultivars in Egypt. Thus, wheat breeders and decision makers in this region might have an idea about the effect of heat stress on wheat production.

5. Conclusions

The results revealed that wheat performance was significantly influenced by environment, genotype, and their interaction. Heat stress had a negative impact on all traits. The late sown condition increased the prevalence and severity of leaf and stem rust which most likely contributed to the overall yield losses. The late sown condition decreased the overall yield production for the studied cultivars by 45%. The cultivars showed high G × E interaction, and the sowing date increased that interaction. Stability measurements were useful in determining the most stable genotypes. However, inconsistency was observed among some measurements. AMMI and GGE biplots were adequate for analyzing and visualizing the patterns of G × E. Sids12 was stable and outperformed the tested materials under both the recommended and late sown conditions. Gemmeiza12 was more stable and outperformed the tested materials under the recommended sown conditions. Gemmeiza9 followed by Sids12 were stable and performed better than the rest of the tested cultivars under the late sown conditions. However, late sown conditions reduced yield by 47.15%, 64.5%, and 59.2% for cultivars Sids12, Gemmeiza9, and Gemmeiza10, respectively. As a result of this work, we recommend importing and evaluating wheat accessions known to be tolerant to heat stress and cross them with the adapted wheat cultivars in this region to boost heat stress tolerance. Even though this study was conducted using cultivars grown mainly in Egypt; we expect that heat stress will have a similar effect on the spring wheat cultivars grown in the Mediterranean region because most of the wheat breeding programs in the Mediterranean region share lines from each other and international wheat breeding organizations.

Conflicts of Interest: The author declares no conflict of interest.

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Opinion

Generating Plants with Improved Water Use Efficiency

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Abstract: To improve sustainability of agriculture, high yielding crop varieties with improved water use efficiency (WUE) are needed. Despite the feasibility of assessing WUE using different measurement techniques, breeding for WUE and high yield is a major challenge. Factors influencing the trait under field conditions are complex, including different scenarios of water availability. Plants with C_3 photosynthesis are able to moderately increase WUE by restricting transpiration, resulting in higher intrinsic WUE (iWUE) at the leaf level. However, reduced CO_2 uptake negatively influences photosynthesis and possibly growth and yield as well. The negative correlation of growth and WUE could be partly disconnected in model plant species with implications for crops. In this paper, we discuss recent insights obtained for *Arabidopsis thaliana* (L.) and the potential to translate the findings to C_3 and C_4 crops. Our data on *Zea mays* (L.) lines subjected to progressive drought show that there is potential for improvements in WUE of the maize line B73 at the whole plant level (WUE_{plant}). However, changes in iWUE of B73 and Arabidopsis reduced the assimilation rate relatively more in maize. The trade-off observed in the C_4 crop possibly limits the effectiveness of approaches aimed at improving iWUE but not necessarily efforts to improve WUE_{plant}.

Keywords: water use efficiency; crop breeding; yield; drought; maize; Arabidopsis; C_4-C_3 comparison; stomatal conductance; abscisic acid (ABA); photosynthesis

1. Introduction

Green Revolution technologies and significant expansion in the use of land, water, and other natural resources for agricultural purposes have led to a tripling in agricultural production between 1960 and 2015 [1]. Despite this success, the high costs to the natural environment that accompany elevated productivity and changes in the food supply chain threaten the sustainability of food production [1]. Global food security is further challenged by climate change, with a predicted increase in frequency of droughts [2,3]. Globally, agriculture accounts for at least 70% of withdrawals from freshwater resources, with large effects on ecosystems [4,5]. Despite this high water deployment, major yield losses due to water deficits are experienced in crops [6]. At the same time, global population growth increases the demand for food, feed, and fuel, which intensifies the pressure to improve water use efficiency (WUE) of crops [7,8]. While better crop and water management practices provide an immediate opportunity to increase crop water productivity, breeding for superior varieties can achieve a medium- and long-term increase [9,10].

Physiologically, water use efficiency can be defined at different scales [11–13]. At the plot level, it represents the ratio of grain or biomass yield to water received or evapotranspired. At the single

plant level (WUE$_{plant}$), it is the ratio of biomass to transpiration. The increase in biomass and amount of water transpired over time can be assessed gravimetrically [14]. However, this is destructive and laborious on a long-term basis, especially regarding large crops like maize and sorghum. Therefore, analyses of intrinsic water use efficiency (*i*WUE) and carbon isotope discrimination (Δ^{13}C) are used as surrogates when evaluating WUE [11,15]. The *i*WUE is assessed at the leaf level as the ratio of net CO_2 assimilation (A$_n$) to stomatal conductance (g$_s$) and can be measured noninvasively with portable gas exchange equipment [16]. As transpiration rate (E) is influenced not only by g$_s$ but also by the leaf-to-air vapor pressure deficit (VPD) of the air [17], *i*WUE usually differs from transpiration efficiency (A$_n$/E). In addition, VPD affects the stomatal aperture and therefore g$_s$ [17,18]. Extrapolation of gas exchange data from single-leaf to whole plant is error-prone due to differences in photosynthesis and transpiration among leaves [19]. Prediction of long-term biomass accumulation and water consumption, WUE$_{plant}$, based on *i*WUE is even more uncertain given the possible differences in VPD and additional physiological processes such as dark respiration and photorespiration influencing the resulting biomass increase [19]. Despite these limitations, analysis of *i*WUE provides a convenient measure for the water efficiency of carbon capture. The throughput of *i*WUE analyses is quite low as only single, time-consuming measurements per plant can be taken, which impedes large-scale phenotyping.

Analysis of stable carbon isotope discrimination (Δ^{13}C) offers a suitable alternative in C$_3$ plants by providing a read-out for transpiration efficiency integrated over time. Discrimination of the heavier isotope is mainly caused by differences in diffusion rates of the isotopes and enzymatic discrimination during carboxylation reactions [20]. Therefore, Δ^{13}C has been used as an indirect trait to select cultivars with improved WUE [21–24]. By combining the analysis with oxygen isotope enrichment Δ^{18}O, an estimation for transpiration rate [25–27], contributions of water loss, and CO_2 assimilation on *i*WUE could be disentangled [28–30]. Stable isotope compositions of leaves or grains, however, represent integrated measures of many processes over a period of plant growth and therefore correlation with *i*WUE can be limited [31]. In C$_4$ plants, CO_2 prefixation, for instance, by phosphoenolpyruvat carboxylase and bundle sheath leakiness restrict the responsiveness of Δ^{13}C to changes in WUE [20,32] and make the relationship between Δ^{13}C, g$_s$ and WUE in C$_4$ species less predictable compared to C$_3$ plants [13].

Improving WUE of crops is considered beneficial in very dry climates and in very severe and terminal drought conditions, while growth maintenance traits are advantageous under milder drought conditions [33–35]. For crops experiencing water deficit early in their development, traits found to be positive for improving WUE are negative for yield [36]. Enhanced water uptake through investments in the root system can result in reduced plant size and water expenditure for growth maintenance can result in increased drought stress experiences if plants are growing at very low soil water availability [33,34,36,37]. Hence, water-conserving traits as imposed by higher WUE would be beneficial, provided growth and yield are not negatively affected.

2. Disconnecting Improved WUE and Growth Trade-Offs

Being a ratio, *i*WUE can be improved by reducing g$_s$ per amount of CO_2 assimilated or by enhancing the assimilation rate at a given g$_s$. Both cases result in lowered intercellular CO_2 concentration (C$_i$) and consequently in an increased stomatal CO_2 gradient (C$_a$ − C$_i$, with external CO_2 concentration C$_a$), which is directly proportional to the ratio of A$_n$ to g$_s$ according to Fick's law applied to carbon assimilation in leaves, A$_n$ = g$_s$ (C$_a$ − C$_i$) [16,38,39]. Increased *i*WUE has been observed in several C$_3$ species under water deficit conditions when plants reduce g$_s$ [11,15,40–42], although a decrease in g$_s$ caused by drought was found to be overridden by heat stress [43]. However, closing stomatal pores to reduce transpiration often results in a reduction of A$_n$ [41,44]. Lowering g$_s$ impinges on C$_i$ and unless this change in C$_i$ is counteracted by an elevated mesophyll conductance (g$_m$), the CO_2 concentration at the site of Rubisco-dependent carboxylation (C$_c$, CO_2 concentration in chloroplasts) will be reduced [39,45,46]. A reduction of C$_c$ affects the carboxylation efficiency of

Rubisco and favors photorespiration [47,48]. Sustaining net photosynthesis under these conditions might require a higher electron transfer rate (ETR) and/or reduced nonphotochemical quenching to support enhanced carboxylation by Rubisco for compensation of enhanced photorespiration [49–52]. There are reports that water deficit results in increased g_m; however, in most analyses, no change or a reduced g_m was observed under drought [44,53–58].

Gains in WUE are often associated with growth trade-offs [59,60]. As pointed out by Blum [61], crops with high CO_2 assimilation and high biomass accumulation per unit land area require high stomatal conductance. This is supported by the observation of a constant WUE on the field level over a broad range of yields [8]. Nevertheless, there might be ways to achieve elevated WUE and high photosynthesis, namely by exploring CO_2 concentrating mechanisms, increased g_m, and increased CO_2 specificity of Rubisco [10].

Interestingly, several reports of C_3 plants have shown enhanced *i*WUE without the expected negative impact on A_n or growth [11,15,62–64], as postulated by plant physiologists [4]. In these studies with transgenic tomato and Arabidopsis plants, g_s was moderately reduced by enhancing the biosynthesis or the responsiveness to the phytohormone abscisic acid (ABA) or by reducing the size and density of leaf stomata [15,63–65]. Plants overexpressing distinct ABA receptors—termed ABA-Binding Regulatory Component (RCAR)/Pyrabactin Resistance 1-(like) (PYR1/PYL)—caused increases of 40% in *i*WUE, integrated WUE based on Δ^{13}C of biomass and cellulose fractions, and WUE_{plant} [15]. Growth rates and biomass accumulation were not significantly different from wild type [15]. Hence, the ABA receptor lines revealed higher water productivity, i.e., WUE per time, both under well-watered growth conditions and under water deficit. Net carbon assimilation was comparable to the wild type, however, at lowered C_i levels and without detectable changes in g_m. This report and other studies show that improving WUE is possible without growth trade-offs. The underlying physiological mechanisms are largely unknown and might involve the root system, as grafting experiments have suggested [15], and enzymes of the C_4 metabolism, such as PEP carboxylase and its regulatory protein kinase PEPC kinase, which are both upregulated in C_3 plants at low CO_2 availability [65].

C_4 and C_3 plants differ in WUE [66–68]. At a given g_s, C_4 plants show higher net carbon assimilation rates and higher WUE [66]. The CO_2 concentrating mechanism involving PEP carboxylase results in saturation of C_4 photosynthesis at relatively low C_i [69,70]; therefore, lower g_s and a steeper CO_2 gradient ($C_a - C_i$) are realized in C_4 plants compared to C_3 plants [66,70]. C_3 plants have C_i values in the range of 300 ± 60 µmol mol^{-1}, while the C_i of the C_4 plants is around 150 ± 40 µmol mol^{-1} [71–73] at ambient CO_2 of 370–400 µmol mol^{-1} in well-watered conditions. Under optimal growth conditions, maize and sorghum with C_4 metabolism therefore have higher yields per water transpired than the C_3 crop wheat [9].

3. Comparative Analysis of Maize and Arabidopsis

The question arises as to whether it is possible to transfer the finding of improved *i*WUE without having the negative impact on growth to crops. The data on g_s-modified tomato plants suggests that it might work for C_3 plants [63], but the lower C_i level of C_4 plants could preclude such an accomplishment in maize.

To explore the relevance of these findings of uncoupling WUE improvement and yield decreases for the C_4 crop maize (*Zea mays* L.), we analyzed gas exchange data obtained from the maize inbred line B73 and compared them to findings in Arabidopsis. In addition, we analyzed the WUE_{plant} of maize lines subjected to drought. B73 is an inbred line that is commonly used in breeding programs, but is known to be drought-sensitive [74]. B73 was included in a progressive drought stress experiment adapted from Yang et al. [15] in which biomass production with a given amount of water was analyzed and WUE_{plant} was determined. In this experiment, B73 showed the lowest WUE_{plant} (Figure 1a) compared to the maize inbred Mo17 and lines derived from an introgression library described by Gresset et al. [75]. In Figure 1, data are shown of the recurrent parent (RP) of the introgression library as well as two introgression lines differing from RP by reduced (IL-05) or elevated kernel Δ^{13}C (IL-81) [75].

A significantly reduced WUE_{plant} compared with the recurrent parent for IL-05 shows the potential of genetic improvement for this trait. However, the largest difference in WUE_{plant} was observed between B73 and Mo17, with an increase of ~27% (Figure 1a). Our data is in accordance with a previous drought stress experiment conducted on seedlings of maize inbred lines, where Mo17 ranked top in yield per plant [76]. The results indicate genetic variation in the efficiencies of water use among maize lines and a potential for genetic improvement of the WUE_{plant} for B73.

(a) (b)
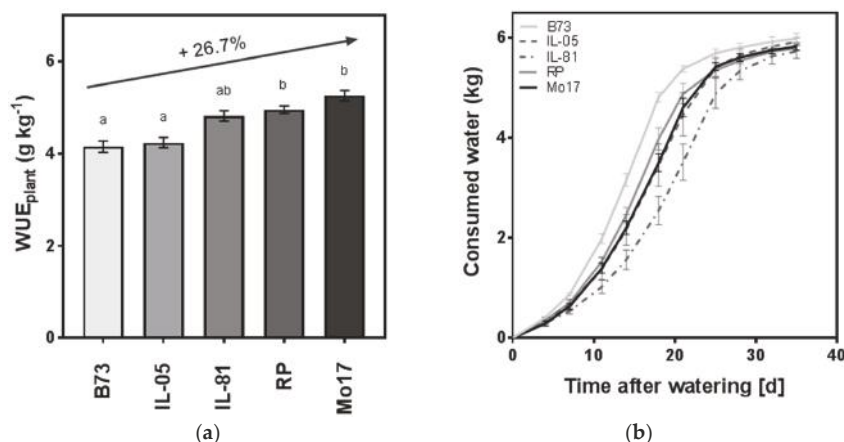
Figure 1. Water use efficiency and water consumption of maize lines under progressive drought. (**a**) Water use efficiency (WUE_{plant}) and (**b**) whole plant water consumption were assessed over the course of a progressive drought stress experiment adapted from Reference [15]. In the greenhouse, 23 maize genotypes were grown in a randomized complete block design, including the two maize inbred lines Mo17 and B73 and introgression lines described by Gresset et al. [75]. The maize inbred line RP and introgression lines derived therefrom (IL-81, IL-05) were kindly provided KWS Saat SE (Einbeck, Germany). Inbred lines B73 and Mo17 were kindly provided by the Chair of Genetics, Technical University of Munich, Freising, Germany. Prior to the experiment, maize seedlings were established in small pots in the growth chamber (16 h day at 25 °C, 650 µE m^{-2} s^{-1} photosynthetically active radiation [PAR], 8 h night at 20 °C; 75% relative humidity [RH]) for two weeks after germination under well-watered conditions. Plants of RP harvested at this age had an aboveground dry matter of 0.62 g ± 0.27 g, and plants of an introgression line derived from IL-05 weighed 0.62 g ± 0.26 g. The influence of initial biomass on the biomass at the end of the experiment (28.74 g ± 2.22 g and 25.5 g ± 2.39 g, respectively) was approximately 2%. The plants were transplanted into 10 L pots containing 8 L water-saturated soil (85% v/v soil water content; CL ED73, Einheitserdewerke Patzer, Germany, particle diameter <15 mm). A cover of polyethylene foil was used to prevent evaporation, and the progressive drought experiment was initiated by no further watering. The experiment was conducted in the greenhouse (Gewächshauslaborzentrum Dürnast in Freising, Germany) in Oct–Nov 2017 at full sunlight plus supplemental light at 25–33 °C, 19–20 °C day/night, 400 µmol m^{-2} s^{-1} PAR, 40% RH. Soil water content declined progressively during the course of the experiment until the plants used all available water. The water consumed was determined gravimetrically (means ± SE of n ≥ 4 biological replicates). WUE_{plant} was calculated as final aboveground biomass per water consumed (means ± SE of n ≥ 4 biological replicates). The increase in WUE in Mo17 compared to B73 is indicated with an arrow. Student's paired *t*-tests of the maize lines were adjusted for multiple comparisons with the Bonferroni method and lines, which did not differ significantly ($p < 0.01$), and are marked with common letters.

Maize lines showed a difference in water consumption over the five weeks of the experiment (ANOVA, $p < 0.001$, Figure 1b). However, differences in water consumption cannot explain the

differences observed in WUE$_{plant}$ and towards the end of the progressive drought, all genotypes included in the experiment had consumed an equal amount of water (5.8 kg $\pm$ 0.02 kg, mean $\pm$ SE).

The way in which the change in soil water content (SWC) during the progressive drought experiment affected photosynthesis and *i*WUE was analyzed by gas exchange measurements. The A$_n$ of leaves was fairly constant for maize B73 plants exposed to high SWC levels up to 40%, then the A$_n$ dropped steadily approaching zero at approximately 20% SWC (Figure 2a). In parallel, g$_s$ changed moderately between 70% and 40% SWC and declined to zero at 20% SWC (Figure 2b). The C$_i$ values were in the range of 80–100 μmol CO$_2$ per mol between 40–60% SWC. They were somewhat higher in plants from water-saturated soil and were lowered to a minimum of approximately 40 μmol mol^{-1} at 25% SWC (Figure 2c). Further reduction of the water content in the soil resulted in the steep rise of C$_i$ values, indicating collapsing photosynthesis at very low g$_s$ of plants experiencing severe drought stress. As the ambient CO$_2$ concentration (C$_a$) surrounding the leaf was maintained at 400 μmol mol^{-1}, the CO$_2$ gradient (C$_a$ – C$_i$) at the stomatal pores increased from approximately 250 μmol mol^{-1} (C$_i$ of 150 μmol mol^{-1}) at soil water saturation to approximately 360 μmol mol^{-1} (C$_i$ of 40 μmol mol^{-1}) at the brink of terminal drought. The SWC also influenced *i*WUE (Figure 2d). Values increased from well-watered conditions to a maximum at 25% SWC, with a plateau around 170 μmol CO$_2$ per mol H$_2$O between 40–60% SWC. Under mild water deficit between 40–60% SWC, there was little variation in A$_n$, and g$_s$ and, consequently, the *i*WUE values.

The results for maize B73 differed from data gained by similar analyses of Arabidopsis plants (Figure 3a–d; Reference [15]). The A$_n$ remained constant between 30–70% SWC, which might be caused by light-limited, but not water-limited, photosynthesis. However, g$_s$ and C$_i$ steadily decreased with decreasing SWC and, concomitantly, the *i*WUE increased by twofold from approximately 35 to 70 μmol mol^{-1} at 30% SWC. The CO$_2$ gradient at stomata increased more than twofold from approximately 80 μmol mol^{-1} at soil water saturation to approximately 170 μmol mol^{-1} at 30%. The data were obtained at light conditions that did not saturate photosynthesis, but analysis at saturating light confirmed the capacity of Arabidopsis to lower C$_i$ and maintain photosynthetic rates unchanged [15]. The improvement in *i*WUE by limiting g$_s$ without major trade-offs in A$_n$ (Figure 3) was observed for the C$_3$ plants Arabidopsis [15,24] and tomato [63]. A twofold enhancement in *i*WUE has been reported in different C$_3$ species under drought [11,15,77–79]. Besides, considerable differences in WUE in the absence of drought stress have been observed among natural variants [80,81].
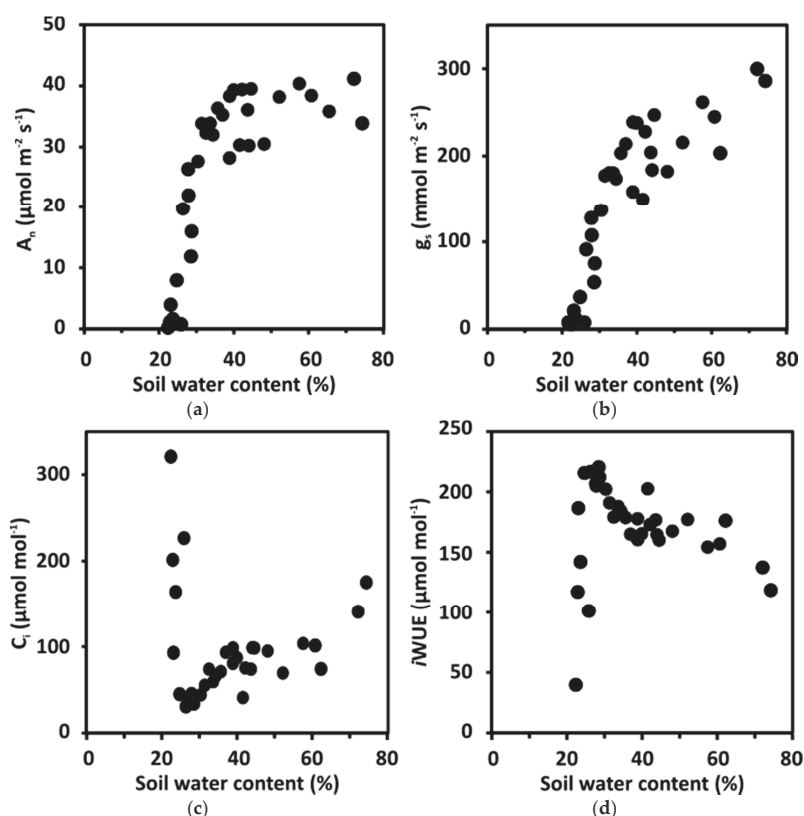
Figure 2. Changes in intrinsic water use efficiency of maize exposed to a progressive depletion of soil water content. (**a**) Net carbon assimilation rate (A_n), (**b**) stomatal conductance (g_s), (**c**) intercellular CO_2 concentration (C_i), and (**d**) intrinsic WUE (*i*WUE; defined as the ratio of A_n to g_s) of B73 plants at different soil water content. Gas exchange measurements using the GFS-3000 gas exchange system (Heinz, Walz GmbH, Effeltrich, Germany) were conducted at a photon flux density of 1000 μmol m^{-2} s^{-1}, an external CO_2 (C_a) of 400 μmol mol^{-1} CO_2, and vapor pressure deficit (VPD) of 26 Pa kPa^{-1} $\pm$ 2 Pa kPa^{-1}. The first fully expanded leaf counting from the top of the plants was clamped into an 8 cm^2 cuvette for measurements, and plants were subjected to progressive drought as detailed in Figure 1. Plants were grown in soil (Classic Profi Substrate Einheitserde Werkverband) as described in Reference [15]. The experiment was conducted in a greenhouse in the Department of Botany in Freising, Germany from June to August. The maize plants were exposed to full sunlight, at an average temperature of 27 °C, and an average relative humidity of 55% in the experimental period. (**a**–**d**) five biological replicates and each data point represents single measurements with five technical replicates.
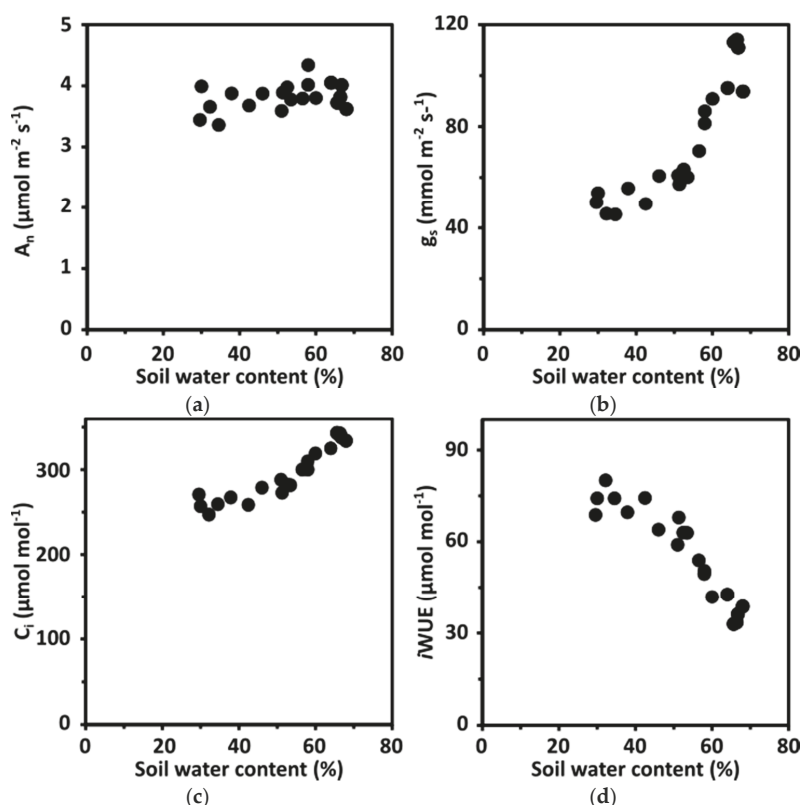
Figure 3. Changes in intrinsic water use efficiency of Arabidopsis exposed to a progressive depletion of soil water content (SWC). (**a**) Net carbon assimilation rate (A_n), (**b**) stomatal conductance (g_s), (**c**) CO_2 concentration in intercellular space (C_i) and (**d**) intrinsic WUE (*i*WUE) of whole leaf rosettes of Arabidopsis accession Columbia (Col-0; kindly provided by the Nottingham Arabidopsis Stock Center, Nottingham, UK). The measurements were performed with the device mentioned in Figure 2 at a photon flux density of 150 µmol m^{-2} s^{-1}, an ambient CO_2 (C_a) level of 420 µmol mol^{-1} and vapor pressure deficit of 13 ± 1 Pa kPa^{-1}. The plants were grown under short day conditions (8 h light/16 h dark photoperiod) at a photon flux density of 150 µmol m^{-2} s^{-1} and 22 °C and 50% relative humidity in the day time and 17 °C and 60% relative humidity at night. Data presented in (**a–d**) consists of three biological replicates and single measurements for each data point consist of 10 technical replicates. Data and the correlation between SWC and water potentials are presented in Reference [15].

Comparing the results from the analysis of maize and Arabidopsis, the potential for increasing *i*WUE in maize was more limited relative to Arabidopsis. Between 30–60% SWC—reflecting mainly mild water deficit [15]—Arabidopsis responded to the mounting water deficit by a 70% increase in *i*WUE, while maize showed an increase of less than 20%. Based on the *i*WUE values obtained at water-saturated soil, the *i*WUE increase in Arabidopsis and maize was approximately 100% and 40%, respectively, but water logging might be an issue at these high SWC levels. Between 30% and 60% SWC, the stomatal CO_2 gradient of maize increased from 300 to 350 µmol mol^{-1}, which corresponded to a 17% increase, whereas in Arabidopsis, the gradient was enhanced by 70%, from 100 to 170 µmol mol^{-1}.

To sum up, mild drought stress (30–60% SWC) had a minor effect on A_n, g_s, and *i*WUE in maize. However, in Arabidopsis, g_s and *i*WUE changed dynamically, while A_n was little affected. A reduction in SWC from 35% to 25% led to a rapid decline of A_n in maize.

These results are in accordance with data from C_3 and C_4 grass species [77,78] but only partly meet the behavior expected for C_3 and C_4 plants based on a meta study [82] where decreases in g_s and A_n under mild drought stress were more pronounced in C_3 relative to C_4 species. Comparing the A_n and g_s curves of maize B73 and Arabidopsis Col-0 (Figure 2a,b and Figure 3a,b), a reduction in stomatal conductance led to an immediate reduction in assimilation rate A_n for maize but not for Arabidopsis. However, not all C_3 plants show the same flat A_n/g_s curve as Arabidopsis [83,84] and therefore the data cannot be translated to C_3 crops in general. A previous study on maize lines found differences in g_s without trade-offs in A_n at well-watered conditions [85], and the author noted that C_i values did not become low enough to limit A_n as it might in high VPD conditions [85] or as it was observed here under drought.

Still, the limitations in improving WUE in maize B73 could be unique to this inbred line, and comparable data from other maize lines is needed before implications can be expanded to maize in general. Additionally, results from maize cannot represent C_4 photosynthesis in general because this pathway evolved independently in 19 angiosperm families [67]. However, other C_4 grass species have also shown a slight increase in *i*WUE with progressive depletion of water followed by a steep decline under very severe drought conditions driven by a pronounced decline in A_n [77,78]. The loss of A_n in the C_4 grass species under drought could partly be attributed to stomatal limitations, while other limitations dominate, including photoinhibition, limitations of CO_2 fixation due to desiccation, and decreases in g_m [73,77].

It has also been shown that for subspecies of *Alloteropsis semialata* (R.Br.) Hitchc., A_n is massively reduced in C_4 subspecies under drought in such a way that C_4 photosynthesis totally loses its advantage over photosynthesis of the C_3 subspecies [73]. This conclusion cannot be drawn from our experiments. However, the observation that C_3 plants become more water use efficient during mild and moderate drought while C_4 plants show more stable WUE [86] is in accordance with our data on *i*WUE.

The less potent improvement in WUE observed in maize under drought might be attributable to a limitation in increasing the CO_2 gradient ($C_a - C_i$) further. Maize, like other C_4 species, possesses a CO_2 concentrating mechanism utilizing precarboxylation of CO_2 by PEP carboxylase, which results in C_i values approximately half compared to C_3 species [87]. Such a mechanism results in advantages in A_n and WUE under non- or mild-water-deficit conditions [9,66,69,73,77,78]. However, these advantages cannot be maintained when the drought gets severe, especially for maize and C_4 grass species [66,69,73,77,78,86], which is in agreement with our observation for maize B73 at SWC below 27%. The differences can also translate to the field level, where maize has been found to be more sensitive to drought than wheat (C_3), with yield reductions of 39.3% compared to 20.6%, respectively, at approximately 40% water reduction [88]. Maize and sorghum are equally or even more sensitive to water stress than many C_3 plants [69,73].

Our results show a potential to increase WUE in maize. The inbred lines displayed a broad variation in WUE_{plant} under progressive drought, and *i*WUE—measured under the same conditions for the least efficient line at whole plant level—still showed a moderate increase in *i*WUE with declining SWC. However, the potential of WUE improvement is limited in this C_4 plant compared to Arabidopsis. This limitation is caused by a very high *i*WUE and low C_i under well-watered conditions, which provides a minor degree of freedom for further lowering the C_i. The C_3 plant is more responsive concerning increases in *i*WUE under mild water deficit compared to maize. Hence, screening C_3 plants for enhanced *i*WUE in combination with efficient growth is a suitable approach to identify crops with improved WUE_{plant}. This approach is less promising for C_4 plants. Establishing higher $C_a - C_i$ gradients in C_3 crops at a given soil water potential, e.g., by biotechnical engineering using ABA receptors, has the potential to increase *i*WUE at the cost of minor reductions in A_n. Moderate reductions in A_n do not necessarily influence yield. In barley, improvements in *i*WUE and WUE_{plant} have been associated with trade-offs in carbon assimilation but without deleterious effects on plant growth or seed yield [89].

4. Conclusions

Our results indicate that the improvement in *i*WUE without trade-offs in carbon assimilation, as observed for tomato and Arabidopsis [15,63], is less promising for maize and possibly other C_4 plants. The large CO_2 gradient established by the CO_2-concentrating mechanisms of C_4 plants limits the potential for further increases in *i*WUE compared to C_3 plants. However, our data show major differences in WUE_{plant} for maize inbred lines and therefore potential for genetic improvement of this trait.

A recent meta-analysis on WUE revealed a tenfold bias in favor of C_3 plant studies compared to analyses on C_4 plants [86]. We therefore see an urgent need for more studies on C_4 crops to shed light on the mechanisms of WUE under water deficit in these important but drought-sensitive crops. Cereals like rice, maize, and wheat contribute largely to global food security [90]; therefore, breeding for and generating water-efficient and high yielding crops are an urgent task to meet future challenges.

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Alleviation of Drought Stress by Nitrogen Application in *Brassica campestris* ssp. *Chinensis* L.

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Abstract: To assess the influence of drought stress on the growth and nitrogen nutrition status of pakchoi (*Brassica campestris* ssp. *Chinensis* L.) at different nitrogen (N) levels, the changes in N accumulation and enzyme activities involved in N assimilation were investigated. The drought was induced by adding polyethylene glycol (PEG) under hydroponic culture conditions. Pakchoi seedlings were exposed to a modified nutrient solution with different nitrogen concentration (N1, N2, and N3 represent 2, 9 and 18 mM NaNO$_3$, respectively) and osmotic potential (W1, W2 and W3 represent 0, 60 and 120 g·L^{-1} PEG 6000) in a full factorial, replicated randomized block design. A short time (seven days) of drought stress caused a significant decline in plant water content, transpiration rate, shoot biomass and shoot nitrogen concentration. Increasing N availability considerably alleviate drought stress by increasing the content of total free amino acids in the roots, promoting the acceleration of root biomass accumulation, and improving the activities of nitrate reductase (NR; EC 1.7.1.1) and glutamine synthetase (GS; EC 6.3.1.2) which would reduce moisture limitations. The results suggested that pakchoi supplied with relative higher N had better growth performance under drought stress.

Keywords: pakchoi; drought stress; nitrogen concentration; enzyme activities; leafy vegetable

1. Introduction

Water and nitrogen (N) are the two main limiting factors for crop growth and productivity, especially those grown under greenhouse conditions. However, tighter water restrictions and competition with other sectors of society are increasing pressure to diminish the share of fresh water for irrigation, thus resulting in the decrease in water diverted for agriculture in China [1,2]. In general, water deficiency depresses N uptake by the root and transport to the aboveground part due to a restricted transpiration rate affecting active transport and membrane permeability [3,4]. N is a critical mineral nutrient for crop growth and involves almost all physiological processes like adenosine triphosphate (ATP) synthesis, photosynthesis and active defense mechanisms in plants [5]. To ensure the high productivity of crops, it is of particular importance to better understand N metabolism, and the utilization of crops in response to water deficit.

Nitrate (NO$_3^-$) is the main N source for the crop in the soil. Once taken up by plants, the NO$_3^-$ is reduced to nitrite (NO$_2^-$) by nitrate reductase (NR; EC 1.7.1.1) then to ammonium (NH$_4^+$) by nitrite reductase (NiR; EC 1.7.2.2). The NH$_4^+$ is incorporated into glutamic acid (Glu) by glutamine synthetase (GS; EC 6.3.1.2) and glutamate synthase (GOGAT; EC 1.4.7.1). Then amino transferases like glutamic oxalacetic transaminase (GOT; EC 2.6.1.1) and glutamate pyruvate transaminase (GPT; EC 2.6.1.2) work on transformation and synthesis among amino acids [6]. It has been well documented that N uptake, allocation, and assimilation in plants were regulated by adverse conditions including water stress [7]. For example, polyethylene glycol (PEG)-induced drought stress reduced the total N content in tall fescue, Italian ryegrass, and cocksfoot [4]. Drought stress has been reported to affect the rates

of NO_3^- absorption in almonds, with harmful effects at Ψ_w below -0.18 MPa [8]. The drought has been reported to restrict the plants growth through enzymes inhibition implicated in N metabolism as well [9–11]. For instance, Pawar et al. [10] have demonstrated that drought stress leads to decreased NR, GS, and GOGAT enzyme activities. It has also been reported that drought can alter the expression level of genes such as *NRT1;2* and *NRT2;5*, *AMT1;3*, *AMT1;1b*, *NR*, *GS2*, and *GS1;2*, which are involved in N uptake and assimilation [12,13].

Previous studies also have demonstrated that higher N metabolism enhanced the drought tolerance of crops [14]. Zhong et al. [15] reported that higher levels of nitrate transport and assimilation contributed to enhancing the drought resistance of *AtTGA4* transgenic *Arabidopsis thaliana* (L.). Chang et al. [16] found that proper N level supports regular plant growth and helps plants to defense stress. Tran et al. [17] demonstrated that higher nitrogen application rate (>75 kg $N \cdot hm^{-2}$) enhances the plasticity expression of the root development in rice under mild drought stress conditions. Shabbir et al. [18] found that combined foliar spray of supplemental nitrogen, phosphorous and potassium influenced the accumulation of osmoprotectants and activity of both N assimilation and antioxidant enzymes for improved yield and drought tolerance potential in wheat. Conversely, Liu et al. [19] suggested that elevated N deposition reduces the tolerance and ability to recover from drought in two moss species. Therefore, the regulatory function of N in drought tolerance of plant depends upon the intensity of stress, N level, and plant species.

Pakchoi (*Brassica campestris* ssp. *Chinensis* L.) is one of the most popular vegetables, cultivated over a large northern to southern range in China. Excessive N fertilizer is often employed to ensure the adequate yield and profitability from vegetable cultivation [20]; leading to most of the N accumulates in soils or is lost by runoff or lixiviation [21–23]. It has been reported the residual soil N in Chinese cabbage production is even high enough to avoid the requirement for additional N in the subsequent season [24]. Many studies have investigated the response of pakchoi to different N levels and N forms at the physiological and molecular levels [5,25–27]. However, there is less information available about the influence of drought stress on the N assimilation process and N nutrition status. In the current study, in order to understand the growth and physiological responses of pakchoi to drought stress at different N levels, we examined morphological (leaf area and root characteristics) and physiological changes (N assimilation enzymes activity, accumulation of NO_3^--N, NH_4^+-N and total free amino acids) associated with N metabolism. We hypothesized that increasing N availability might contribute to the enhancement of nitrogen assimilation ability and the accumulation of an osmotic substance in pakchoi suffering from water deficiency, which alleviates the growth restriction.

2. Materials and Methods

2.1. Plant Cultivation and Treatments

The experiment was carried out in a glass greenhouse at School of Agriculture and Biology, Shanghai Jiao Tong University, China. The pakchoi (cv. Hua wang) seeds were sown in the vegetable seedling substrate (Agriculture Technology Co., Ltd., Huaian, China) on 27 February 2017. After twenty-one days of germination, the seedlings were transferred to black containers (54 cm $\times$ 28.5 cm $\times$ 6 cm) and cultivated hydroponically. The seedlings received distilled water, half-strength nutrient solution and total nutrient solution (pH 5.6–5.8) for 3, 3, and 6 days, respectively (acclimating hydroponics environment). The total nutrient solution contained 2.5 mM $MgSO_4$, 2.0 mM KH_2PO_4, 5.0 mM $CaCl_2$, 5.0 mM KCl, 20 μM EDTA-Fe, 46 μM H_3BO_3, 9.1 μM $MnCl_2$, 1.0 μM $ZnSO_4$, 0.32 μM $CuSO_4$ and 0.1 μM Na_2MoO_4 (all the reagents were purchased from Sinopharm Chemical Reagent Co., Ltd, Shanghai, China), and the pH was 5.6~5.8. On 2 April 2017, similar seedlings were selected and transferred to a modified nutrient solution with different N concentration and osmotic potential. For N treatment, the plants were supplied with nutrient solution containing 2 mM $NaNO_3$ (N1, low nitrogen), 9 mM $NaNO_3$ (N2, moderate nitrogen) and 18 mM $NaNO_3$ (N3, high nitrogen). The osmotic potential of the solution was manipulated by adding PEG 6000. The plants received a

third of the dose of PEG 6000 every 6 h until it was up to the set amount. The doses of PEG 6000 applied were 0 (W1, control), 60 (W2, mild drought stress) and 120 (W3, moderate drought stress) g·L^{-1}, respectively. Totally there were nine treatments (W1N1, W1N2, W1N3, W2N1, W2N2, W2N3, W3N1, W3N2, and W3N3), three blocks, 21 repetitions per block for 567 plants. Drought stress lasted for one week; the old leaves of pakchoi were turning yellow and even fell off. The NO_3^- concentration in the nutrient solution was measured using the optical method described by García-Robledo et al. [28], and $NaNO_3$ was added on day 4 to restore the mineral concentration to its initial value. The moisture loss was evaluated by a weighing method and supplemented every evening throughout the period.

The greenhouse environmental data were monitored every 5 min by using an automatic data logging system (PM-11 Phyton monitor, Bio Instruments S.R.L., Chisinau, Moldova), and it was showed in Table S1. During the whole experimental period, the air temperature ranged from 2.88 °C to 42.95 °C with an average of 15.45 °C. The average relative humidity was 73.9%, with the highest at 92.8% and lowest at 21.4%. Mean solar radiation in the greenhouse was 2.18 MJ·m^{-2}·day^{-1}.

2.2. Growth Parameter Assays

The growth parameter (dry weight of the shoots and roots, total leaf area, and root morphology) of four plants in each container was determined, and the mean was considered as one repetition. The transpiration of newest leaves was measured between 9:00 am and 11:00 am on the seventh day of drought. The transpiration rate (Tr) was measured using a portable photosynthetic measurement system (Li-3000) coupled with a light-emitting diode (LED) light source (400 µmol photon m^{-2} s^{-1}). The air flow was 750 µmol·s^{-1}.

Experimental plants were harvested and divided into shoots and roots after seven days of treatment. Fresh and dry weights were determined on an electronic scale (AUY220; SHIMADZU, Kyoto, Japan) and plant water content was calculated. Leaves were scanned by an EPSON scanner (EPSON PERFECTION V700 PH07, Nagano, Japan) and total leaf area was calculated by Image J freeware (National Institutes of Health, Bethesda, MD, USA). Whereas the roots were scanned and analyzed by a WinRHIZO root analyzer system (WinRHIZO version 2007d, RegentInstruments Canada, Montreal, QC, Canada).

2.3. Nitrogen Concentration Determination

The fresh shoot and root samples were heated at 105 °C for 20 min and dried at 75 °C for 72 h. The dried samples were ground and sifted (60 mesh), and nitrogen concentration was measured using an Elementar (ELIII, Hanau, Germany).

2.4. NO_3^--N, NH_4^+-N, Total Free Amino Acids Measurements

The NO_3^--N content in the roots and leaves was spectrophotometrically analyzed as described earlier [29]. Briefly, a total of 1.00 g fresh sample was added to 5 mL of deionized water and incubated at 95 °C for 30 min. After filtration, the filtrate was diluted to 25 mL with water. And an appropriate solution (0.1 mL) was thoroughly mixed with 0.4 mL of 5% (*w/v*) salicylic acid in concentrated H_2SO_4 followed by incubation at room temperature for 20 min. The NO_3^- concentration was measured at 410 nm.

For ammonium quantitation, 1.00 g of the fresh sample was ground with 8 mL of distilled water. The homogenates were then centrifuged (12,000× *g* for 10 min) at 4 °C. The resulting supernatant was diluted 1:30 (*v/v*) in deionized water, and 3 mL of the solution was mixed with 0.5 mL of 1% (*w/v*) phenol-0.01% (*w/v*) sodium nitroprusside solution and 0.5 mL of 1% (*v/v*) sodium hypochlorite-0.5% (*w/v*) sodium hydroxide solution (in water). After incubating the mixture at 37 °C for 30 min, 5.9 mL of the deionized water and 0.1 mL of 8% (*w/v*) potassium sodium tartrate, 2% (*w/v*) disodium ethylenediamine tetraacetate, and 0.08% (*w/v*) sodium hydroxide solution (in water) were added, and absorbance was measured at 625 nm [9].

Total free amino acids were determined according to the method described by Shabbir R. N. et al. [18] with slight modifications. For this, sample mixture containing 0.3 mL of the extract, 1 mL of pyridine (10%, w/v) and 1 mL of ninhydrin solution (3%, w/v), was heated in boiling water bath for 15 min. After cooling to room temperature, 5 mL of ethyl alcohol (95%) was added and optical density was recorded at 570 nm. Total free amino acids were estimated by a standard curve constructed with alanine.

2.5. Nitrogen Metabolic Enzyme Activity Assays

NR activity was determined as reported by Foyer et al. [30]; whereas the activities of GS and GOGAT were measured in accordance with the method described by Lin C. C. et al. [31]. Activities of GOT and GPT were assayed, adopting the method used by Wu L. H. et al. [32].

2.6. Data Processing and Statistical Analysis

All the statistical analyses were carried out using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA). A two-way analysis of variance (ANOVA) was employed to examine the effect of drought and nitrogen treatments on the experimental variables. Differences between means were calculated on the basis of least significant differences ($p < 0.05$).

3. Results

3.1. Plant Water Content

After seven days of exposure to drought stress, plant water content significantly decreased in the moderate drought group, but there were no differences between mild drought group and blank at three N levels (Figure 1). Simultaneously, plant water content was regulated by N levels, and the values in the groups of moderate and high N were dramatically higher than that in the low N treatment.
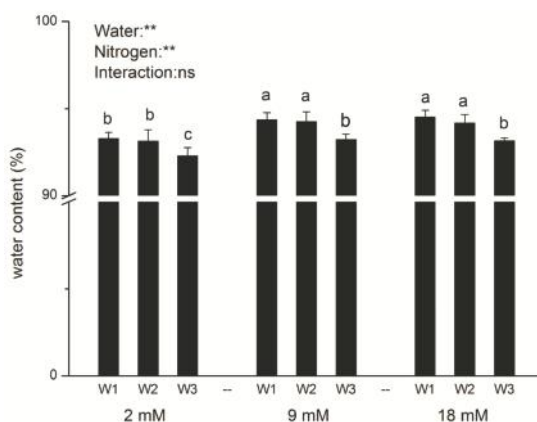
Figure 1. Plant water content of pakchoi at the end of the experiment. Bars with the different letters indicate significant difference ($p < 0.05$). W1: 0 g·L^{-1} polyethylene glycol (PEG) 6000, blank; W2: 60 g·L^{-1} PEG 6000, mild drought stress; W3: 120 g·L^{-1} PEG 6000, moderate drought. Error bars represent the standard deviation. p-values of the ANOVAs of drought, nitrogen supply, and their interaction are indicated. ns, not significant. ** $p < 0.01$.

3.2. Growth Parameters

After seven days of exposure to drought stress, the aerial parts of pakchoi exhibited marked growth inhibition, resulting in a diminished leaf area and shoot biomass (Figure 2 and Table 1).

Drought stress also significantly decreased ($p < 0.05$) the transpiration rate as well. The availability of N had a prominent effect on shoot growth under drought stress and blank groups. Under drought stress, the pakchoi supplied with higher N had higher leaf area and shoot biomass compared with the pakchoi supplied with low N.

However, root growth was promoted by the drought-induced by PEG 6000. Drought stress increased both the root biomass and root/shoot ratio (Figure 2). Total root length and root surface areas were evidently increased under drought stress conditions, and the effect was only remarkable under low nitrogen rate (Table 1). Drought stress also significantly increased the root volume and mean root diameter, and the degree of elevation positively correlated to the nitrogen rate (Table 1).
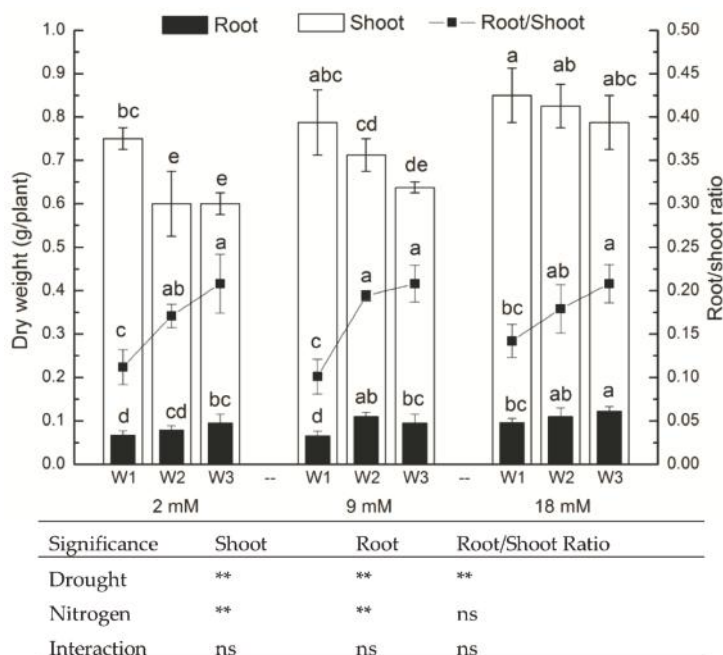
Figure 2. Shoot biomass and root biomass (bars) of pakchoi at the end of the experiment. Root/Shoot ratio is also represented (continuous line). W1: 0 g·L^{-1} PEG 6000, blank; W2: 60 g·L^{-1} PEG 6000, mild drought stress; W3: 120 g·L^{-1} PEG 6000, moderate drought. For the dry weight of shoot and root, bars with the different letters indicate significant difference ($p < 0.05$). For Root/Shoot ratio, lines with the different letters indicate significant difference ($p < 0.05$). Error bars represent the standard deviation. *p*-values of the ANOVAs of drought, nitrogen supply, and their interaction are indicated. ns, not significant. ** $p < 0.01$.

Table 1. Growth parameters and transpiration of pakchoi under different drought and nitrogen levels.

Nitrogen Level	Drought	Total Leaf Area (cm²)	Transpiration Rate (mmol m⁻² s⁻¹)	Total Root Length (cm)	Total Root Surface Area (cm²)	Total Root Volume (cm³)	Mean Root Diameter (mm)
2 mM	Blank	118.5 ± 11.6 [d]	2.6 ± 0.5 [a]	265.2 ± 10.2 [b]	28.6 ± 4.1 [d]	0.29 ± 0.05 [e]	0.35 ± 0.04 [e]
	Mild drought	134.1 ± 10.9 [cd]	2.3 ± 0.8 [a]	310.7 ± 24.0 [a]	46.5 ± 2.4 [a]	0.44 ± 0.04 [bc]	0.44 ± 0.02 [cd]
	Moderate drought	83.2 ± 9.3 [e]	1.2 ± 0.2 [c]	325.3 ± 6.4 [a]	36.8 ± 1.3 [bc]	0.39 ± 0.04 [cd]	0.44 ± 0.01 [cd]
9 mM	Blank	163.2 ± 8.8 [b]	2.7 ± 0.3 [a]	258.4 ± 7.3 [bc]	35.0 ± 2.9 [bcd]	0.30 ± 0.03 [e]	0.35 ± 0.02 [e]
	Mild drought	156.1 ± 13.0 [bc]	1.8 ± 0.9 [abc]	261.0 ± 9.0 [bc]	39.1 ± 2.8 [bc]	0.48 ± 0.04 [b]	0.49 ± 0.05 [bc]
	Moderate drought	92.9 ± 16.7 [e]	1.7 ± 0.5 [bc]	261.6 ± 22.5 [b]	33.6 ± 2.2 [bcd]	0.44 ± 0.02 [bc]	0.52 ± 0.02 [b]
18 mM	Blank	197.4 ± 15.6 [a]	2.2 ± 0.8 [ab]	225.4 ± 34.3 [c]	32.5 ± 2.4 [cd]	0.34 ± 0.03 [de]	0.41 ± 0.05 [de]
	Mild drought	173.8 ± 14.8 [b]	1.9 ± 0.5 [abc]	245.2 ± 19.6 [bc]	40.1 ± 7.5 [b]	0.57 ± 0.03 [a]	0.53 ± 0.02 [b]
	Moderate drought	139.7 ± 13.6 [cd]	1.7 ± 0.5 [bc]	245.2 ± 16.8 [bc]	29.5 ± 3.9 [d]	0.44 ± 0.05 [bc]	0.60 ± 0.09 [a]
Significance							
Drought		**	*	*	**	**	**
Nitrogen		**	ns	**	ns	**	**
Interaction		*	ns	ns	*	ns	ns

(1) Blank: 0 PEG 6000, mild drought stress: 60 g·L⁻¹ PEG 6000, moderate drought: 120 g·L⁻¹ PEG 6000; (2) Mean values ± SD with different letters in the column are significantly different according to the Duncan test at $p < 0.05$; (3) ns: non-significant, * $p < 0.05$, ** $p < 0.01$, The statistics is based on two-way analysis of variance (ANOVA).

3.3. Nitrogen Content

Nitrogen accumulation responses to drought stress were measured, and the results were displayed in Figure 3 and Table S2. The N concentration was decreased in shoots with increasing drought level under normal and high nitrogen supply (Figure 3A). Mild and moderate drought stresses resulted in shoot N concentration decreased by 6.00% and 6.87% under normal nitrogen application rate. However, high nitrogen application rate could alleviate the drought stress. Under high nitrogen rate (18 mM), shoot N concentration increased by 11.61% and 23.75%, respectively, in mild and moderate drought stresses compared with the normal nitrogen application rate (9 mM). In addition, root nitrogen concentration was regulated by exogenous nitrogen application rate under the drought stress (Figure 3B). Drought stress decreased root nitrogen concentration under low nitrogen supply (2 mM) but increased under normal nitrogen supply (9 mM). On the other hand, no significant difference was observed among the high nitrogen supply (18 mM).

Figure 3. Nitrogen concentration (**A**,**B**) in shoots and roots, nitrogen content (**C**,**D**) in shoots and roots of pakchoi at the end of the experiment. W1: $0 \text{ g} \cdot \text{L}^{-1}$ PEG 6000, blank; W2: $60 \text{ g} \cdot \text{L}^{-1}$ PEG 6000, mild drought stress; W3: $120 \text{ g} \cdot \text{L}^{-1}$ PEG 6000, moderate drought. Bars with the different letters indicate significant difference ($p < 0.05$). Error bars represent the standard deviation. *p*-values of the ANOVAs of drought, nitrogen supply, and their interaction are indicated. ns, not significant. * $p < 0.05$. ** $p < 0.01$.
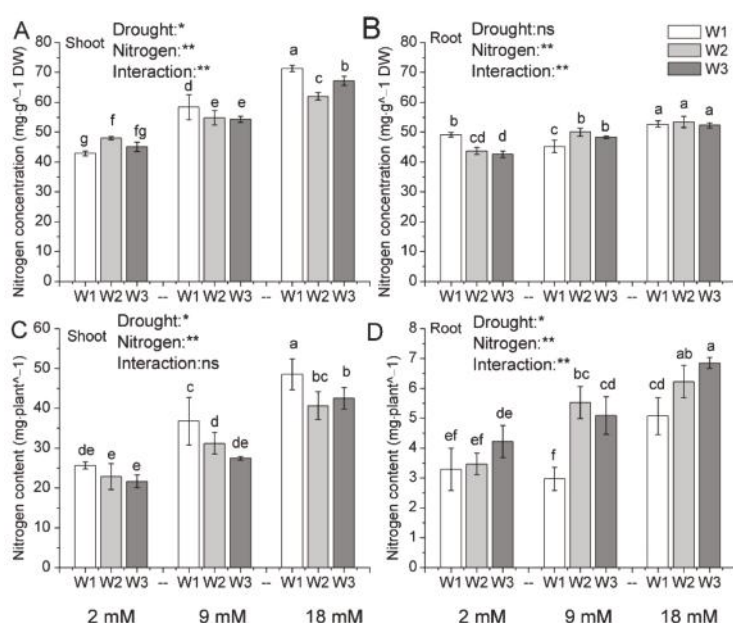
The total N content was decreased in shoots with increasing drought level, but it increased in roots (Figure 3C,D). Mild and moderate drought stresses resulted in total N content in shoots decreasing by 15.09% and 25.32% under normal nitrogen application rate. However, total N content in shoots would go up with increased nitrogen application rate under the drought stress. Under high nitrogen rate (18 mM), total N content in shoots increased by 30.24% and 54.92%, respectively, in mild and moderate drought stresses compared with the normal nitrogen application rate (9 mM). In addition, nitrogen accumulation in roots was regulated by both exogenous nitrogen application rate and drought stress (Figure 3D). Drought stress significantly increased root nitrogen content under normal and high

nitrogen supply, but no significant change was found under low nitrogen supply. In addition, nitrogen accumulation in roots increased with the increased nitrogen supply under the drought stress.

3.4. Accumulation of NO_3^--N, NH_4^+-N, and Total Free Amino Acids

The NO_3^--N, NH_4^+-N, and free amino acid content in the roots and leaves of pakchoi were determined and the data were showed in Table S2. Results showed that there was a significant interactive effect of drought stress and N on NO_3^--N content in leaves (Figure 4A). Especially in the high N treatments, NO_3^--N content significantly reduced with drought stress. Simultaneously, NO_3^--N content in roots also decreased with the drought, and mild and moderate drought stress resulted in roots NO_3^--N content decreased by 5.56%–53.49% and 74.42%–86.11%, respectively (Figure 4B). Similarly, NO_3^--N content in roots displayed interactive effect of drought and nitrogen as well. Figure 4C indicated that drought stress increased the accumulation of NH_4^+-N in leaves, especially the moderate drought stress. The increment was more pronounced in the higher nitrogen supply (9 and 18 mM), displaying interaction effect of water and nitrogen. The accumulation of NH_4^+-N in roots was also affected by exogenous nitrogen and water supply (Figure 4D). Under the same level of nitrogen condition, drought significantly increased the NH_4^+-N accumulation in roots. Simultaneously, the NH_4^+-N accumulation increased with the increasing nitrogen rate under both control and drought stress condition. The drought stress increased the accumulation of free amino acids in shoots and roots, respectively (Figure 4E,F). Free amino acids were regulated by nitrogen application rate as well. Regardless of mild or moderate drought stress, normal and high nitrogen supply increased the accumulation of free amino acids.
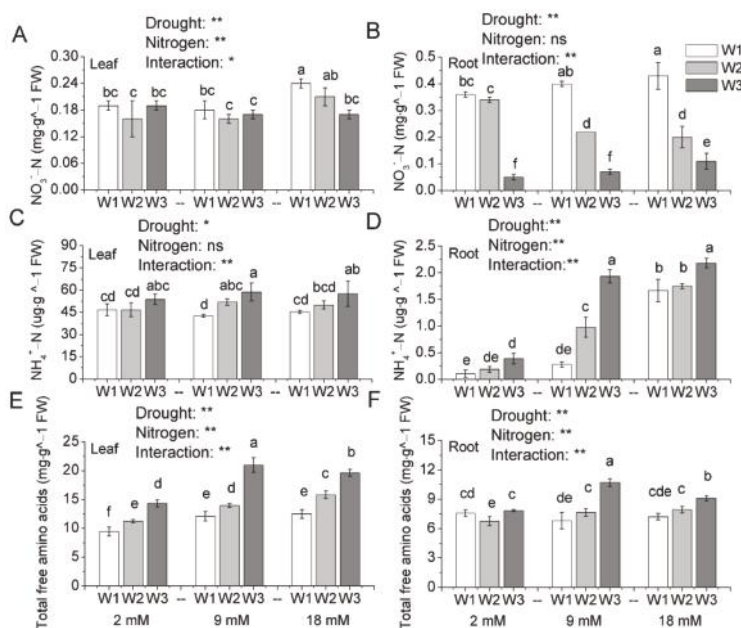
Figure 4. NO_3^--N content (**A,B**), NH_4^+-N content (**C,D**) and total free amino acids content (**E,F**) in leaves and roots of pakchoi at the end of the experiment. W1: 0 g·L^{-1} PEG 6000, blank; W2: 60 g·L^{-1} PEG 6000, mild drought stress; W3: 120 g·L^{-1} PEG 6000, moderate drought. Bars with the different letters indicate significant difference ($p < 0.05$). Error bars represent the standard deviation. p-values of the ANOVAs of drought, nitrogen supply, and their interaction are indicated. ns, not significant. * $p < 0.05$. ** $p < 0.01$.

3.5. Nitrogen Metabolic Enzyme Activity

N accumulation was disturbed by drought stress, so we measured the enzyme activity involved in N assimilation (Figure 5 and Table S3). Results evidenced that there existed a significant effect of both drought and N on NR activity (Figure 5A,B). Under low N condition, drought decreased the NR activity in leaves and in roots. However, under normal and high N condition, the effect of mild drought stress on NR activity was not significant, moderate drought stress significantly enhanced NR activity in leaf and root.
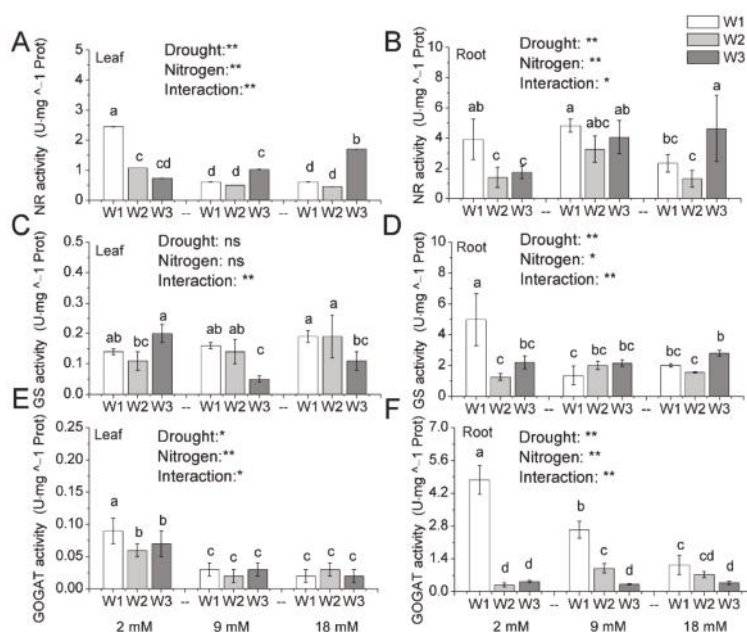
Figure 5. The activity of nitrate reductase (**A**,**B**), glutamine synthetase (**C**,**D**) and glutamate synthetase (**E**,**F**) in leaves and roots of pakchoi by different drought and nitrogen supply. W1: 0 g·L^{-1} PEG 6000, blank; W2: 60 g·L^{-1} PEG 6000, mild drought stress; W3: 120 g·L^{-1} PEG 6000, moderate drought. Bars with the different letters indicate significant difference ($p < 0.05$). Error bars represent the standard deviation. *p*-values of the ANOVAs of drought, nitrogen supply, and their interaction are indicated. ns, not significant. * $p < 0.05$. ** $p < 0.01$.

Figure 5C exhibits that the effect of drought and N on the GS activity in leaves. Mild drought stress had no significant effect on GS activity in leaves. However, under moderate drought stress, normal and high N application rates significantly decreased the GS activity, but not at low N application rate. Similarly, drought and N played critical roles in the regulation of the GS activity in roots (Figure 5D). Under low N condition, drought significantly decreased the GS activity in roots. However, under normal and high N condition, the negative effect of drought stress on GS activity was not significant. The results in Figure 5E revealed that mild and moderate drought stress significantly decreased the GOGAT activity in leaves under low N application rate. However, drought stress has no significant effect on the GOGAT activity under normal and high N application rate. In roots, GOGAT activity declined with the drought stress, and high N application rate decreased the drop degree (Figure 5F). Generally, the interactive effect of drought and N on GOGAT activity was significant both in leaves and roots.

GOT and GPT are two key enzymes for transamination among amino acids in the plant. Results in Figure 6A showed that drought stress significantly increased the GOT activity in leaves under low nitrogen condition, but no significant effect was observed at normal and high N application rate. However, drought stress significantly decreased the root GOT activity, and the results were observed in all of the three N treatments (Figure 6B). The result also indicates that the normal and high nitrogen application rate decreased GOT activity in roots. Similarly, drought was found to be the critical factors in the regulation of GPT activity in leaves (Figure 6C). Under low N condition, drought significantly increased the GPT activity. However, under normal and high N condition, the positive effect of drought stress on GPT activity was not significant. Differently, the GPT activity of root was just controlled by drought stress, and the N effect was not significant (Figure 6D). For example, moderate drought stress decreased GPT activity under whatever the N application rate. Moreover, the interaction effect of drought and N on GOT and GPT activity was significant in leaves and roots, respectively.
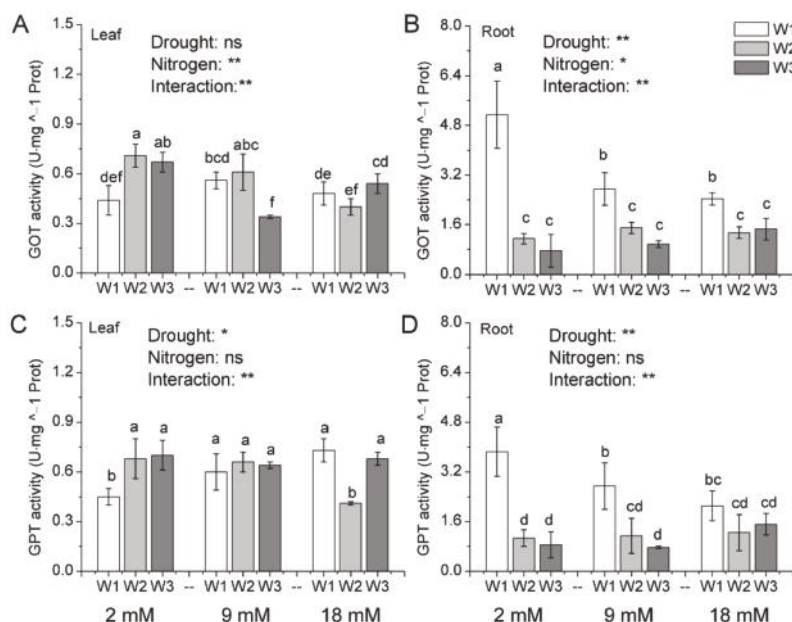
Figure 6. The activity of glutamic oxalacetic transaminase (**A**,**B**) and glutamic pyruvic transaminase (**C**,**D**) in leaves and roots of pakchoi as affected by drought and nitrogen supply. W1: 0 g·L^{-1} PEG 6000, blank; W2: 60 g·L^{-1} PEG 6000, mild drought stress; W3: 120 g·L^{-1} PEG 6000, moderate drought. Bars with the different letters indicate significant difference ($p < 0.05$). Error bars represent the standard deviation. *p*-values of the ANOVAs of drought, nitrogen supply, and their interaction are indicated. ns, not significant. * $p < 0.05$. ** $p < 0.01$.

4. Discussion

4.1. Drought Restricted the Transport of Nitrogen from Root to Aboveground

The size and configuration of roots are directly related to the absorption of moisture and nutrients because they are in direct contact with the nutrients in growth medium [33–35]. Generally, larger root systems with greater fine root surface area have a higher uptake rate of moisture and nutrients [36,37]. The results indicated that moderate drought stress considerably promoted the increase of total root volume and dry weight in pakchoi (Table 1 and Figure 2). Nitrate is absorbed by the root, but only a fraction of it remains in roots and participate in the building of amino acids and proteins. Most of the

N is transported aboveground through the xylem by transpiration pull, and is involved in various physiological and metabolic processes in the leaves [38,39]. The drought stress significantly decreased the leaf transpiration (Table 1) and shoot N concentration (Figure 3), and this finding was consistent with Delhon et al. [40], who observed that experimental decrease in transpiration rate resulted in a lower N uptake, accompanied by an increase in root N concentration. On wheat, Nicolas et al. [41] showed that water deficit limits N translocation from roots to shoots by drastically reducing water xylem flux. Gonzalez-Dugo [4] reported that restricted root-to-shoot translocation and decreased sink size are responsible for limited nitrogen uptake in three grass species under water deficit. Therefore, drought might restrict the translocation of N from roots to shoots, and the restriction may further contribute to growth limitation. In addition, root-shoot ratios increased with drought, largely due to a stronger negative effect of water deficit on shoot growth than on root growth, which was in agreement with earlier reports [42,43].

4.2. Drought Disturbed the Nitrogen Metabolism Process

Drought might alter N metabolism and N allocation through the regulation of enzymes involved in N assimilation. In this study, water deficit alters the activities of NR, GS, and GOGAT (Figure 5), and this result agrees with Wang et al. [13] who observed that water deficit alters N uptake of maize. Since drought decreases crop growth, due to high sensitivity to leaf area expansion to plant water status, water deficit therefore also affects N demand [1]. Leaf area could act as an indicator of N demand [4]. In this study, we found that total leaf area and plant water content were decreased with drought, indicating that water limitation leads to a reduced shoot N demand, and thus a decrease in enzyme activity.

Drought also leads to an accumulation of N assimilation pathway intermediates. Ammonium from nitrate reduction is incorporated into amino acids by glutamine synthetase (GS) and glutamate synthase (GOGAT). In this study, moderate drought caused an increased accumulation of NH_4^+ (Figure 4), an increment in NR activity, and a reduction in GS activity (Figure 5) under moderate and high N conditions, revealing that an increased accumulation of NH_4^+ under drought might be attributed to the enhanced nitrate reduction. In addition to nitrate reduction, ammonia may also be released for re-assimilation by sink tissue from nitrogen transport compounds (e.g., asparagine, arginine and the uredine) and through breakdown of other nitrogenous compounds [3,44].

Plants antagonize drought stress mainly depend on osmotic regulation, and the ability of osmotic adjustment directly reflects drought resistance [45]. Amino acids, as an important osmotic substance, play a vital role in maintaining the cell turgor pressure and in the reduction of tissue dehydration [46,47]. In this paper, moderate drought stress significantly increased the accumulation of total free amino acids (Figure 4E,F), which contributed to reduce the cell osmotic potential and maintain cell turgor pressure. Additionally, GOT and GPT are two key enzymes for transamination among amino acids in the plant. The different activities of GOT and GPT (Figure 6) suggested that drought affect the transformation of amino acids. However, Liu et al. reported that amino N content was not affected by drought and drought did not alter the composition of amino N in the xylem and phloem in *Robiniapseudoacacia* L. [48]. So the composition of amino compounds responded to drought depend upon plant species, therefore the drought-mediated changes in amino N in pakchoi need to be elucidated further.

4.3. Higher Nitrogen Application Could Alleviate the Adverse Effect of Drought Stress on Pakchoi

Our data showed that drought stress decreased the leaf area, biomass and nitrogen concentration in the shoot, while higher nitrogen application mitigated the negative effects of drought stress (Table 1, Figures 1 and 2). Simultaneously, the accelerated accumulation of total free amino acids in the roots and dry weight was observed in the high N supply under moderate drought stress (Figures 2 and 4). Thus, increasing N availability may help alleviate drought stress by increasing osmotic regulators in the roots and promoting the acceleration of root biomass accumulation which would reduce moisture limitations. We also found that NR activity in leaves and roots were increased with the increasing

nitrogen application rate under moderate drought stress, our results are in a good agreement with those reported earlier [13]. Zhong et al. reported that higher levels of nitrate transport and assimilation contributed to enhancing the drought resistance of *AtTGA4* transgenic plants [15]. So we assumed that the enhancement of nitrogen assimilation ability also alleviates the growth limitation caused by water deficiency in pakchoi. However, the expressions of genes related to nitrogen transport and assimilation should be determined to reveal whether nitrogen transport and assimilation contribute to enhanced drought resistance of pakchoi from the molecular level.

5. Conclusions

PEG 6000-induced drought stress limited pakchoi development and nitrogen nutrition. Increasing N availability may alleviate drought stress by increasing osmotic regulators in the roots, promoting the acceleration of root biomass accumulation, and improving nitrogen assimilation ability which would reduce moisture limitations. Thus, our results proposed that pakchoi supplied with relative higher N had better growth performance under drought stress.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/5/66/s1, Table S1: The greenhouse environmental data, including solar radiation (w/m^2), temperature (°F), relative humidity (RH, %), photosynthetically active radiation (PAR, μE). Table S2: Nitrate-N (NO$_3^-$-N) content, Ammonium-N (NH$_4^+$-N) content, free amino acid-N content, plant nitrogen concentration and plant nitrogen content in leaves and roots of pakchoi at the end of the experiment. Table S3: The activity of nitrate reductase (NR), glutamine synthetase (GS), glutamate synthetase (GOGAT), glutamic oxalacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) in leaves and roots of pakchoi by different drought and nitrogen supply.

Author Contributions: X.X. and D.H. conceived and designed the experiments; X.X. performed the experiments; X.X. and L.C. analyzed the data and produced the figures. X.X. and M.K. wrote the manuscript. L.C., J.Z. and D.H. supervised and coordinated work. All authors reviewed the manuscript.

Conflicts of Interest: The authors have declared that no competing interests exist.

Abbreviations

N	nitrogen
PEG	polyethylene glycol
NO$_2^-$	nitrite
NO$_3^-$	Nitrate
NH$_4^+$	ammonium
NiR	nitrite reductase
NR	reductase
GS	glutamine synthetase
Glu	glutamic acid
GOGAT	glutamate synthase
GOT	goxalacetic transaminase
GPT	glutamate pyruvate transaminase
W1	control
W2	mild drought stress
W3	moderate drought stress
Tr	transpiration rate

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Article

Halotolerant Bacterial Diversity Associated with *Suaeda fruticosa* (L.) Forssk. Improved Growth of Maize under Salinity Stress

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Abstract: Halotolerant bacterial strains associated with the rhizosphere and phytoplane of *Suaeda fruticosa* (L.) Forssk. growing in saline habitats were isolated to mitigate the salinity stress of *Zea mays* L. 16S rRNA gene sequencing confirmed the presence of strains that belong to *Gracilibacillus, Staphylococcus, Virgibacillus, Salinicoccus, Bacillus, Zhihengliuella, Brevibacterium, Oceanobacillus, Exiguobacterium, Pseudomonas, Arthrobacter*, and *Halomonas* genera. Strains were screened for auxin production, 1-aminocyclopropane-1-carboxylate (ACC)-deaminase, and biofilm formation. Bacterial auxin production ranged from 14 to 215 µg mL^{-1}. Moreover, several bacterial isolates were also recorded as positive for ACC-deaminase activity, phosphate solubilization, and biofilm formation. In pot trials, bacterial strains significantly mitigated the salinity stress of *Z. mays* seedlings. For instance, at 200 and 400 mM NaCl, a significant increase of shoot and root length (up to onefold) was recorded for *Staphylococcus jettensis* F-11. At 200 mM, *Zhihengliuella flava* F-9 (45%) and *Bacillus megaterium* F-58 (42%) exhibited significant improvements for fresh weight. For dry weight, *S. jettensis* F-11 and *S. arlettae* F-71 recorded up to a threefold increase at 200 mM over the respective control. The results of this study suggest that natural plant settings of saline habitats are a good source for the isolation of beneficial salt-tolerant bacteria to grow crops under saline conditions.

Keywords: antioxidant enzymes; bacterial auxin production; halotolerant bacteria; halophytes; maize; plant-growth-promoting rhizobacteria; salinity stress; *Suaeda fruticose* (L.) Forssk.

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1. Introduction

Soil salinity is one of the most severe environmental factors limiting agricultural productivity in arid or semiarid regions around the world. Salinity adversely affects the physical and chemical parameters of the soil as well as crop production to a significant extent [1]. Halophytes are the vegetation of saline habitats. Halophytes possess special morphological, anatomical, and physiological characteristics that are well suited to cope with saline habitats. *Suaeda fruticosa* (L.) Forssk. is a plant species that belongs to the family Amaranthaceae. It is a halophyte that occurs in arid and semiarid saline habitats or salt marches. *S. fruticosa* has a strong ability to accumulate and sequester NaCl [2]. It can be used for soil reclamation to reduce salinity and contamination of heavy metals. Halophytes have a number of adaptations to mitigate salinity stress, including salt discharge from roots, accumulation of organic acids, reduced stomatal conductance, lower water potential, and uptake of inorganic ions [3]. Moreover, specific types of amino acids, carbohydrates, and glycine betaine are accumulated as compatible solutes under abiotic stress [4].

High salt content in soil and irrigation water is a major threat to the sustainability of agriculture around the world. The presence of excess salts in the rhizosphere can cause severe injury to the root system followed by their gradual accumulation in other plant tissues. It can cause heavy damage to

plant metabolism, which can lead to stunted plant growth and reduced yield [5]. Salinity can disrupt the water uptake and ion equilibrium of plants. It can lead to oxidative damage to plants due to the production of reactive oxygen species (ROS). This includes the production of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH) etc. Halophytes have the ability to keep the level of these ROS at minimal levels due to the presence of an antioxidant system that consists of enzymes like catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) [6,7].

Rhizobacteria associated with the root system of halophytes can enhance the growth, development, and stress tolerance of plants growing in saline soils [8]. It has been demonstrated that beneficial microorganisms play a significant role in alleviating salt stress in plants, which results in increased crop yield. Plant-growth-promoting rhizobacteria (PGPR) are a group of microorganisms that colonize the root system of plants and trigger plant growth and development under stress conditions. PGPR can employ several direct or indirect mechanisms to enhance plant growth and productivity. Auxin production, 1-aminocyclopropane-1-carboxylate (ACC)-deaminase, siderophores production, and phosphate solubilization are well documented in the literature [9,10]. Indole-3-acetic acid (IAA) represents one of the most extensively studied auxins in plants. It mediates multidimensional developmental processes including apical dominance, tropic responses, cellular differentiation, and pattern formation [11]. IAA is also quantitatively the most abundant phytohormones secreted by rhizobacteria as secondary metabolites. Auxin production has been reported for several halotolerant bacterial genera including *Bacillus*, *Enterobacter*, *Arthrocnemum*, *Agrobacterium*, *Ochrobactrum*, *Pseudomonas*, and *Pantoea* in NaCl-amended medium [8,9]. Moreover, soil microorganisms possess the enzyme ACC-deaminase that converts ACC (precursor of ethylene) to α-ketobutyrate and ammonia. When plants are exposed to stress conditions, ethylene is synthesized, which results in retardation of root growth and senescence [12,13]. Although ethylene mediates growth responses in plants, it can inhibit plant growth at elevated levels (under stress), leading to physiological changes in plant tissues. Tolerance to biotic or abiotic stresses can be increased by treating plant roots with ACC-deaminase-producing rhizobacteria [14]. Halotolerant PGPR have been reported to confer upon plants the ability to mitigate environmental or abiotic stresses [7]. For instance, *Bacillus aquimaris*, *Bacillus thuringiensis*, *Enterobacter cloacae*, *Enterobacter asburiae*, *Ochrobactrum anthropi*, and *Pseudomonas stutzeri* significantly increased vegetative and yield parameters under saline conditions [9,10,15]. Maize (*Zea mays* L.) is the third most important cereal crop that is grown under a wide range of climatic conditions. Salt-tolerant PGPR isolated from halophytes have been reported to enhance the vegetative growth parameters of maize under induced salinity [16].

Soil salinity affects approximately 6.3 million hectors of irrigated land in Pakistan and has caused considerable losses to the agriculture sector [17]. There is a need to devise environmentally friendly strategies that can ameliorate soil salinity by the application of resident halotolerant bacteria. In the present study, salt-tolerant bacteria associated with halophytic plants have been evaluated to mitigate the salinity stress of maize. In Pakistan, *S. fruticosa* is distributed in coastal and inland saline habitats [6]. Little is known about the association of halotolerant bacterial communities with this plant. Therefore, the purpose of this study was to evaluate the bacterial diversity of the rhizosphere and phytoplane of *S. fruticosa* growing in saline habitats of the Photohar Plateau, a region of northeastern Pakistan. Bacterial communities associated with the surfaces of *S. fruticosa* may have naturally adapted to tolerate high salt concentrations. Therefore, screening of resident bacterial flora may indicate potential candidates for agricultural applications under saline habitats.

2. Materials and Methods

2.1. Isolation of Bacterial Strains

Suaeda fruticosa (L.) Forssk. is a halophytic plant that grows in the saline habitats of the Photohar Plateau, a region of northeastern Pakistan. Halotolerant bacterial strains were isolated from the rhizosphere and phytoplane of *S. fruticosa*. For rhizospheric samples, 1 g of soil was dissolved in 99 mL

of sterilized distilled water. Then, the serial dilutions of the samples were prepared successively as described in Cappuccino and Sherman [18]. For endophytes, leaves were sterilized by soaking in 70% ethanol for 2–3 min and then washed with autoclaved distilled water. One gram of leaves was grounded to paste and transferred to a test tube containing 9 mL of autoclaved distilled water. About 50 μL from soil and leaf suspensions was plated on L-agar supplemented with 0.5 M and 1 M NaCl. Plates were incubated at 30 °C for 24 h. Discrete bacterial colonies were selected and purified by many rounds of streaking.

2.2. 16S rRNA Gene Sequencing

Bacterial strains were identified by 16S rRNA gene sequencing. Total genomic DNA from overnight grown bacterial cultures was extracted using the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan). PCR amplification of 16S rRNA gene sequencing was accomplished by using forward primer 27f (5′-AGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1522r (5′-AAGGAGGTGATCCA (AG)CCGCA-3′) [19]. PCR amplification was performed in 50 μL of Dream Taq™ Green PCR Master Mix (Fermentas, Waltham, MA, USA) with 0.5 μg of chromosomal DNA template and 0.5 μM of each primer. The reaction mixtures were incubated in a thermocycler Primus 96 (PeQLab, Erlangen, Germany) at 94 °C for 5 min and passed through 30 cycles: Denaturation for 20 s at 94 °C, primer annealing for 20 s at 50 °C, and extension at 72 °C for 2 min. The amplified products were purified by using the FavorPrep™ Gel Purification Mini Kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan). Purified PCR products were sent to First Base Laboratories, Singapore for sequencing. Sequences were aligned with a multiple sequence alignment program (Clustal W) by using Molecular Evolutionary Genetics Analysis (MEGA) 6. software) [20]. The phylogenetic relationships among halotolerant bacterial strains were studied by constructing phylogenetic trees by the neighbor-joining method [21].

2.3. Halophility Assay

L-broth medium supplemented with 0, 0.5, 1, and 1.5 M NaCl was used to evaluate the salt tolerance of bacterial strains. After inoculation, strains were incubated overnight at 37 °C for 24 h at 130 rpm/min. For all treatments, three replicates were placed for comparison. The optical density of the cultures was recorded at 600 nm by a spectrophotometer (CECIL CE 7200, Cecil Instruments Limited, Cambridge, UK). Proline analysis of bacterial strains grown under salt stress was also accomplished by following the method of Cha-Um and Kirdmanee [22].

2.4. Plant-Growth-Promoting Traits

For auxin quantification, strains were inoculated in triplicate in L-broth supplemented with 1 M NaCl and 500 μg mL^{-1} filter sterilized solution of L-tryptophan. After incubation at 37 °C for 72 h, cells were removed from stationary phase cultures by centrifugation. One milliliter of culture supernatant was mixed with 2 mL of Salkowski reagent [23]. Afterwards, samples were incubated in the dark for the development of pink color. Optical density was recorded at 535 nm with a spectrophotometer (CECIL CE 7200). The standard curve was constructed by using different concentrations of authentic auxins to calculate the auxin production by different bacterial strains. The phosphate solubilization potential of bacterial isolates was evaluated by streaking on Pikovskaya's agar plates [24].

The 1-aminocyclopropane-1-carboxylate (ACC)-utilization ability of the strains was evaluated by following the method described in [25]. Bacterial strains were inoculated in 5 mL of L-broth in triplicate and incubated overnight at 28 °C on a shaker at 200 rpm/min. Two milliliters of each culture were harvested in a 2-mL Eppendorf tube by centrifugation at 8000× *g* for 5 min. The cell pellet was washed twice with 1 mL of liquid Dworkin-Foster(DF) medium. Afterwards, the pellet was suspended in 2 mL of DF-ACC medium in a 12-mL culture tube. The tubes were incubated at 28 °C on a shaker at 200 rpm/min for 24 h. A 2-mL sample of DF-ACC medium without inoculation was incubated in parallel. One milliliter of each culture was centrifuged in a 1.5 mL Eppendorf tube as mentioned

above. One hundred microliters of each supernatant were diluted to 1 mL with liquid DF medium in a 1.5-mL Eppendorf tube. Sixty microliters of each tenfold-diluted supernatant was used for the 96-well PCR-plate ninhydrin-ACC assay and mixed with 120 μL of ninhydrin reagent. The PCR plate was then heated on a boiling water bath for 30 min. The DF medium was used as a blank. Each diluted supernatant was run in triplicate. After transfer of 100 μL of the remaining reaction solution, the absorbance of the samples was measured at 570 nm with a microplate spectrophotometer (Epoch, BioTek, Winooski, VT, USA).

2.5. Biofilm Formation

The biofilm-forming ability of bacterial strains was evaluated by following the method of [26]. Bacterial strains were inoculated in triplicate in Tryptic Soy Broth (TSB) supplemented with 0, 200, and 400 mM NaCl and incubated at 37 °C for 24 h. Following incubation, 20 μL of the cultures was transferred to the wells of a 96-well flat bottom microtiter plate that contained 180 μL of TSB supplemented with the abovementioned NaCl concentrations. Negative controls contained only 200 μL of the TSB medium and the assay was performed in triplicate. To promote biofilm formation, the plates were incubated on a shaker at 37 °C for 72 h. After incubation, the well contents were discarded and washed thrice with 250 μL of sterile distilled water to remove any nonadherent and weakly adherent cells. Plates were air dried for 30 min. The biofilm formed in the wells was fixed with 250 μL/well 98% methanol for 15 min. After air drying, the fixed bacterial cells were stained with 200 μL of 0.1% v/v crystal violet solution for 5 min. Excessive stain was removed by placing the plate under low-running tap water and the plate was air dried. Resolubilization of crystal violet with the adherent cells was done by adding 200 μL/well of 33% v/v glacial acetic acid. The optical density was measured at 570 nm using a microplate spectrophotometer (Epoch, BioTek, Winooski, VT, USA).

2.6. In Vitro Plant Bioassay

Seeds of maize (*Zea mays* L. Var. Sahiwal-2002) were obtained from Punjab Seed Corporation (Lahore, Pakistan). Seeds were surface sterilized by treatment with 0.1% $HgCl_2$ for 2–3 min followed by repeated washing with sterilized distilled water. Rooting assay was performed with single or mixed bacterial cultures. Single bacterial cultures included F-9, F-11, F-12, F-35, F-37, F-58, F-71, F-72, F-81, F-83, F-84, and F-87. Mixed culture combinations C-1 (F-9, F-11, F-12, F-87), C-2 (F-35, F-71, F-81, F-83), and C-3 (F-37, F-58, F-72, F-84) were also included for seed inoculation. For inoculum preparation, strains were cultivated in L-broth for 24 h. Then, cultures were harvested by centrifugation and the cell pellet was washed with autoclaved distilled water. The optical densities of the cultures were adjusted to a final concentration of 10^7 colony forming units (CFU)/mL in sterilized distilled water. Sterilized seeds were soaked in a bacterial suspension for 30 min and placed in Petri plates containing two sterilized filter papers. Water-treated seeds were used for comparison. For each strain and combination, five seeds were inoculated in duplicate. Petri plates were incubated in an Environmental Test Chamber (MRL-350H; Sanyo, Osaka, Japan) at 25 °C. After 7 days, shoot length, root length, number of roots, and fresh and dry weights of seedlings were recorded.

2.7. Pot Trials with Salt Stress

Pot experiments were conducted to evaluate the growth-promoting effect of bacterial strains on maize under different salt-stress conditions. Seeds were surface sterilized as mentioned earlier and placed on moistened autoclaved double-filter paper in Petri plates. Healthy germinated seeds were selected for bacterization with single and mixed cultures of bacterial strains as mentioned above. Germinated seeds were sown in pots containing 100 g of autoclaved soil. Five seeds were sown in each pot and experiments were carried out in triplicate. Three salt-stress conditions, i.e., 0, 200, and 400 mM, were applied up to the field capacity of soil. Salt stress was applied twice—at the time of sowing and to 7-day-old seedlings. Water-treated seeds were used as a control. Pots were kept under conditions of 12 h of photoperiod and a temperature of 25 °C in the Environmental Test Chamber (MRL-350H;

Sanyo, Osaka, Japan). After two weeks, plant-growth parameters such as shoot length, root length, number of roots, and fresh weight of seedlings were recorded. For dry weight, plants were incubated in an oven at 80 °C for 24 h.

2.8. Biochemical Analysis of Plants

The proline content of plants grown under 0, 200, and 400 mM NaCl was measured as described by Cha-Um and Kirdmanee [22]. Similarly, the method of Racusen and Foote [27] was used for the quantitative estimation of peroxidases from fresh leaves. Acid phosphatase was extracted from plant leaves following the method of Iqbal and Rafique [28].

2.9. Statistical Analysis

Data for bacterial auxin production and plant-growth parameters were subjected to statistical analysis by using SPSS 20 software (IBM Corporation, New York, NY, USA). The data were also subjected to analysis of variance (ANOVA). Finally, mean values were separated by using Duncan's multiple range test ($p \leq 0.05$). Correlation analysis between bacterial growth and NaCl concentrations was also performed ($p = 0.05$).

3. Results

3.1. Strains Identification

One hundred salt-tolerant bacterial strains were isolated from the rhizosphere and phytoplane of *Suaeda fruticosa* (L.) Forssk. *fruticosa* growing in saline habitats. 16S rRNA gene sequences were compared with already-deposited sequences in GenBank. The comparison indicated around 99% similarity with respective identified species. Analysis showed that the strains belong to the genera *Gracilibacillus*, *Staphylococcus*, *Virgibacillus*, *Salinicoccus*, *Bacillus*, *Zhihengliuella*, *Brevibacterium*, *Oceanobacillus*, *Exiguobacterium*, *Pseudomonas*, *Arthrobacter*, and *Halomonas*. Sequences were submitted to GenBank under accession numbers KT027652–KT027742. Rhizospheric soil samples recorded the presence of 45 salt-tolerant rhizobacteria and were represented by 9 bacterial genera (Table 1). Similarly, the phytoplane recorded the presence of 46 endophytic bacterial strains represented by 8 bacterial genera (Table 2). Figure 1 shows the phylogenetic relationships among different salt-tolerant bacteria isolated from the rhizosphere and phytoplane of *S. fruticosa*. Analysis showed that the majority of the Gram-positive strains (firmicutes) clustered in one major group. This included strains from the genera *Bacillus* and *Staphylococcus*. A few Gram-positive strains were also represented by the genera *Oceanobacillus*, *Salinococcus*, *Gracilibacillus*, *Brevibacterium*, and *Exiguobacterium*. In addition to that, Gram-positive *Arthrobacter* and *Zhihengliuella* clustered in a separate group. Gram-negative strains were represented by the genera *Pseudomonas* and *Halomonas* and occupied a separate cluster in the phylogenetic tree.

Table 1. 16S rRNA gene sequencing of halotolerant bacterial strains isolated from the rhizosphere of *Suaeda fruticose* (L.) Forssk.

S. No.	Strains	Identified as	Accessions
1	F-1	*Gracilibacillus saliphilus* F-1	KT027652
2	F-2	*Staphylococcus petrasii* F-2	KT027653
3	F-3	*Virgibacillus salarius* F-3	KT027654
4	F-4	*G. saliphilus* F-4	KT027655
5	F-5	*Salinicoccus sesuvii* F-5	KT027656
6	F-6	*Bacillus licheniformis* F-6	KT027657
7	F-7	*B. subtilis* F-7	KT027658
8	F-8	*B. mojavensis* F-8	KT027659

Table 1. *Cont.*

S. No.	Strains	Identified as	Accessions
9	F-9	*Zhihengliuella flava* F-9	KT027660
10	F-10	*B. licheniformis* F-10	KT027661
11	F-11	*S. jettensis* F-11	KT027662
12	F-12	*S. arlettae* F-12	KT027663
13	F-13	*B. sonorensis* F-13	KT027664
14	F-14	*B. subtilis* F-14	KT027665
15	F-15	*B. aerius* F-15	KT027666
16	F-16	*B. licheniformis* F-16	KT027667
17	F-17	*B. subtilis* F-17	KT027668
18	F-18	*B. atrophaeus* F-18	KT027669
19	F-19	*Brevibacterium halotolerans* F-19	KT027670
20	F-20	*B. subtilis* F-20	KT027671
21	F-21	*B. licheniformis* F-21	KT027672
22	F-22	*B. axarquiensis* F-22	KT027673
23	F-23	*B. amyloliquefaciens* F-23	KT027674
24	F-24	*B. subtilis* F-24	KT027675
25	F-25	*B. subtilis* F-25	KT027676
26	F-26	*G. thailandensis* F-26	KT027677
27	F-27	*B. subtilis* F-27	KT027678
28	F-28	*Oceanobacillus picturae* F-28	KT027679
29	F-29	*S. devriesei* F-29	KT027680
30	F-30	*S. jettensis* F-30	KT027681
31	F-31	*S. petrasii* F-31	KT027682
32	F-32	*B. subtilis* F-32	KT027683
33	F-33	*B. infantis* F-33	KT027684
34	F-34	*B. subtilis* F-32	KT027685
35	F-35	*Exiguobacterium mexicanum* F-35	KT027686
36	F-36	*B. subtilis* F-36	KT027687
37	F-37	*S. caprae* F-37	KT027688
38	F-38	*S. devriesei* F-38	KT027689
39	F-39	*S. hominis* F-39	KT027690
40	F-83	*B. stratosphericus* F-83	KT027734
41	F-84	*B. pumilus* F-84	KT027735
42	F-85	*B. pumilus* F-85	KT027736
43	F-86	*B. marisflavi* F-86	KT027737
44	F-87	*Pseudomonas japonica* F-87	KT027738
45	F-89	*Sal. sesuvii* F-89	KT027740

Table 2. 16S rRNA gene sequencing of halotolerant bacterial strains isolated from the phytoplane of *S. fruticosa.*

S. No.	Strains	Identified as	Accessions
1	F-40	*Bacillus subtilis* F-40	KT027691
2	F-41	*Exiguobacterium* sp. F-41	KT027692
3	F-42	*Staphylococcus jettensis* F-42	KT027693
4	F-43	*Salinicoccus sesuvii* F-43	KT027694
5	F-44	*Oceanobacillus kapialis* F-44	KT027695
6	F-45	*B. flexus* F-45	KT027696
7	F-46	*B. pumilus* F-46	KT027697
8	F-47	*S. devriesei* F-47	KT027698
9	F-48	*B. malacitensis* F-48	KT027699
10	F-49	*B. malacitensis* F-49	KT027700
11	F-50	*E. aquaticum* F-50	KT027701
12	F-51	*O. polygoni* F-51	KT027702
13	F-52	*O. kimchii* F-52	KT027703

Table 2. *Cont.*

S. No.	Strains	Identified as	Accessions
14	F-53	*B. mojavensis* F-53	KT027704
15	F-54	*B. megaterium* F-54	KT027705
16	F-55	*B. pumilus* F-55	KT027706
17	F-56	*Sal. roseus* F-56	KT027707
18	F-57	*B. subtilis* F-57	KT027708
19	F-58	*B. megaterium* F-58	KT027709
20	F-59	*B. aryabhattai* F-59	KT027710
21	F-60	*B. tequilensis* F-60	KT027711
22	F-61	*B. malacitensis* F-61	KT027712
23	F-62	*B. subtilis* F-62	KT027713
24	F-63	*S. warneri* F-63	KT027714
25	F-64	*Arthrobacter bergerei* F-64	KT027715
26	F-65	*A. ardleyensis* F-65	KT027716
27	F-66	*A. arilaitensis* F-66	KT027717
28	F-67	*B. subtilis* F-67	KT027718
29	F-68	*B. cereus* F-68	KT027719
30	F-69	*S. arlettae* F-69	KT027720
31	F-70	*S. cohnii* F-70	KT027721
32	F-71	*S. arlettae* F-71	KT027722
33	F-72	*S. jettensis* F-72	KT027723
34	F-73	*Pseudomonas rhizosphaerae* F-73	KT027724
35	F-74	*S. gallinarum* F-74	KT027725
36	F-75	*S. petrasii* F-75	KT027726
37	F-76	*S. lugdunensis* F-76	KT027727
38	F-77	*S. capitis* F-77	KT027728
39	F-78	*S. pasteuri* F-78	KT027729
40	F-79	*S. jettensis* F-79	KT027730
41	F-80	*S. equorum* F-80	KT027731
42	F-81	*Halomonas nanhaiensis* F-81	KT027732
43	F-82	*B. safensis* F-82	KT027733
44	F-88	*O. kimchii* F-88	KT027739
45	F-90	*E. mexicanum* F-90	KT027741
46	F-91	*Exiguobacterium* sp. F-91	KT027742

3.2. Halophility Assay

Bacterial isolates showed variable growth responses at different NaCl concentrations (Figure 2). The majority of the isolates that included *S. jettensis* F-11, *S. arlettae* F-12, *S. caprae* F-37, *S. arlettae* F-71, *S. jettensis* F-72, and *B. safensis* F-83 showed good growth up to 1.5 M NaCl. A few strains recorded sensitivity against increasing levels of NaCl. For example, *Z. flava* F-9, *B. megaterium* F-58, and *B. pumilus* F-84 recorded poor growth as compared to other halotolerant bacterial strains. Proline is produced as an osmoprotectant under osmotic stress induced at high salinity. It accumulates as a compatible solute in living cells to counter the effects of NaCl toxicity. Bacterial proline content did not show a significant difference with the increasing salt content from 0 to 1.5 M NaCl. The highest concentration of proline was observed with *S. jettensis* F-11 (228 μg g^{-1}) and *S. jettensis* F-72 (216 μg g^{-1}) at 1.5 M NaCl (Figure 3).
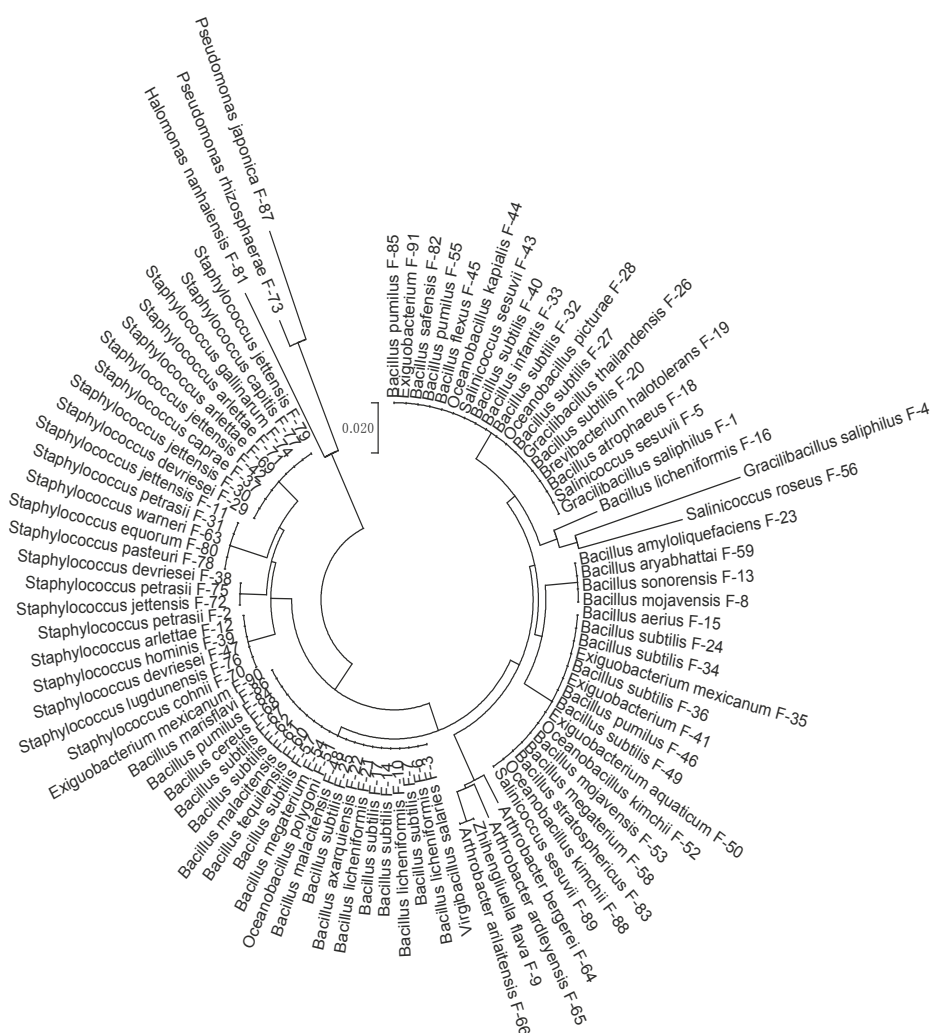
Figure 1. Phylogenetic tree showing the relationships among different halotolerant bacterial strains isolated from rhizosphere and phytoplane of *S. fruticosa*. (L.) Forssk. Scale bar represents mutations per nucleotide position.
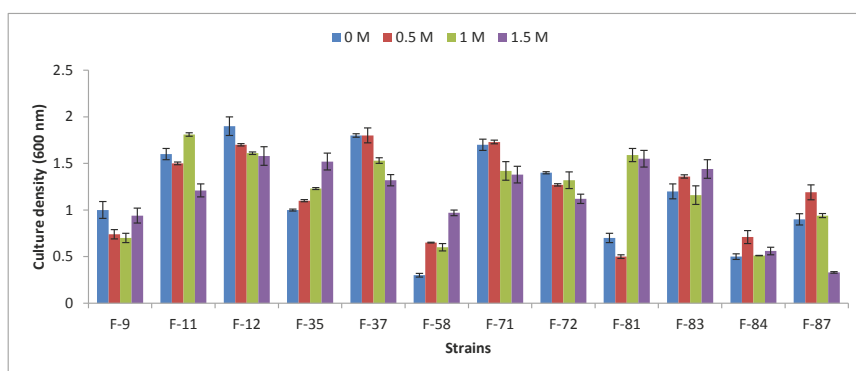
Figure 2. Halophility assay of salt-tolerant rhizobacteria in the presence of different concentrations of NaCl. Bar represents mean ± SE of three replicates.
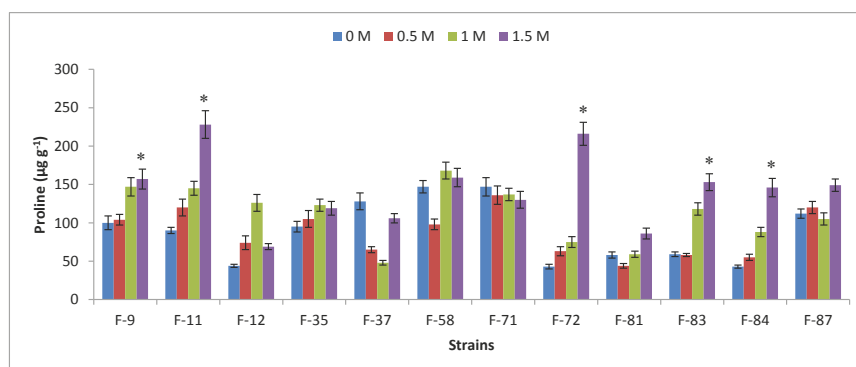
Figure 3. Effect of NaCl concentrations on proline content of halotolerant bacteria. Bars represent mean ± SE of three replicates. Treatments followed by * indicate significant difference over control using Duncan's multiple range test ($p \leq 0.05$).

3.3. Plant-Growth-Promoting Traits

One hundred bacterial strains were screened for in vitro auxin production by colorimetric analysis. Screening indicated significant auxin production by 30 bacterial strains (Table 3). In the absence of L-tryptophan, bacterial auxin production ranged from 5.70 to 87.90 µg mL^{-1}. Bacterial strains produced significant levels of auxin in culture supernatant in the presence of 500 µg mL^{-1} L-tryptophan as compared to the nonamended control. For instance, *S. petrasii* F-2, *V. salarius* F-3, *Z. flava* F-9, *S. jettensis* F-11, *S. arlettae* F-12, and *S. arlettae* F-71 recorded 10-, 2-, 5-, 3-, 3-, and 2-fold increases in auxin production over the control. Overall, auxin production ranged from 14 to 215 µg mL^{-1} in L-tryptophan-amended medium. On the other hand, 14 isolates were recorded as positive for phosphate solubilization. *S. jettensis* F-11, *S. arlettae* F-12, and *S. jettensis* F-72 were the most efficient as compared to other strains.

Bacterial isolates showed variable potential for ACC-deaminase activity. Highly significant activity was observed for *S. caprae* F-37, *S. arlettae* F-71, *B. subtilis* F-14, *E. mexicanum* F-35, and *O. kapialis* F-44. For biofilm formation, *S. caprae* F-37 and *B. pumilus* F-84 recorded good production at 200 mM NaCl. However, at higher NaCl levels, poor biofilm formation was recorded for the majority of the strains (Figure 4).

Table 3. L-tryptophan-dependent bacterial auxin production in the presence of 1 M NaCl.

S. No.	Strains	L-tryptophan (µg mL^{-1})	
		0	500
		Auxin (µg mL^{-1})	
1	*Gracilibacillus saliphilus* F-1	6.20 (a)	15.70 (a)
2	*Staphylococcus petrasii* F-2	9.30 (a)	113.50 (o)
3	*Virgibacillus salarius* F-3	87.90 (i)	150.00 (p)
4	*Zhihengliuella flava* F-9	44.70 (d–h)	215.00 (r)
5	*S. jettensis* F-11	63.20 (g–i)	185.70 (qr)
6	*S. arlettae* F-12	51.40 (e–i)	174.30 (pq)
7	*Exiguobacterium* sp. F-41	6.40 (a)	43.50 (a–f)
8	*S. jettensis* F-42	33.60 (d–g)	78.70 (l–o)
9	*Salinicoccus sesuvii* F-43	16.50 (abc)	28.40 (abc)
10	*S. devriesei* F-47	78.30 (hi)	28.00 (abc)
11	*Bacillus megaterium* F-58	20.80 (abc)	48.70 (a–g)
12	*B. aryabhattai* F-59	25.90 (d–g)	61.20 (i–n)
13	*S. warneri* F-63	6.40 (a)	14.00 (a)
14	*Arthrobacter bergerei* F-64	13.40 (ab)	25.50 (abc)
15	*A. ardleyensis* F-65	9.10 (a)	29.60 (abc)
16	*S. arlettae* F-69	14.30 (abc)	54.20 (h–n)
17	*S. cohnii* F-70	15.70 (abc)	42.10 (a–f)
18	*S. arlettae* F-71	53.60 (f–i)	88.60 (no)
19	*S. jettensis* F-72	30.60 (d–g)	59.80 (i–n)
20	*Pseudomonas rhizosphaerae* F-73	36.80 (d–g)	67.70 (g–n)
21	*S. gallinarum* F-74	15.40 (abc)	23.00 (abc)
22	*S. pasteuri* F-78	10.40 (a)	36.90 (a–c)
23	*S. jettensis* F-79	5.70 (a)	24.50 (abc)
24	*S. equorum* F-80	7.60 (a)	32.20 (a–d)
25	*Halomonas nanhaiensis* F-81	21.10 (abc)	83.30 (mno)
26	*B. safensis* F-82	9.50 (a)	43.90 (a–f)
27	*B. stratosphericus* F-83	23.60 (def)	72.70 (k–n)
28	*B. pumilus* F-84	7.90 (a)	18.10 (ab)
29	*B. pumilus* F-85	32.60 (a–d)	59.80 (i–n)
30	*B. marisflavi* F-86	22.60 (abc)	38.60 (a–e)

Mean of three replicates. Different letters followed by numerical values within same column indicate significant difference between treatments using Duncan's multiple range test ($p \leq 0.05$).

Figure 4. Effect of different NaCl concentrations on biofilm-forming ability of bacterial strains. Bars represent mean ± SE of three replicates. Treatments followed by * indicate significant difference over control using Duncan's multiple range test ($p \leq 0.05$).
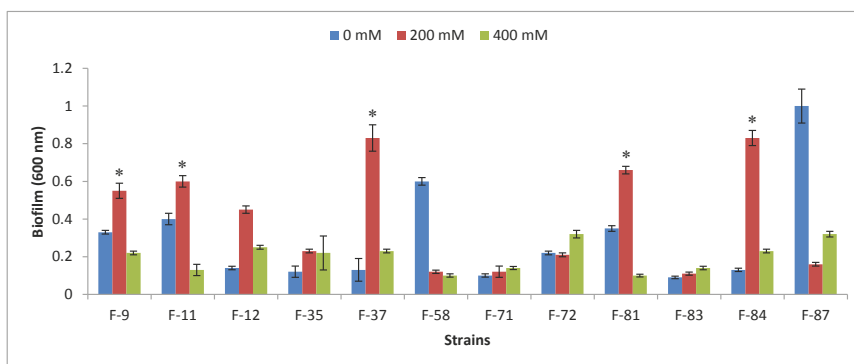
3.4. In Vitro Plant Bioassay

Bacterial strains as single or mixed cultures significantly enhanced the growth of *Z. mays* under in vitro conditions. Maximum increases in shoot length over the water-treated control were observed for *S. jettensis* F-72 (61%), *S. arlettae* F-71 (48%), and *S. arlettae* F-12 (48%). Similarly, for root length, *S. arlettae* F-12, *S. jettensis* F-72, and *B. safensis* F-83 showed 92%, 85%, and 78% increases, respectively, over the control. A statistically significant increase in the number of roots per plant was recorded for *S. jettensis* F-72 and mixed culture C-2. For fresh and dry weights, *S. arlettae* F-12 and mixed culture combination C-1 were the most promising (Table 4).

Table 4. Effect of bacterial strains on growth parameters of *Zea mays* under in vitro conditions.

Strains	Shoot Length (cm)	Root Length (cm)	Roots/Plant	Fresh Weight (g)	Dry Weight (g)
Control	23 (a)	13 (b)	6.4 (de)	4.6 (a)	0.7 (ab)
F-9	28 (bcd)	20 (cd)	4.8 (a)	5.3 (ab)	0.9 (abc)
F-11	32 (defg)	20 (cde)	5.6 (abcd)	6.8 (a–e)	1.1 (bc)
F-12	34 (fgh)	25 (f)	6.2 (bcde)	8.8 (ef)	1.3 (c)
F-35	31 (c–g)	21 (de)	5.7 (abcd)	5.7 (abc)	0.8 (ab)
F-37	29 (cd)	20 (cde)	5.5 (abcd)	10.2 (f)	0.9 (abc)
F-58	33 (efgh)	20 (cde)	6.0 (bcd)	7.4 (b–f)	0.9 (abc)
F-71	34 (gh)	22 (def)	5.7 (abcd)	8.1 (cdef)	1.1 (bc)
F-72	37 (h)	24 (f)	7.1 (e)	7.8 (b–f)	1.0 (abc)
F-81	30 (cdef)	20 (cd)	5.0 (ab)	7.5 (b–f)	1.3 (c)
F-83	29 (cde)	23 (ef)	6.2 (cde)	4.2 (a)	0.6 (a)
F-84	31 (c–g)	22 (def)	5.1 (abc)	6.0 (abcd)	1 (abc)
F-87	28 (bc)	19 (cd)	5.4 (abcd)	8.6 (def)	1.1 (bc)
* C-1	22 (a)	10 (a)	5.6 (abcd)	9.1 (ef)	1.1 (bc)
* C-2	24 (ab)	14 (b)	6.5 (de)	7.8 (b–f)	1.3 (c)
* C-3	23 (a)	17 (c)	5.6 (abcd)	10.1 (f)	1.4 (ab)

Mean of 10 plants. Different letters within same column indicate significant difference between treatments using Duncan's multiple range rest ($p \leq 0.05$); * Mixed culture combinations: C-1 (F-9, F-11, F-12, F-87), C-2 (F-35, F-71, F-81, F-83), C-3 (F-37, F-58, F-72, F-84).

3.5. Pot Trials under Salt Stress

Halotolerant bacterial strains were evaluated to mitigate the effects of salinity on the plants of *Z. mays*. Bacterial strains were used as single or mixed cultures as mentioned above. Bacterization of seeds significantly enhanced growth parameters at 0 mM NaCl. A highly significant response for shoot and root length was shown by *S. arlettae* F-12 and *S. arlettae* F-71 over the water-treated control. For fresh weight, *Z. flava* F-9 was the most effective. For dry weight, *S. jettensis* F-11, *S. caprae* F-37, and *H. nanhaiensis* F-81 recorded up to threefold increases. Increases in salt content (0–400 mM NaCl) negatively affected the growth of plants in control treatments (Table 5). However, inoculations by halotolerant rhizobacteria significantly improved different growth parameters under saline conditions. At 200 mM NaCl, significant increases in shoot length were observed with *S. jettensis* F-11 (59%), *S. arlettae* F-12 (55%), and *S. arlettae* F-71 (50%) over the respective control. At higher stress (400 mM NaCl), *S. jettensis* F-11, *S. arlettae* F-12, and *B. marisflavi* F-87 recorded around a onefold increase for shoot elongation over the respective control. For root length, *S. jettensis* F-11, *S. arlettae* F-12, *S. arlettae* F-71, and *H. nanhaiensis* F-81 gave a promising response at 200 mM NaCl. Similarly, at 400 mM salt stress, the highest response for root elongation was observed with *S. jettensis* F-11 (85%) and *S. arlettae* F-12 (69%). For number of roots, *S. jettensis* F-72 (98%) and *E. mexicanum* F-35 (52%) recorded significant improvements over the control. At 400 mM stress, a 46% increase in root number was observed with *S. arlettae* F-12. Seed bacterization also significantly influenced plant weight under saline conditions. For instance, at 200 mM salt stress, *Z. flava* F-9 (45%) and *B. megaterium* F-58 (42%) demonstrated statistically significant improvements for fresh weight. Similarly, at higher NaCl content (400 mM), *S. jettensis* F-11 (44%) was the most effective (Table 5). On the other hand, for dry weight, *S. jettensis*

F-11 and *S. arlettae* F-71 recorded two- to threefold increases over the 200 mM control. At 400 mM salt stress, the mixed culture C-1 gave good results for dry weight.

Table 5. Effect of halotolerant bacterial strains on the growth of *Z. mays* in salt amended soils.

Strains	NaCl (mM)	Shoot Length (cm)	Root Length (cm)	Roots/Plant	Fresh Weight (g)	Dry Weight (g)
Control	0	22 (b–g)	13 (abc)	5.0 (jklm)	0.9 (abcd)	0.1 (a)
	200	22 (bcde)	16 (bcde)	4.2 (i–m)	1.2 (c–j)	0.1 (abcd)
	400	14 (a)	13 (abc)	4.3 (a–e)	1.1 (c–g)	0.1 (ab)
F-9	0	29 (i–o)	19 (e–k)	5.0 (b–j)	2.0 (r)	0.3 (e–l)
	200	29 (i–p)	21 (g–n)	5.2 (b–l)	1.7 (pqr)	0.2 (abcd)
	400	25 (d–i)	21 (f–m)	4.6 (a–f)	1.6 (l–q)	0.2 (c–h)
F-11	0	32 (n–s)	20 (e–k)	6.0 (e–l)	1.8 (qr)	0.4 (nop)
	200	35 (rs)	25 (m)	6.0 (f–l)	1.6 (m–r)	0.4 (op)
	400	32 (n–r)	24 (klmn)	5.0 (b–k)	1.6 (n–r)	0.2 (e–j)
F-12	0	34 (pqrs)	25 (mn)	6.2 (h–m)	1.6 (n–r)	0.3 (j–p)
	200	34 (pqrs)	23 (j–n)	6.0 (f–m)	1.4 (g–n)	0.3 (d–j)
	400	30 (k–r)	22 (h–n)	6.3 (jklm)	1.3 (f–l)	0.3 (e–k)
F-35	0	31 (l–r)	21 (g–n)	6.0 (f–l)	1.5 (j–p)	0.2 (c–h)
	200	26 (f–l)	21 (g–n)	6.4 (klm)	1.2 (c–i)	0.2 (c–g)
	400	25 (e–i)	21 (g–m)	5.0 (b–j)	1.0 (b–f)	0.2 (e–j)
F-37	0	29 (i–o)	20 (f–l)	5.5 (e–l)	1.0 (a–e)	0.4 (k–p)
	200	27 (g–m)	21 (g–n)	5.3 (b–l)	0.7 (ab)	0.1 (abc)
	400	23 (b–g)	16 (bcde)	5.1 (b–k)	1.4 (g–n)	0.3 (i–o)
F-58	0	33 (o–s)	21 (f–m)	6.0 (g–m)	1.2 (d–k)	0.3 (e–j)
	200	29 (i–o)	21 (f–m)	5.0 (b–i)	1.7 (opqr)	0.2 (c–i)
	400	23 (b–g)	21 (f–m)	3.4 (a)	1.1 (c–g)	0.4 (l–p)
F-71	0	34 (qrs)	22 (h–n)	6.0 (f–l)	1.4 (h–p)	0.3 (f–m)
	200	33 (o–s)	23 (j–n)	6.0 (g–m)	1.5 (l–q)	0.3 (e–k)
	400	23 (c–h)	18 (d–i)	5.0 (b–i)	1.5 (k–p)	0.3 (g–m)
F-72	0	37 (s)	24 (lmn)	7.1 (m)	1.7 (opqr)	0.3 (f–m)
	200	32 (n–r)	18 (d–h)	8.6 (n)	1.6 (m–r)	0.2 (c–h)
	400	25 (e–i)	21 (g–n)	5.0 (b–h)	1.3 (g–m)	0.3 (i–o)
F-81	0	30 (i–q)	19 (e–j)	5.0 (b–k)	1.4 (i–p)	0.4 (p)
	200	28 (i–o)	20 (f–l)	5.0 (b–g)	1.5 (j–p)	0.2 (bcde)
	400	20 (bcd)	16 (cdef)	4.6 (a–g)	1.0 (b–f)	0.1 (abc)
F-83	0	29 (i–o)	23 (i–n)	6.2 (i–m)	1.0 (a–f)	0.2 (c–g)
	200	29 (i–o)	22 (g–n)	5.5 (e–l)	1.0 (abcd)	0.2 (c–h)
	400	21 (bcde)	19 (e–k)	4.6 (a–f)	0.7 (a)	0.2 (b–f)
F-84	0	31 (m–r)	22 (h–n)	5.1 (b–k)	1.1 (c–g)	0.2 (d–j)
	200	28 (i–n)	18 (d–h)	5.0 (b–i)	1.1 (c–g)	0.2 (b–f)
	400	20 (bcd)	18 (d–h)	4.1 (abcd)	1.4 (g–o)	0.3 (h–n)
F-87	0	28 (i–n)	19 (e–j)	5.4 (d–l)	1.2 (c–h)	0.2 (d–j)
	200	32 (n–s)	22 (g–n)	6.0 (f–m)	1.2 (e–l)	0.3 (e–l)
	400	28 (h–n)	20 (f–l)	5.0 (b–k)	1.4 (g–n)	0.3 (e–j)
* C-1	0	22 (b–f)	10 (a)	6.0 (e–l)	1.3 (f–l)	0.2 (c–h)
	200	19 (bc)	12 (ab)	5.2 (b–l)	1.4 (g–n)	0.2 (b–f)
	400	25 (e–i)	18 (d–h)	4.0 (ab)	1.6 (m–r)	0.4 (mnop)
* C-2	0	24 (d–i)	14 (abcd)	6.5 (lm)	1.4 (g–n)	0.2 (c–h)
	200	19 (b)	15 (bcde)	5.4 (c–l)	1.4 (g–n)	0.2 (c–g)
	400	26 (e–k)	15 (bcde)	4.6 (a–f)	1.4 (g–o)	0.2 (c–h)
* C-3	0	23 (c–h)	17 (defg)	5.6 (e–l)	1.1 (c–g)	0.2 (abcd)
	200	22 (bcde)	18 (d–h)	4.6 (a–g)	1.0 (abc)	0.2 (c–g)
	400	19 (bc)	21 (f–m)	4.0 (abc)	1.2 (c–i)	0.3 (i–o)

Mean of 15 plants. Different letters within same column indicates significant difference between treatments using Duncan's multiple range rest ($p \leq 0.05$); * Mixed culture combinations.

3.6. Antioxidant Analysis

Bacterial inoculations recorded high proline accumulation in plants. At 200 mM NaCl, *S. arlettae* F-12, *B. safensis* F-83, and C-1 (mixed culture) recorded the highest accumulation of proline. Similarly,

at 400 mM salt stress, *S. arlettae* F-12 and *S. arlettae* F-71 resulted in considerable increases in proline concentrations over water-treated control plants (Figure 5a). Regarding peroxidases, plants produced a very low content in water-treated seeds. However, with increasing salinity (200–400 mM), considerable enhancements in peroxidase activity was observed (Figure 5b). Acid phosphatase production significantly increased at 200 mM NaCl with *Z. flava* F-9, *S. arlettae* F-12, and *S. jettensis* F-72. At 400 mM salt stress, *Z. flava* F-9, *S. jettensis* F-72, and *B. safensis* F-83 recorded up to twofold increases for acid phosphatase content over the control (Figure 5c).
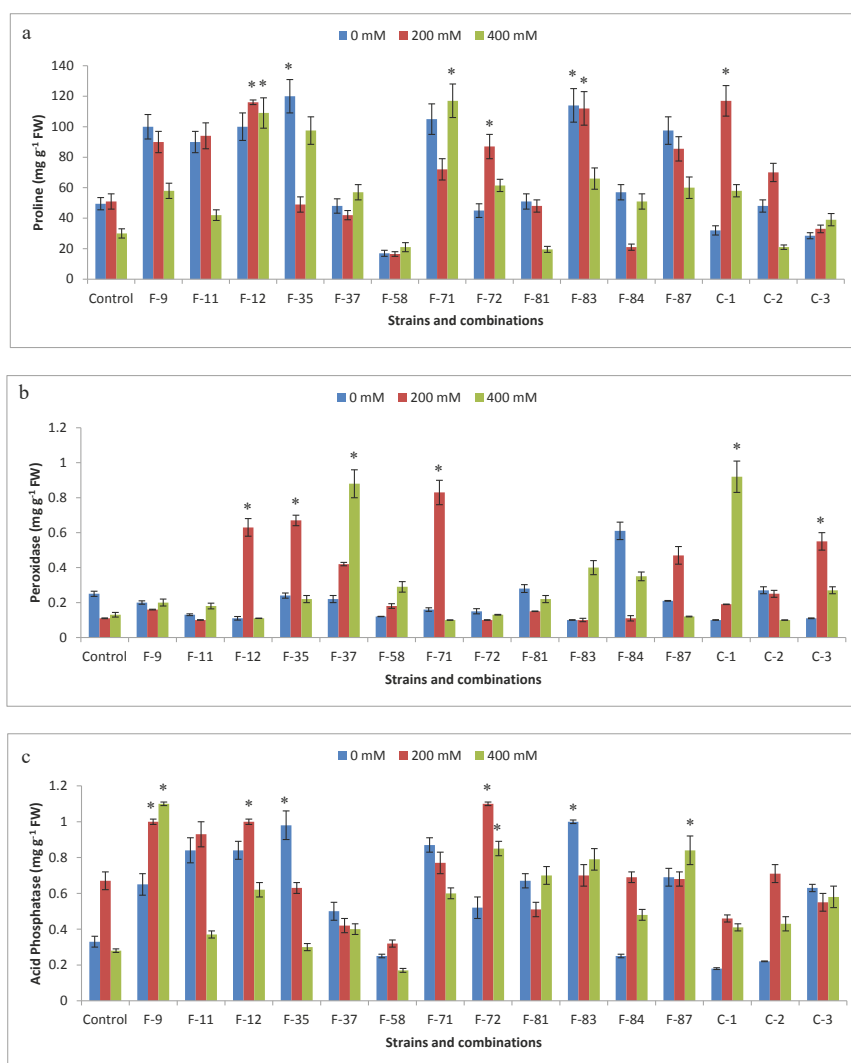
Figure 5. Effect of single and mixed bacterial inoculations and different salinity levels on the activities of antioxidant metabolites or enzymes: (**a**) Proline; (**b**) Peroxidase; (**c**) Acid phosphatase. Bars represent mean ± SE of three replicates. Treatments followed by * indicate significant difference over respective control using Duncan's multiple range test ($p \leq 0.05$).

4. Discussion

Soil salinity severely affects crop productivity and resident soil microbial communities. In Pakistan, soil salinity results in approximately 26% losses for the agroeconomy because it affects the germination, growth, and respiration of seeds [17]. The present study was carried out to examine the bacterial diversity of *S. fruticosa* growing in saline habitats of northeastern Pakistan. Extreme habitats have been reported to be colonized by a variety of microorganisms that are well adapted to these environments [29–31]. Natural plant settings growing in these localities may harbor beneficial microbes for agricultural applications under saline conditions [9]. *S. fruticosa* is a halophytic plant that mainly colonizes saline habitats. Therefore, we targeted the isolation of plant-growth-promoting rhizobacteria (PGPR) associated with the surfaces and rhizosphere of this plant. Analysis of 16S rRNA gene sequences confirmed the presence of several strains of halotolerant bacteria. Since little is known about the bacterial diversity associated with *S. fruticosa*, we therefore reported in detail the diversity and agricultural significance of bacterial communities colonizing the surfaces of this halophytic plant.

Bacterial strains showed good potential for salt tolerance and production of proline up to 1.5 M NaCl stress. Especially, *E. mexicanum* F-35 recorded a significant positive correlation ($r = 0.967$; $p = 0.05$) with increasing NaCl concentration in culture media. Inoculation of *Z. mays* by halotolerant rhizobacteria also influenced the production of antioxidant enzymes. For instance, plants grown under salinity stress exhibited the accumulation of proline as a compatible solute to retain water that otherwise might be lost due the surrounding hypertonic environment. Similarly, bacterial inoculations also influenced the peroxidase and acid phosphatase contents of plants. Peroxidases and acid phosphatases are considered very important enzymes to mitigate salinity-induced stresses. Exposure of plants to environmental stresses can lead to the production of reactive oxygen species (ROS) that can damage plant macromolecules. In addition to plants, microbes can augment the supply of enzymatic and nonenzymatic metabolites to detoxify the impact of ROS [32,33]. Previously, bioassays for the antioxidant system have also been reported with different plants and microorganisms [34,35].

In the present study, halotolerant rhizobacteria also showed good potential to produce a variety of plant-growth-promoting attributes. For example, strains recorded up to a 10-fold increase in auxin content in the presence of L-tryptophan over the unamended control. Under in vitro conditions, strains of genus *Staphylococcus* (F-12, F-71, F-72) were the most promising to enhance the rooting or shooting of inoculated maize plants. In pot trials, strains belonging to *Bacillus*, *Staphylococcus*, *Zhihengliuella*, and *Halomonas* genera were very effective at enhancing plant growth in salt-amended soils. However, strains of *S. arlettae* (F-12, F-71) recorded consistent results under both sets of experiments. Auxin-producing PGPR have been reported to improve plant growth under different sets of conditions. Charcoal-based formulations of rhizobacteria with auxin-production ability have been shown to enhance the vegetative and yield parameters of wheat [36]. Previously, we also reported the growth and yield improvement of plants grown under different salinity and water stresses by auxin-producing rhizobacteria [9,33]. Defez et al. [37] demonstrated the role of bacterial auxin in the upregulation of nitrogenase activity of inoculated rice plants. Inoculation of maize seeds with auxin-producing *B. megaterium* and *Azotobacter chroococcum* significantly enhanced the rooting and shooting compared to control seedlings [38]. Bacterial auxin has also been shown to alleviate the salt stress of plants grown under saline conditions. For instance, halotolerant bacteria from saline habitats stimulated plant growth by mitigating osmotic stress [9,39]. Similarly, salt-tolerant rhizobacteria exhibiting multiple plant-growth-promoting traits stimulated the growth of wheat in salt-affected soils [40]. In another study, strains of *Bacillus* facilitated the growth of maize under water-stress conditions [41].

High salinity levels in soil or in irrigation water are a major threat to the sustainability of agricultural production. The accumulation of salts in the vicinity of a plant's rhizosphere can cause severe injury to the root system. High concentrations of salts can result in the production of a gaseous hormone ethylene after seed germination. Elevated levels of ethylene can suppress the root system, which can result in compromised plant growth and development. Salt-tolerant rhizobacteria have the

ability to produce the enzyme ACC-deaminase, which can degrade the substrate (ACC) of ethylene into α-ketobutyrate and ammonia [13,32]. Hence, the ACC-deaminase activity of rhizobacteria mitigates deleterious levels of stress-induced ethylene. In the present study, highly significant ACC-deaminase activity was exhibited by *S. caprae* F-37, *S. arlettae* F-71, and *B. subtilis* F-14. PGPR with ACC-deaminase activity can protect inoculated plants from the deleterious effects of abiotic stressors. Orhan et al. [42] reported the salt-stress alleviation of wheat by halotolerant and halophilic PGPR that exhibited ACC-deaminase activity. Halotolerant *Klebsiella* with ACC-deaminase activity has also been shown to promote the vegetative growth parameters and chlorophyll content of plants under salt stress [43]. Similarly, Tank and Saraf [44] reported the positive effects of ACC-deaminase-containing bacteria on plant growth against salinity stress.

Biofilm formation is another important trait of PGPR that can help plants tolerate abiotic stress. It is a complex association of bacterial cells attached to the plant's root system and plays a very critical role in retaining moisture and protecting against different biotic or abiotic stresses. In this study, *S. caprae* F-37 and *B. pumilus* F-84 demonstrated good production of biofilm at 200 mM NaCl. The biofilm-forming ability of halotolerant rhizobacteria has been shown to enhance plant biomass under saline conditions [45]. Inoculation of barley plants with biofilm-forming *B. amyloliquefaciens* ameliorated salt stress and also stimulated different plant-growth parameters [46].

5. Conclusions

Finally, it can be concluded that *S. fruticosa* growing in saline areas harbor a range of beneficial plant-growth-promoting rhizobacteria. Bacterial strains showed a good ability to grow in up to 1.5 M NaCl. Halotolerant bacterial strains showed good auxin-production potential, ACC-deaminase activity, and biofilm formation. In pot trials, strains recorded good results for the amelioration of salt stress of maize plants. Especially, *S. jettensis* F-11, *S. arlettae* F-12, *B. marisflavi* F-87, *Z. flava* F-9, and *H. nanhaiensis* F-81 were shown to exhibit multiple plant-growth-promoting traits. In pot trials, strains were also very effective at mitigating the salinity stress of maize plants. For instance, *S. jettensis* F-11, F-12, *S. arlettae* F-71, *B. marisflavi* F-87, *H. nanhaiensis* F-81, *Z. flava* F-9, and *E. mexicanum* F-35 enhanced plant-growth parameters under salt stress. The data presented in this work are very encouraging for the use halotolerant rhizobacteria to enhance plant growth under salinity stress. The results of this study also indicated that the natural plant settings of a saline habitat are a good source for isolating beneficial PGPR to grow crops under saline conditions.

Author Contributions: F.A. conducted the lab experiments related to the isolation, identification of bacterial strains and pot trials. B.A. developed the experimental layout, performed statistical analysis and wrote this manuscript.

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Analysis of Stress Resistance Using Next Generation Techniques

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Abstract: Food security for a growing world population remains one of the most challenging tasks. Rapid climate change accelerates the loss of arable land used for crop production, while it simultaneously imposes increasing biotic and abiotic stresses on crop plants. Analysis and molecular understanding of the factors governing stress tolerance is in the focus of scientific and applied research. One plant is often mentioned in the context with stress resistance—*Chenopodium quinoa*. Through improved breeding strategies and the use of next generation approaches to study and understand quinoa's salinity tolerance, an important step towards securing food supply is taken.

Keywords: stress resistance; abiotic and biotic stresses; genetic diversity; *Chenopodium quinoa*; omics

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1. Introduction—Strategies to Improve Crop Yield

How to feed the world's population is still one of the most challenging questions. A United Nations report expects the current world population of 7.6 billion to reach 8.6 billion in 2030, 9.8 billion in 2050, and 11.2 billion in 2100 [1]. At the same time, the size of arable land is dramatically reducing. Erosion rates from ploughed fields are, on average, 10 to 100 times greater than rates of soil formation, with the result that the world has lost nearly a third of its farmable land to erosion or pollution in the last 40 years [2]. Crops are also exposed to different biotic and abiotic stresses: Biotic stresses include pathogen infection and herbivore attack [3], while abiotic stresses are environmental factors that compromise plants and reduce their productivity. These factors include extreme changes in temperature, water, nutrients, gases, wind, radiation and other environmental conditions. Rapid climate change also intensifies abiotic stresses and limits the time for a crop to adapt to new environmental conditions. For breeding programs, improvements in tolerance to drought, salinity and heat as well as the analysis of water economy in plants are most important [4]. Besides traditional breeding approaches, next generation techniques are also used to identify and study stress-tolerant plants. This includes "omics" approaches, which are very effective molecular methods to investigate biochemical, physiological and metabolic strategies of plants exposed to biotic and abiotic stresses. These include genomics (study of genome), transcriptomics (structural and functional analysis of coding and non-coding RNA), proteomics (protein and post-translational protein modification) and metabolomics (analysis of metabolites). Together, omics provide a powerful tool to identify the complex network of stress tolerance [5]. New insights promise to deliver new breeding targets for the stress adaptation of traditional crops by exploiting the allelic but also core- and pan-genomic reservoir. Finally, precision editing tools promise tailored adaptation of molecular circuits in future crop generations. In this article, influences of abiotic and biotic stresses are summarized, next generation analyses are introduced, and potential routes to increase food production are discussed.

2. Abiotic and Biotic Stresses and Responses

Both biotic and abiotic stresses affect crop plants and severely reduce their yield. As plants are sessile organisms, they are not able to escape these stresses but developed a range of strategies to adopt. Depending on the respective stress factors, these defense mechanisms can include morphological, biochemical and molecular modifications, such as altering certain signaling pathways, changes in cell wall structure, etc. A complete and in-depth understanding of these mechanisms is seen as important contributor for future breeding targets and for sustainable agriculture [3,6,7].

Biotic stress is triggered by interactions with other organisms like pests, parasites and pathogens, which are responsible for plant diseases [8]. To withstand, plants use different strategies such as passive barriers and active recognition systems. They produce chemical compounds against herbivores and pathogens, and use thickened cuticles and waxy layers as physical defense against intruders [9]. Plants have established an effective immune response to counteract biotic stress. By so-called pattern recognition receptors (PRRs), they can recognize microbial- or pathogen-associated molecular patterns (MAMPs or PAMPs) like flagellin, inducing PAMP-triggered immunity (PTI). Furthermore, plants possess disease resistance or R genes, which are encoding NB-LRR (nuclear binding—leucine rich repeat) proteins. These NB-LRR proteins recognize pathogen effectors and induce the effector-triggered immunity (ETI) [10]. PTI and ETI induce a first response against biotic stress which leads to an increase in cytoplasmic calcium concentration, the production of reactive oxygen species (ROS) and the activation of mitogen-activated protein kinases (MAPKs). Both PTI and ETI also induce several downstream signaling pathways in which phytohormones, mostly salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), play an essential role [6].

Abiotic stresses are caused by non-living factors that have an impact on growth conditions. Already in 1982, it was suggested that environmental factors limit crop production by 70%. A quarter century later, in 2007, it was reported that 96.5% of worldwide land area is influenced by abiotic stress [11]. Plants have developed different strategies to face these environmental changes. The stress responses can be both elastic (reversible) and plastic (irreversible), and are mostly very complex. Plant cells are able to sense environmental changes, which are subsequently reflected by specific changes in their gene expression, metabolism and physiology. Until today, only a few sensors have been identified, maybe due to functional redundancy in sensor protein encoding genes or to their essentiality, meaning mutations in these genes are lethal [3]. The phytohormone abscisic acid (ABA) seems to play a central role as endogenous messenger in abiotic stress responses. It was shown that especially under drought and salinity stress, increased ABA levels in combination with highly altered gene expression are detectable. In 2009, a small protein family, which is able to bind ABA, was identified as ABA receptors. These findings initiated the analysis of ABA pathways and ABA-induced gene transcription. Furthermore, the understanding of stomatal closure regulation due to ABA signals, which are controlling ion channels in guard cells, was improved [12].

In addition, abiotic stresses have an effect on the occurrence and spread of biotic stressors. Alterations of environmental conditions also directly influence pest-plant interactions by affecting physiological and defense responses. Apparently, the combination of stress factors is more harmful, but not always additive. Plants are able to pyramid responses to combined stress factors. The identification and development of crops with enhanced stress tolerance to combined biotic and abiotic stresses is in the focus of research [13].

3. Drought and Salinity—The most Affecting Abiotic Stresses

During the next decades, climate change will impose increased abiotic stresses—mainly drought, heat, and salinity. It is expected, that drought will be most influential on crop productivity, as by the end of the twentieth century, 30% of land will be extremely dry [4]. Salinity rises constantly since irrigation with brackish water increases the worldwide area of salt-damaged arable land. Every minute, three hectares of land become unusable for crop production [14,15].

More than 50 years ago, plant drought responses were grouped in three categories—drought escape, drought avoidance and drought tolerance. Plants often combine these strategies and their viability depends on how effective the composition of these changes is [4]. Drought escape is an adaptive mechanism including faster development to complete the plant's life cycle before the drought period starts, such as through early flowering. Drought avoidance describes better water uptake due to deeper roots and decreased water evaporation through thicker waxy layers. Drought tolerance is induced after stress occurrence and enables the plant to grow under water deficiency due to biochemical changes. The decrease in osmotic potential by osmolyte accumulation is defined as osmotic adjustment (OA), a typical physiological mechanism against dehydration [4,16,17]. Typical osmolytes, also often called osmoprotectants, are betaine, proline and fructans. These substances do not take part in biochemical responses but influence the osmotic behavior of cells. The accumulation of osmolytes affects gene expression in order to regulate the production of relevant enzymes [18].

In land plants, different strategies evolved to handle high salt (NaCl) concentrations in the soil. Non-salt tolerant glycophytes actively transport salt from the roots back into the environment. This is only effective when facing low salt concentrations. Only about 2% of all plant species are halophytes with high salt tolerance. Halophytes can tolerate NaCl concentrations comparable to seawater and developed two strategies to cope with increased salt concentrations. Succulent halophytes have large internal vacuoles in which they store sodium (Na^+) in order to protect the core plant from toxic salt loads. Another possibility to exclude NaCl from sensitive tissues is the ability to sequester large quantities of salt to so-called epidermal bladder cells (EBCs), which are present in 50% of all halophytes. The diameter of EBCs is about 10-times larger than normal epidermal cells resulting in a 1000-times larger storage volume for Na^+ compared to vacuoles of normal leaf cells [15].

4. Sequence Diversity in Crops—Searching for Tolerant Plants

Structural gene variants like presence/absence variants (PAVs) and copy number variants (CNVs) are contributing to the diversity genepool [19]. Often different crop varieties are adopted and optimized for growth in different habitats. The optimal development of these ecotypes is influenced by their allelic diversity, which is reduced in elite cultivars as a consequence of intense breeding and selection for particular characteristics often ignoring others [20]. Consequently, the complete gene and allele pool diversity cannot be captured by an individual variant, but requires the analysis of a broader set of cultivars. To include all existing genes, contribution to phenotypic and agronomic trait diversity, the construction and analysis of pan-genomes is necessary. This pan-genome contains the complete gene set, including the core-genome, in which all genes are present in all members of a species, and variable genes, which occur only in some variants [21]. Since one reference genome represents only one variety, increasing awareness is put towards the fact that a range of genomes need to be sequenced completely to generate high resolution pan-genomes. For a number of species, including wheat, maize, rice, soybean and cabbage, pan-genomes have been analyzed. The analysis of the cabbage pan-genome, for example, revealed that 20% of genes are affected by presence/absence variation. Some of these were related to important agronomical factors like stress resistance, flowering time and vitamin biosynthesis [19,20].

The genomes of many traditional crops, such as tomato, barley, wheat, sorghum and wild emmer, were sequenced in the last years [20,22–26]. Several consortiums are working on the assembly of additional genomes to gain insights into the pan-genomes of all important crops. The allelic diversity in the gene pool will aid in analyzing different stress resistances in detail. However, also non-traditional crops with high stress tolerances need to be sequenced to study their (pan-) genomes, in order to understand their particular molecular mechanisms to eventually learn and profit for adjusted breeding goals and solutions. Quinoa (*Chenopodium quinoa*), a plant reputed for its high salinity tolerance, has yet a relatively unimportant role compared to traditional crops. Quinoa has the potential to serve as model plant for stress resistance. Besides the recently published reference genome, a variety of quinoa ecotypes exist that seem to grow under nearly all climate conditions.

5. *Chenopodium quinoa*—A Salinity Tolerant Crop

A halophyte plant often used to study salt tolerance is *Chenopodium quinoa*. Quinoa is a highly nutritious crop and is supposed to have been domesticated more than 7000 years ago by pre-Columbian cultures in the Andean region. It was called "mother grain" during the Incan Empire. It is a pseudocereal crop of the family *Amaranthaceae*, also including the important economic plants *Beta vulgaris* (sugar beet), *Spinacia oleracea* (spinach) and *Amaranthus hypochondriacus* (amaranth) [15,27]. Quinoa has become a plant of interest: It is called a "superfood" as its seeds contain a high amount of essential amino acids and vitamins but no gluten. This makes quinoa an alternative to replace wheat-based products in cases of celiac disease. The seeds are rich in several minerals and, in comparison to other grains, have an excellent ratio of proteins, lipids, fiber and carbohydrates. Because of these characteristics, the United Nations Food and Agricultural Organization (FAO) declared 2013 as the "International Year of Quinoa", an award which plants only received three times. In its origin, the Andean region, quinoa is used to grow in several harsh environmental conditions. It adapted to the salty coast as well as to the highlands 3500 m above sea level, with extreme differences in abiotic factors like temperature, precipitation and salt concentrations. Due to this broad adaptation combined with the nutritious characteristics, the number of quinoa-growing countries has increased 10-fold during the last 30 years [28–30].

6. Omic Approaches Using Quinoa

C. quinoa has not only reached public attention as food of the future but also its salinity tolerance is in the focus of several research groups. Next to traditional growth experiments, in order to decipher the salt concentration tolerated by quinoa, the studies were supported by omic approaches, namely genomics, transcriptomics, proteomics and metabolomics.

The quinoa genome was published recently [28,29] and the evolution of quinoa and its salt tolerance were studied. The analysis of ABA-related genes showed that the key factors of ABA biosynthesis, transport and perception were expanded in the quinoa genome, contributing to salinity tolerance [28,29]. In further studies, these genomes were used for resequencing approaches [31,32]. One example is the detection of genomic variations, like single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels), to distinguish among different ecotypes which can be used in further breeding programs [31].

For transcriptomics, the gold standard approach is RNA sequencing (RNAseq). This method is applied in several approaches, e.g., to identify differentially expressed genes (DEGs) between different conditions, tissues or ecotypes. Several studies on salt tolerance and its sequestration in EBCs were performed [28,33,34]. In combination with the quinoa reference genome, Zou and colleagues analyzed the transcriptome of EBCs and leaves under salt-treated and non-treated conditions. Out of totally 54,438 protein-coding genes present in the quinoa genome, they identified 8148 DEGs between bladder and leaf cells. In EBCs, genes involved in abiotic stress response and cell wall synthesis were upregulated, while those related to photosynthesis were downregulated. These findings underlined the functions of bladders being cells that sequester salt but are inactive in anabolic functions. Furthermore, several ion transporters were found to be upregulated in EBCs which seem to be involved in salt secretion [28]. Another study analyzed RNAseq data from several closely related *Chenopodium* species (*C. quinoa*, *Chenopodium berlandieri*, *Chenopodium hircinum*) differing in tolerated salt concentration. Investigating DEGs between the species, the group identified 15 genes encoding for putative transmembrane proteins that potentially contribute to a higher salinity tolerance [27].

Several analyses of quinoa seeds using proteomics approaches were performed. Aloisi and colleagues investigated the changes in the amino acid and protein profiles of seeds from several quinoa ecotypes grown under salt-treatment. They were able to show that salinity influences proteins which belong to functional categories like stress-protein, metabolism and storage [35]. Another group analyzed 16 grains from different crops, including quinoa. The comparison of the different proteomes showed that over 90% of detected proteins from extensively studied cereals like wheat, barley, maize

and rice are registered in the Uniprot database. In the case of the quinoa proteome, only 3% of detectable proteins showed an entry in this database. Thus, quinoa opens a so far largely unexplored territory also with respect to the proteome and protein composition of the seeds [36].

Since plants contain the largest metabolome of all life forms, metabolomics is an important part of the omic approaches. Under normal conditions and especially under stress conditions, plants produce a high and diverse amount of primary and secondary metabolites. While primary metabolites directly regulate growth, development and reproduction, secondary metabolites have other important ecological functions like protecting the plant from stresses. In quinoa and other halophytes, metabolites contributing to salinity tolerance were studied [37]. More than half of all metabolites were significantly affected by salinity—e.g., the osmoprotectant proline was about 17-fold increased. As a next experimental step, quinoas EBCs were mechanically removed prior to the salt treatment resulting in the loss of plant's salt tolerance. This procedure dramatically altered the metabolite composition demonstrating that EBCs also serve as metabolite storage [33].

7. Conclusions and Perspectives

While the world population is massively growing, farmers will increasingly face harsh environmental conditions affecting agricultural productivity, accelerated by the rapid climate change. Biotic and abiotic stresses are associated with crop loss and are in the focus of active research and breeding programs. The analysis of plant's stress response revealed that phytohormones play a key role as messengers in downstream signaling pathways [6,38,39]. A lot of studies were performed to unravel all details of hormone production, transport and its characteristics. Their impact in stress response is still not completely understood but further discoveries will reveal new possibilities to increase tolerance of traditional crops. Available data on completely sequenced plant and crop genomes increase continuously, and pan-genomes of crops become available for the detection of allelic variants, stress-associated alleles and tolerant phenotypes.

As about 22% of worldwide agricultural land is saline, tolerant plants and detailed knowledge about the ability to grow efficiently in salt-contaminated environments are urgently needed. The investigation of halophytes has advanced the knowledge about salinity tolerance as these plants are able to grow even when watered with seawater. In the recent years, the halophyte *C. quinoa* was studied intensively by the use of next generation omic approaches. New insights in the function of quinoa's EBCs were gained, also demonstrating the storage capacity for metabolites in EBCs and a model of how ions are transported into these salt dumpers. These findings and further studies will help to understand the molecular mechanisms of salt tolerance and the engineering of salt-tolerant crops. Additional genome sequences of quinoa varieties and close relatives will trigger insights into the pan-genome. Combining the study and understanding of stress resistance, targeted breeding, potential application and engineering in other crops as well as the use of tolerant ecotypes in areas useless for traditional crop production, a step towards securing food supply will be undertaken.

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Improving Flooding Tolerance of Crop Plants

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Abstract: A major problem of climate change is the increasing duration and frequency of heavy rainfall events. This leads to soil flooding that negatively affects plant growth, eventually leading to death of plants if the flooding persists for several days. Most crop plants are very sensitive to flooding, and dramatic yield losses occur due to flooding each year. This review summarizes recent progress and approaches to enhance crop resistance to flooding. Most experiments have been done on maize, barley, and soybean. Work on other crops such as wheat and rape has only started. The most promising traits that might enhance crop flooding tolerance are anatomical adaptations such as aerenchyma formation, the formation of a barrier against radial oxygen loss, and the growth of adventitious roots. Metabolic adaptations might be able to improve waterlogging tolerance as well, but more studies are needed in this direction. Reasonable approaches for future studies are quantitative trait locus (QTL) analyses or genome-wide association (GWA) studies in combination with specific tolerance traits that can be easily assessed. The usage of flooding-tolerant relatives or ancestral cultivars of the crop of interest in these experiments might enhance the chances of finding useful tolerance traits to be used in breeding.

Keywords: hypoxia; waterlogging; submergence; flooding; maize; soybean; barley; aerenchyma

1. Introduction

In times of changing climate, agriculture faces increasing problems with extreme weather events leading to considerable yield losses. In combination with a growing population and higher food demand, this presents a challenge to scientists and breeders to maintain the current food supply. Certainly, more effort is required to develop stress-resistant crops and improve agricultural practices in order to cope with these problems.

Plants are, due to their sessile nature, exposed to all changes in abiotic and biotic factors occurring at their habitat. Water availability, for example, is always problematic and can change from periods of drought to periods of flooding after a heavy rainfall. Plants can adapt to changing environmental conditions, but this comes at the cost of reduced growth and reproduction. If stress duration or severity exceeds the plant's ability to adapt, it will eventually die.

Most crop plants are rather sensitive to stresses since they were selected for high yield. In order to improve crop plant resistance to stresses, and to improve their productivity and survival, two research strategies are required. First, mechanisms of tolerance against stresses have to be understood. This requires analyses at the molecular level (transcriptomics, proteomics, metabolomics) in order not only to get to know adaptive mechanisms but also to understand their activation and regulation. The best approach for these analyses is the usage of a stress-resistant plant species closely related or even ancestral to the crop of interest, that potentially has lost some adaptational responses.

Second, due to the long history of breeding all over the world, a huge number of cultivars has become available for many crop plants. These breeding processes often focused on high yield and food quality, concomitant with a loss of genetic diversity and stress resistance. However, older cultivars with low productivity might still contain tolerance loci for a certain stress condition that could be

transferred to modern, highly productive cultivars. Strategies to achieve this goal are (1) screening of a wide range of cultivars under a specific stress condition; (2) selection of cultivars with low and high resistance; (3) understanding the physiological basis for resistance, i.e., the tolerance trait; and (4) the genetic analysis of those cultivars by quantitative trait locus (QTL) analysis and other molecular methods in order to find the genetic locus that underlies the tolerance trait. If a genetic locus has been discovered and characterized, it subsequently can be transferred into modern varieties using marker-assisted breeding technology to achieve stress-tolerant cultivars.

In this review, the current progress in crop resistance to flooding will be presented. First, overall plant responses and survival strategies under flooding conditions will be summarized. Then, breeding approaches in different temperate crops will be presented. The focus of this article will be on temperate plants, rather than rice and other tropical crops like cotton or sorghum. Flooding research on rice and its flooding-tolerance traits has been reviewed and discussed in several publications previously [1–5].

2. Plant Responses to Flooding

Besides the low availability of water leading to drought stress, plants can also be affected by too much water. Flooding primarily restricts gas diffusion between the plant and its surroundings due to physical properties (e.g., [6–8]). Oxygen as well as CO_2 cannot be easily exchanged via stomata and cell walls under water. This leads to a lack of oxygen inside flooded plant parts, and mainly limits heterotrophic energy production in mitochondria. Furthermore, low CO_2 availability in flooded leaves restricts photosynthesis. Therefore, flooding causes an energy crisis within plant cells.

Flooding events can be classified by two versions, (1) waterlogging, where only the root system inside the soil is affected; and (2) submergence, where also parts or the whole shoot are under water [9]. In flooded plant parts without ongoing photosynthesis, the oxygen concentration quickly declines and leads to hypoxic conditions (e.g., [10,11]).

Several plant species have developed mechanisms to cope with flooding stress, which enable them even to grow and reproduce in wet soil or under water. But also non-wetland plants can survive flooding, at least for a short period of time. Survival strategies can be divided into two major forms, (1) avoidance of oxygen deficiency within plant tissues; and (2) adaptation to oxygen deficiency. These strategies are described below.

2.1. Avoidance of Oxygen Deficiency by Morphological Modifications

The first strategy, the avoidance of oxygen deficiency inside the flooded plant parts, mainly involves anatomical and morphological modifications that improve gas exchange with the surroundings [8,12]. These modifications are largely mediated by the gaseous plant hormone ethylene that naturally accumulates in flooded plant parts [13].

One of the most prominent modifications is the increase of intercellular gas spaces, the so-called aerenchyma formation, to improve gas transport and distribution inside submerged plant tissues. Aerenchyma can develop in the root cortex as well as in stems and leaves. They are inducible by flooding conditions in several non-wetland species (e.g., wheat, maize [14,15]), or constitutive in many wetland species (e.g., rice, *Zea nicaraguensis* H.H.Iltis and B.F. Benz 2000 [12,15]). The formation of shoot-born adventitious roots has been observed in some plant species under water, for example in rice [16,17] and *Solanum dulcamara* L. [18,19]. Those roots also contain large aerenchyma. In order to restrict gas loss through the root surface, many roots of wetland plants also develop a barrier against radial oxygen loss (ROL) surrounding the aerenchyma-containing tissue (e.g., rice [20,21]; *Z. nicaraguensis* [12,22]).

When the whole plant is under water, some plants have developed the ability to move their leaves up in order to reach the water surface and to restore contact to air. This is achieved by hyponastic growth, meaning the change of the leaf angle to a more upright position, which can be observed in non-wetland (e.g., *Arabidopsis thaliana* (L.) Heynh. [10]) as well as in wetland plants species (e.g., *Rumex palustris* Sm. [23]). Some plant species can go one step further and enhance the shoot growth under

water to get their leaves out of the water. This so-called "escape strategy" can be achieved either by growth acceleration in petioles (e.g., *Rumex palustris* Sm. [24,25]; *Ranunculus sceleratus* L. [26]), or by enhanced growth of stems (e.g., rice [27,28]).

2.2. Adaptation to Oxygen Deficiency by Metabolic Modifications

If a plant species is not able to induce morphological modifications, or if water levels are too high to be outgrown, they have to cope with restricted gas exchange, mainly with low-oxygen concentrations. This adaptation involves metabolic modifications (summarized in [7,29]). A first response of plant cells under oxygen deficiency is the induction of fermentation. Since the mitochondrial ATP production is limited by oxygen availability, plants are dependent on glycolytic ATP production. During glycolysis, NADH accumulates and needs to be re-oxidized to NAD in order to maintain the glycolytic process. This is done by lactic acid fermentation, but mainly by ethanolic fermentation via alcohol dehydrogenase and pyruvate decarboxylase.

The higher transcription of genes encoding fermentative enzymes under oxygen deficiency is largely regulated by a group of oxygen-labile transcription factors, group VII of the ethylene-response factor family (groupVII-ERFs). The Arabidopsis groupVII-ERFs AtRAP2.2, AtRAP2.12, and AtRAP2.3 are constitutively expressed, but the proteins are degraded under normoxia by the Arg-branch of the N-end rule pathway. Under hypoxia, they can accumulate and act as transcriptional activators for example for genes encoding fermentative enzymes, but also for other metabolic and regulatory proteins [30–32].

While all plant species analyzed so far are able to induce fermentative enzymes under oxygen deficiency, the availability of carbohydrates and the efficiency to cope with lower energy production (2 Mol ATP per Mol glucose in glycolysis versus 30–36 Mol ATP per Mol glucose in mitochondrial respiration) restrict plant productivity and survival. Sensitive plant species often die from energy deficiency due to exhaustion of fermentable substrates, before the flooding period ends.

However, certain plant species and organs can consume large amounts of carbohydrates from sources such as starch that are otherwise difficult to access under oxygen deficiency. This is achieved by specific amylases and manipulation of the regulatory pathways (e.g., rice coleoptiles [33,34]; *Potamogeton pectinatus* (L.) Böerner tubers [35]). This high carbohydrate availability enables the strong elongation growth in plants exerting the "escape strategy". Other species, among them certain cultivars of rice, restrict metabolism and growth under water and apply the so-called "quiescence strategy", which enables them to survive for longer times with restricted carbohydrate supply. Some plant species might also make use of alternative energy pathways (e.g., the utilization of pyrophosphate instead of ATP for phosphorylation), but this has still not been fully explored [36–38].

2.3. Tolerance Traits for Flooding Survival and Their Usage in Breeding

The overview presented above of adaptational mechanisms employed by plants in response to flooding conditions makes it obvious that there is not only one mechanism or trait of tolerance in a tolerant plant. Hence, the contribution of individual metabolic pathways and morphological modifications to overall flooding tolerance has to be deciphered. This knowledge should enable scientists to focus on specific adaptational mechanisms, and to discover the underlying genetic basis for tolerance traits, which subsequently can be used for breeders in order to improve a crop's tolerance to flooding.

The most prominent example of successful agronomical application of knowledge on a flooding-tolerance trait and its transfer to crops comes from rice. Although this crop is naturally flooding-tolerant, most rice cultivars cannot survive more than one week of complete submergence [39]. Cultivars with the "quiescence strategy" can survive deep floods for up to 14 days, by restricting growth and carbohydrate consumption. On the other hand, deepwater-rice can outgrow a flood within a short time, using the "escape strategy", and thus restores contact to air, enabling long-term survival [27,28]. For both traits, QTL analyses and subsequent molecular investigations have revealed

the underlying genes. In both cases transcription factors related to groupVII-ERFs have been made responsible for either restriction of growth under water (SUB1A-1 [39,40]), or for enhanced growth (SNORKEL1/2 [28]). However, only the first genetic trait, the ability to induce quiescence, has been successfully used for crop improvement [5,41], because the second strategy has negative effects on crop stability once the floods recede.

In the next sections, we discuss what progress has been made in improving the flooding tolerance of major temperate crops, and what strategies are currently being applied in research. Among temperate crops, several species have been used for tolerance screenings and QTL analyses. Among the cereals, maize, wheat, and barley are well studied, and some data exist on the pasture grass *Lolium perenne* L. Hardly any data exist on other cereals such as oat and rye (Table 1). Among dicot plants, soybean and rape have been used in several studies, while others like potato or sugar beet have been seldom analyzed. Here, the model species *Arabidopsis thaliana* (L.) Heynh. is the best studied dicot plant with a wealth of expression and metabolic data, and an extended analysis comparing ecotype performances under submergence [11]. However, even here, the underlying mechanisms and genes responsible for tolerance are only now starting to emerge [42–44].

Table 1. Overview over crop species referred to in this review article.

Species	Cultivar Differences in Flooding Tolerance	Quantitative Trait Loci (QTL) Associated with Flooding Tolerance	Genome Sequence	-Omics Data on Flooding/Low-Oxygen Response
Monocots				
Zea mays	yes	yes (Table 2)	[45,46]	available
Triticum aestivum	yes	yes	[47]	not available
Hordeum vulgare	yes	yes (Table 3)	[48]	not available
Avena sativa	unknown	no	not available	not available
Secale cereale	unknown	no	[49]	not available
Lolium perenne	yes	yes	[50]	not available
Dicots				
Glycine max	yes	yes (Table 4)	[51]	available
Brassica napus	yes	yes	[52]	available
Helianthus annuus	unknown	no	[53]	not available
Beta vulgaris	unknown	no	[54]	not available
Solanum tuberosum	unknown	no	[55]	not available

3. Waterlogging Tolerance of Maize and Teosinte

The field crop maize (*Zea mays* L.) is not only a major human food source, but can also be used for animal feed as well as bioethanol production. However, it is relatively flooding-sensitive. Interestingly, maize has several close relatives with higher flooding tolerance, among them the teosinte species *Z. nicaraguensis*, *Z. luxurians*, and *Z. mays* ssp. *huehuetenangensis*. These species have been employed in the past as a basis to improve flooding tolerance of maize, similarly as a huge panel of maize cultivars, as described in detail below.

3.1. Morphological Adaptations of Teosinte as Tolerance Traits

The flooding tolerance of teosinte species has been largely associated with morphological modifications, namely the growth of adventitious roots, the formation of aerenchyma under non-flooded conditions as well as the establishment of a barrier against ROL [22,56,57]. Even under non-waterlogged conditions, *Z. nicaraguensis* and *Z. luxurians* are able to form large aerenchyma in the root cortex. These mechanisms improve aeration of flooded roots and thus enhance waterlogging tolerance [22,56,58]. Multiple QTL analyses revealed several loci associated with the constitutive aerenchyma formation ([59–62], summarized in [63], see also Table 2). Similar studies have been done

with another maize relative, *Z. luxurians* [64]. However, the gene(s) that are responsible for constitutive aerenchyma formation have not yet been identified.

Table 2. Overview over promising QTLs to improve flooding tolerance of maize (*Zea mays* L.).

Crossed Cultivars	Treatment	Trait for Tolerance	QTL	Position (Chr, cM)	Reference
Leaf traits					
Z. mays cv. F1649 × cv. H84	14 days waterlogging with starch solution	Leaf chlorosis	1.03-4	Chr 1	[68]
Z. nicaraguensis CIMMYT 13,451 × *Z. mays* Mi29	16 days waterlogging with starch solution	Leaf chlorosis	Qft-rd4.07-4.11	Chr 4	[69]
Z. mays cv. Mo18W × B73	48 h submergence	Leaf senescence	Subtol6	Chr 6 (162 Mb)	[70]
Adventitious root formation					
Z. mays ssp. *huehuetenangensis* × *Z. mays* B64	14 days waterlogging	Adventitious root formation	Qarf8.05 Qarf8.03 Qarf5.03	Chr 8 Chr 8 Chr 5	[66]
Z. mays cv. Na4 × B64	14 days waterlogging	Adventitious root formation	Qarf8.05	Chr 8	[71]
Z. mays ssp. *huehuetenangensis* × *Z. mays* Mi29	14 days waterlogging	Adventitious root formation	Qarf8.05 Qarf5.03	Chr 8 Chr 5	[67]
Z. nicaraguensis CIMMYT 13,451 × *Z. mays* Mi29	14 days waterlogging	Adventitious root formation	Qarf3.04 Qarf8.03	Chr 3 Chr 8	[67]
Constitutive aerenchyma formation					
Z. nicaraguensis CIMMYT 13,451 × *Z. mays* B64	none	Constitutive aerenchyma formation	Qaer1.07 Qaer1.02-3 Qaer5.09 Qaer8.06-7	Chr 1, 144 Chr 1, 35 Chr 5, 138 Chr 8, 97-101	[59]
Z. nicaraguensis CIMMYT 13,451 × *Z. mays* Mi29	none	Constitutive aerenchyma formation	Qaer1.06 Qaer1.11 Qaer5.09n	Chr 1 Chr 1 Chr 5	[60]
Z. nicaraguensis CIMMYT 13,451 × *Z. mays* Mi29	none	Constitutive aerenchyma formation	Qaer1.05-6 Qaer8.05	Chr 1, 45 Chr 8, 0	[61]
Z. luxurians × *Z. mays* B73	none	Constitutive aerenchyma formation	Qaer2.06 Qaer5.05-6	Chr 2, 88 Chr 5, 96	[64]
Formation of a barrier against radial oxygen loss (ROL)					
Z. nicaraguensis CIMMYT 13,451 × *Z. mays* Mi29	14 days stagnant nutrient solution	ROL formation		Chr 3	[65]

The formation of a barrier against ROL has been observed in *Z. nicaraguensis* under stagnant conditions (i.e., hypoxic nutrient solution), but not in *Z. mays* [22]. Even though maize can form aerenchyma under waterlogging, and therefore transport oxygen-rich air into the roots, root tips usually remain hypoxic. This is due to leaking of oxygen to the outside medium along the whole root. This radial oxygen loss is prevented in teosinte species by a tight barrier in the outer root layers. A locus on chromosome 3 of teosinte was involved in this formation and was sufficient to facilitate barrier formation in maize [65], but more studies need to be done to reveal the responsible gene.

Another close relative, *Z. mays* ssp. *huehuetenangensis*, has a larger potential to form adventitious roots than *Z. mays* cultivars [66,67] (see also Table 2). A QTL analysis suggested loci on chromosomes 4, 5, and 8, but the underlying gene(s) have not been identified yet. Also in *Z. nicaraguensis*, QTLs associated with adventitious root formation have been discovered on chromosomes 3 and 8 [67].

These studies suggest that there is a potential to improve maize flooding tolerance by manipulating its anatomical and morphological responses. It is now important to transfer more

of these traits from teosinte into elite maize cultivars in order to improve their flooding tolerance, without negatively affecting yield and food quality. However, it remains to be elucidated, which and how many genes are required for this approach.

3.2. QTL Analyses of Maize Cultivars with Contrasting Flooding Tolerance

Several studies have compared waterlogging or submergence tolerance of different maize cultivars, for example of Chinese origin [72,73], tropic cultivars [74–76], or of a wide range of lines [70]. Thereby, analyses have either focused on metabolic changes, or on anatomical differences associated with tolerance.

Many of these experiments have been complemented with subsequent QTL analyses. For example, Mano et al. [71] determined QTLs associated with adventitious root formation between cultivars B64 and Na4 on chromosomes 3, 7, and 8, the latter potentially linked to a locus identified previously during a species comparison between maize and teosinte ([66], Table 2). Another QTL on chromosome 1 from the tolerant inbred line F1649 compared to sensitive H84 was associated with waterlogging tolerance under reducing conditions that often occur in flooded soils [68]. A cross between the tolerant cultivar HZ32 and the intolerant cultivar K12 [72] identified several gene loci associated with waterlogging tolerance [77–79]. Another cross of the tolerant HZ32 with sensitive Mo17 suggested the gene Cyp51 (of the Cytochrome P450 family) as a potential hypoxia tolerance gene [80]. Among tropical cultivars, tolerant CAWL-46-3-1 was compared to the sensitive line CML311-2-1-3, which again revealed several QTLs [81]. All of these QTLs await further characterization and a link to specific tolerance traits.

Growing knowledge of the molecular response of maize and teosinte to flooding might help during the identification of responsible genes for sensitivity and tolerance traits. The transcriptomic response of maize has been studied under several circumstances and with different goals. Laser microdissection in combination with microarray analysis has been used on root sections to study aerenchyma formation mechanisms [82,83], which could be used in future to improve elite cultivars, in combination with QTL analyses and the comparisons to teosinte as described above.

A comparative study of four maize cultivars with contrasting tolerance (Mo18W & M162W as tolerant and B97 & B73 as sensitive lines) analyzed the transcriptional response to submergence [70]. A major QTL on chromosome 6 was associated with submergence tolerance, Subtol6, but the underlying gene remains to be characterized. Another RNAseq analysis of several tropical maize lines with contrasting waterlogging tolerance also revealed many candidate genes that might be associated with flooding tolerance [76], among them one gene, GRMZM2G055704, that lies in a region on chromosome 1 that had been previously identified by other screens [78].

A comparison of the tolerant maize cultivar HKI1105 and the sensitive cultivar V372 under waterlogging observed the differential expression of many genes [84]. Subsequently, Arora et al. [85] studied the root transcriptional response under waterlogging of this tolerant cultivar and found metabolism-associated genes as well as genes related to aerenchyma formation that could be important for tolerance. Especially cell-wall-related genes could be important for maize tolerance [86], which is again associated with anatomical properties rather than with metabolic adaptations. Another set of cultivars, sensitive Mo17 and tolerant Hz32, was also studied at the transcript level [87–89]. In these experiments, many differentially expressed genes were observed, yet no gene was clearly associable with tolerance.

In summary, so far no gene or gene variant has been verified to be important for maize waterlogging tolerance. The complex regulatory network and multiple responses in morphology as well as metabolism make it unlikely to find the one gene that determines tolerance. Recent progress in sequencing technologies might help to speed up this process. Rather than laborious crosses between two genotypes with contrasting tolerance and subsequent screening of the progeny, information of many genotypes can be included into one analysis. For such genome-wide association studies (GWAS), tolerance traits are correlated with single nucleotid polymorphisms (SNPs). This approach requires knowledge on the genomic sequence of the species (for an overview, see Table 1) as well

as genotype-specific sequence information. This technique is now also used in order to improve waterlogging tolerance of maize [76,90].

4. Waterlogging Tolerance of Barley and Other Hordeum Species

Another major cereal for human food production is barley (*Hordeum vulgare* L.), used for brewery and animal feed. Barley is more flooding-sensitive than other cereals [91]. As maize, barley has flooding-tolerant relatives such as *H. marinum* and *H. spontaneum*, which have been utilized in studying flooding-tolerance mechanisms.

Several screens for waterlogging tolerance have been performed, for example with large cultivar collections [92,93] at the germination stage. However, smaller screens at later developmental stages might be more effective and practicable [91,94]. In such screens, scientists analyzed selections of Chinese cultivars [95,96], Australian cultivars [91], Nordic cultivars [97], or selections from bigger collections [98]. Thereby, screening methods and parameters observed differed considerably, leading to very different results.

Subsequent QTL analyses have been performed, but in many cases the association with a specific tolerance trait is still missing, making it hard to functionally study them. From the Nordic cultivars, several major QTLs were revealed by different crosses [99], but the underlying mechanisms or genes have not yet been identified. After the screening of the Chinese cultivars [95,96,100], several crosses were performed to do QTL analyses for tolerance traits. Crosses were done between tolerant TX9425 and sensitive Franklin [94], or with sensitive Naso Nijo [96]. Another QTL analysis was done between tolerant Yerong and sensitive Franklin [94,101,102]. Next, the tolerant line YYXT was used for a cross with the cultivar Franklin [103]. These studies revealed several major and minor QTLs that might be used in breeding (for an overview, see Table 3). Three examples that went further and focused on specific tolerance traits are described below.

Table 3. Promising QTLs from barley (*Hordeum vulgare*) and related species associated with waterlogging tolerance.

Tolerant Cultivar	Treatment	Parameters Analyzed	Name of QTL	Location of QTL (Chr, cM)	References
		Hordeum vulgare **L.**			
cv. Yerong	9 weeks waterlogging	Survival rate	QWL.YeFr.4H QWL.YeFr.2H.2	4H, 108–117 2H, 113–118	[101]
cv. Yerong	7 days waterlogging	Aerenchyma formation		4H, 80.95–99.08	[104]
cv. YYXT	9 weeks waterlogging	Survival rate	QWl.YyFr.2H QWl.YyFr.3H QWl.YyFr.4H QWl.YyFr.6H	2H, 76.1 3H, 5.2 4H, 121.1 6H, 78.4	[103]
cv. YYXT	21 days stagnant solution	Root porosity		4H, 116	[105]
cv. Psaknon	18 days waterlogging	Chlorophyll fluorescence (ΦPSII)	QY1 QY2	6H, 114 7H, 59	[99]
cv. TX9425	2 days stagnant	Root membrane potential	QMP.TxNn.2H	2H, 8.85	[106]
		H. spontaneum			
cv. TAM407227	7 days waterlogging	Aerenchyma formation	AER.4H	4H, 98.8	[107]

4.1. Morphological Adaptations in Hordeum Genotypes

Waterlogging tolerance of *H. marinum* is mediated by anatomical properties, namely high root porosity of adventitious roots as well as a barrier against ROL [108,109]. A study on 35 Hordeum species and genotypes revealed strong variation in the ability to anatomically adapt to waterlogging, by formation of aerenchyma or a barrier against ROL [110]. However, so far *H. marinum* has not been used in breeding processes to improve barley flooding tolerance, probably due to significant genetic variation between both species [110].

Also in some *H. vulgare* cultivar screens, scientists focused on anatomical differences between accessions. A cross between tolerant YYXT and sensitive Franklin was analyzed in respect of aerenchyma formation in adventitious roots leading to higher root porosity and therefore better survival [111]. The first study, still focusing on overall waterlogging tolerance, revealed four major QTLs [103], while a subsequent analysis exposed a major QTL on chromosome 4H associated with root porosity [105]. This QTL was confirmed again in another cross between tolerant Yerong and sensitive Franklin [104]. Moreover, in a cross between sensitive Franklin and tolerant *H. spontaneum*, this QTL was discovered besides several others [107]. Fine mapping narrowed down a region of 58 genes that are candidates underlying this waterlogging-tolerance trait [112]. Further studies are required to identify the responsible gene.

4.2. Root Ion Transport as a Tolerance Trait

Another comparison between two barley varieties focused on differences in root ion transport, namely the function of K^+ channels [113]. Root K^+ content was negatively affected under waterlogging in the sensitive variety Naso Nijo, but remained stable in the tolerant variety TX9425. Uptake of ions required for growth and metabolism is energy-dependent, and an energy deficiency under oxygen deficiency should negatively influence ion uptake processes [114]. Recently, a higher K^+ loss through the membrane under oxygen deficiency was associated with lower viability of the root cells [115]. A subsequent QTL analysis of a cross between the two cultivars revealed a major QTL on chromosome 2H underlying this tolerance trait [106]. However, it is currently not clear which gene is responsible for this trait, and whether proton pumps or K^+ channels are involved in the observed tolerance.

Although QTL analyses of barley under waterlogging have been extensively done, hardly anything is known on the transcriptional response to waterlogging as well as on proteomic and metabolomic changes. Very recently, proteomic changes under waterlogging were studied in different tolerant and sensitive barley cultivars, revealing more protection against ROS and higher fermentation capacity in the tolerant varieties [116]. More work is needed here in order to understand and interpret flooding responses in barley.

5. Analysis of Waterlogging Tolerance in Wheat

Wheat is one of the major cereals in Europe, and it is rather waterlogging-sensitive (e.g., [117]). Since wheat is a hexaploid species, the genetic analysis of this cereal is difficult. Furthermore, spring and winter wheat cultivars are available, making this species even more complex.

5.1. Variation in Wheat Waterlogging Tolerance

A number of waterlogging tolerance screens has been performed over many years. For example, van Ginkel et al. [118] tested 1344 lines of spring wheat from the Mexican CIMMYT (Centro Internacional de Mejoramiento de Maiz y Trigo) germplasm collection. A subsequent study re-analyzed six of them and included more lines, confirming the high waterlogging tolerance of the cultivar Ducula [119]. Some of these lines were further examined by crossing tolerant and sensitive lines, and suggested at least four genes to be involved in the tolerance mechanism(s) [120]. One mechanism could be the higher root porosity in waterlogged roots of tolerant lines [121]. This observation was also made

during a screen of Australian cultivars [122]. Additionally, a small analysis of only three lines revealed the importance of adventitious (seminal) roots and their porosity for waterlogging survival [117].

A screen of 34 winter wheat cultivars not only considered flooding, but also winter hardiness [123]. On a smaller scale, Huang et al. [124] identified Bayles as a sensitive and Savannah as a tolerant genotype out of six winter wheat cultivars. The usage of UK cultivars, however, did not result in superior genotypes [125]. Other screens tested further winter wheat cultivars, with different physiological parameters analyzed (e.g., mineral content [126], grain yield [127], root length [128,129]).

After identification of cultivars with different flooding tolerance, subsequent physiological studies tried to link the tolerance with certain morphological or metabolic traits. In one analysis, Savannah was more tolerant than Bayles partially due to higher root porosity [130]. A recent evaluation of Norwegian genotypes identified tolerant ones that showed specific root traits (e.g., stele and aerenchyma area) in comparison to sensitive cultivars [131,132]. Another experiment demonstrated that the tolerant cultivar Jackson was more tolerant than Coker 9835 probably because of a lower respiration rate [133]. Recently, the same cultivar Jackson was compared to sensitive Frument, and several metabolic differences were reported in leaves between both cultivars, but no clear tolerance mechanism has been found yet [134]. Another experimental set-up also considered different temperatures during a rather artificial anoxia treatment, demonstrating that genotypic differences were more pronounced at higher temperatures, but also here a single tolerance trait was not discovered [135].

Scientists have started to reveal underlying genes responsible for waterlogging tolerance by QTL analyses. A cross between two winter wheat cultivars, USG3209 and Jaypee, exposed two major QTLs on chromosomes 1B and 6D, to be used in further experiments [136]. The implication of the synthetic genotype W7984 together with the cultivated genotype Opata85 described 32 QTLs associated with waterlogging tolerance, which need to be studied further [137]. Another QTL analysis between SHWL1 and Chuanmai 32 discovered ten QTLs [138].

However, these diverse screens with different genotypes and at different locations also revealed a low reproducibility of tolerant and sensitive cultivars, as summarized in Setter et al. [139], pointing to multiple tolerance mechanisms that could be superior at one site, but not at other locations [91]. This could also be the reason for the lack of confirmed QTLs associated with waterlogging tolerance from different studies. The best strategy to continue would be the selection of one specific tolerance trait and its genetic analysis, as has already been done for barley varieties (see above).

5.2. Can Related Species be Used to Improve Wheat Waterlogging Tolerance?

So far, the classical QTL approach has not revealed single tolerance genes or loci in wheat. A different interesting approach was developed in Australia: An amphiploid from wheat with the tolerant grass *H. marinum* was created in order to produce flooding-tolerant wheat cultivars [140]. This approach resulted in some lines with a stronger barrier against ROL [141]. However, those lines show lower growth and grain yield, and are therefore not yet suitable for agriculture. So far, this morphological trait could not be transferred to wheat by use of disomic chromosome addition lines [142]. More analyses are required in order to be successful with this approach.

Potentially, also in wheat there are more flooding-tolerant relatives such as *Triticum macha* L. or *T. dicoccum* cv. Pontus [143], or *T. spelta* [144] that could be used in improving flooding tolerance of wheat. The latter species was included in a QTL analysis that revealed several loci associated with flooding tolerance at the germination state, among them five that were related to enhanced coleoptile growth [145]. However, this developmental stage does not necessarily help in improving waterlogging tolerance in the field.

Despite the wealth of greenhouse and field trials, there is not much progress yet in understanding wheat molecular responses to waterlogging, or in improving its waterlogging tolerance. Studies at the transcriptomic, proteomic, and metabolomic level are required to first understand the reason for the sensitivity of wheat cultivars in order to use this as a base for breeding. Probably the most promising direction would be the introduction of morphological changes that could be transferred from

related species. Recent experiments have begun to understand how aerenchyma formation in wheat is regulated [146,147], but also knowledge from other grass species such as rice, maize, and barley should be used.

6. Flooding Tolerance of Ryegrass

One of the most important pasture grasses, *Lolium perenne* L., is often grown on soils not suitable for cereals, for example due to poor drainage. Therefore, it is also a target species to improve its waterlogging tolerance.

One comparison of two genotypes, Aurora6 and Nth African6, together with two F1 lines, was done after four weeks of waterlogging, and Aurora6 was more tolerant than the other genotypes [148]. This cross was subsequently used for a QTL analysis, and 37 loci were identified that were associated with waterlogging tolerance [149]. Another four varieties were studied after one week of waterlogging [150]. Here, antioxidant activity was correlated with waterlogging tolerance. However, more work needs to be done to find loci or genes that are associated with *Lolium perenne* L. waterlogging tolerance.

Another set of studies analyzed the submergence tolerance of 94 to 99 genotypes. In one publication, the behavior of the genotypes was evaluated after seven days of submergence and seven days of recovery, and differential responses of genotypes were observed, ranging from sensitive cultivars to tolerant cultivars with either the quiescence or the escape strategy [151]. This study is an exciting start point for further experiments. In another study, submergence tolerance was correlated with simple sequence repeat (SSR) markers across all genotypes [152]. Finally, a targeted approach was used, and candidate genes were selected from previous physiological experiments and analyzed for SNPs to be related to submergence tolerance [153]. However, verification of these candidate genes is still required.

In summary, work on *Lolium perenne* L. waterlogging and submergence tolerance has only just started. More cultivar screens and QTL analyses are needed, preferentially with specific traits in morphology and metabolism. Furthermore, little is known on the molecular response of ryegrass to waterlogging and submergence, and transcriptomic as well as metabolomic studies need to be done in order to build a basis for breeders and scientists.

7. Soybean Tolerance under Waterlogging

Soybean is a very important crop that can be used as protein-rich food for humans, but is mostly utilized as animal feed. As most other crop species, it is very waterlogging sensitive. In some regions of the world, for example in the mid-south of the US or in Asia, it is grown in rotation with rice on fields that are often flood-prone [154,155]. An improvement of its waterlogging tolerance is therefore of great importance.

Soybean plants are of special importance for food production since they are able to fix nitrogen from air in their nodules with the help of rhizobia, and can facilitate soil enrichment with organic nitrogen compounds. Although nitrogenase is sensitive to oxygen and is therefore protected inside the bacteroids in the nodules, the nitrogen fixation process is very energy-demanding. Therefore, nitrogen fixation quickly stops after waterlogging of soybean roots and nodules, even before roots become fully hypoxic [156].

7.1. Screening for Waterlogging Tolerance in the US and Asia

A first screen on 84 Northern soybean cultivars revealed great variation in waterlogging tolerance [157]. Using one selected tolerant cultivar, Archer, two recombinant inbred line (RIL) populations were created with the sensitive northern cultivars Noir 1 and Minsoy. These were used in a QTL analysis, which exposed one major locus, Sat_064, located on chromosome 18, to be involved in the tolerance [158]. This locus was crossed into two southern genotypes to create near isogenic lines (NILs), but their waterlogging tolerance could not be related to the presence or absence

of this locus [154]. Therefore, further analyses tried to identify other QTLs related to waterlogging tolerance in Archer in RILs emerging from crosses with two southern soybean cultivars, A5403 and P9641. These efforts revealed at least five more markers, for example on chromosomes 5 and 13, pointing to several genes involved in stress tolerance [159,160] (see also Table 4). Further crosses of these genotypes produced some RILs and NILs with improved waterlogging tolerance [161], but no distinct gene has yet been associated with the waterlogging tolerance of the cultivar Archer.

Another line combination was used to study flooding tolerance and resistance to the pathogen *Phytophthora sojae*, namely the susceptible elite cultivar S99-2281 and the tolerant exotic cultivar PI 408105A [162]. This analysis identified four QTLs associated with flooding tolerance on chromosomes 11 and 13, of which one overlapped with a QTL for resistance to *Phytophthora sojae* (see also Table 4). Subsequently, physiological features between these two lines were compared that might underlay differences in tolerance [163–166]. Multiple differences were observed between the two genotypes studied, for example in respect to adventitious root and aerenchyma formation (more in the tolerant genotype), gene expression of SUB1-like transcription factors, as well as abscisic acid networks. However, the major contributing factor remains to be determined. Interestingly, differences in adventitious root formation were also observed between Vietnamese genotypes with contrasting tolerance [167], hinting at an importance of morphological traits, as also seen for cereals (see above). Crosses of the susceptible line S99-2281 and another tolerant line, PI 561271, revealed two more QTLs on chromosomes 3 and 10 [160,168] (see also Table 4). The QTL at chromosome 3 was narrowed down to a region of 23 genes, and research is ongoing to identify the responsible tolerance gene [168].

Marker-assisted selection was used to transfer those QTLs into high-yield cultivars, resulting in three new flooding-tolerant germplasm lines for application in breeding programs [160]. Still, further screens with up to 722 cultivars are ongoing, in order to further improve und understand soybean waterlogging tolerance [169,170].

Also in Asia, soybean is increasingly grown on rice paddy fields. Therefore, also Japanese soybean cultivars were used for tolerance screens. A cross between the tolerant cultivar Misuzudaizu and the sensitive cultivar Moshidou Gong 503 was evaluated for waterlogging tolerance of young soybean plants, revealing several QTLs [155]. Other screens were performed with 92 Japanese cultivars [171], 400 Korean cultivars [172], 21 Vietnamese cultivars [173], and 16 Indonesian cultivars [174], or mixtures of different origin [175,176].

Table 4. QTLs associated with waterlogging tolerance in soybean (*Glycine max* (L.) Merr.).

Crossed Cultivars	Treatment	Parameters Analyzed	Name of QTL	Location of QTL	References
cv. Archer × northers lines	14 days waterlogging	Plant growth, seed yield	Gm18	Chr 18, Sat_064	[158]
cv. Archer × southern lines	14 days waterlogging	Damages and survival	Gm5 Gm13	Chr 5, Satt385 Chr 13, Satt269	[159,160]
cv. Misuzudaizu × cv. Moshidou Gong 503	21 days waterlogging	Seed yield	ft1	Chr 6, Satt100	[155]
cv. S99-2281 × cv. PI 408105A	14 days waterlogging	Damages and survival	FTS11 FTS13	Chr 11 Chr 13	[162]
cv. S99-2281 × cv. PI 561271	4–6 days waterlogging	Damages and survival	qWT_Gm03 qWT_Gm10	Chr 3 Chr 10	[160,168]
cv. Iyodaizu × cv. Tachinagaha	7 days 0.1% stagnant agar solution	Root traits	Qhti-12-2	Chr 12, Satt052-Satt302	[177]

In all of those screens, differences in waterlogging tolerance were observed, but physiological and genetic factors underlying these tolerance differences could be studied further only in a few cases. For example, the tolerant Japanese variety Iyodaizu was used for a QTL analysis with sensitive Tachinagaha. Scientists identified 11 QTLs, of which a QTL region on chromosome 12 was most promising, as shown also with NILs [177]. Next, as for other species, soybean seed germination is likewise oxygen dependent. Therefore, seed germination was also studied in several Asian cultivars, revealing four QTLs related to a high germination rate under water [178].

Recently, GWAS have emerged as another strategy to identify genomic loci associated with waterlogging tolerance, and has been used to study another legume, *Phaseolus vulgaris* L. (e.g., [179,180]). These experiments discovered an interesting overlap between *Phaseolus vulgaris* L. flooding tolerance loci with soybean QTLs [179], namely with Sat_064 [158] and Satt187 [178]. Furthermore, Soybean (*agronomy-328455*) has also a close relative that is more waterlogging-tolerant, namely *G. sojae*, which could be included in breeding programs for higher tolerance [160].

7.2. Physiological and Molecular Responses of Soybean to Waterlogging

During one screen of soybean cultivars, scientists discovered spongy white roots in the cultivar Manokin, pointing to an aerenchyma-like structure, but this was not observed in the most tolerant cultivar Delsoy 4710 [181]. Such secondary aerenchyma around roots, stems, and nodules, emerging from phellem, has been observed before in waterlogged soybean, and might improve aeration of the flooded tissues [182,183]. In contrast to several grasses, soybean develops only small primary aerenchyma in its root cortex [184]. Whether secondary aerenchyma formation or their extension is generally related to waterlogging tolerance, remains to be studied. Some experiments suggest that morphological parameters such as adventitious root formation or root porosity might be associated with higher tolerance also in soybean [163,164,167].

The molecular response of soybean to flooding has been well studied already, especially at the proteome level, using different organs, developmental stages, and treatment conditions (summarized in [185]). In one study, several tolerant and sensitive Asian lines, classified at the seedling stage, were analyzed at the proteome level [175]. These experiments revealed multiple differences in protein expression among genotypes but no obvious trend, pointing to multiple tolerance factors.

Also the transcriptional response has been studied in several experiments, providing a rich base for future functional analyses. For example, the response of leaves to seven days of waterlogging treatment of the roots was explored by RNAseq, showing a negative impact of root stress on leaf photosynthesis [186]. Also roots were investigated directly after root hypoxia [187,188]. The same group also developed a flooding-tolerant soybean mutant, by gamma-irradiation [189] which was compared to wildtype soybean. This study revealed many genes that were differentially expressed between both genotypes [190], but a subsequent analysis of candidate genes responsible for tolerance is required, as well as the identification of the mutation.

8. Waterlogging Tolerance of *Brassica napus* L. and Relatives

Rape (*Brassica napus* L.) is an important oil crop and can also be used as animal feed. It has a complex genetic structure since it is an allotetraploid species that originated from the two diploid species *B. rapa* and *B. oleracea*. Despite its rather recent origin, a wide range of cultivars exists ranging from winter to spring types, but also semi-winter types can be found for example in China (e.g., [191]). Rape is very sensitive to waterlogging, even if compared to other Brassica species [192]. This is at least in part due to its inability to form aerenchyma [193], similar to its parent *B. rapa* [194].

8.1. Chinese Semi-Winter Rape Cultivars Show Contrasting Waterlogging Tolerance

So far, most data available for rape waterlogging tolerance come from Chinese semi-winter cultivars. The interest in flooding tolerance of Chinese cultivars is due to the fact that rape in China is often used as a rotation crop on rice paddy fields, and therefore it is often affected by waterlogging.

A comparison of 18 cultivars, with different seed coat color, tested germination ability after 24 h of submergence. Interestingly, yellow-colored cultivars were mainly sensitive to submergence at the seed stage, including GH01, and dark-colored cultivars were mainly tolerant, including Zhongshuang 9 and 10 [195]. These cultivars also came up in other screens for waterlogging tolerance [196]. Further screens with more Chinese lines were performed at later developmental stages [197–199], confirmed previous tolerant lines (Zhongshuang 9 and 10), and identified further lines to be tolerant (Xiangyou 13, Huayouza 9, Ningyou 12) and sensitive (Yuhuang 1, Zhongyouza 3, Zhongshuang 8).

A subsequent genetic analysis suggested two genes to be involved in waterlogging tolerance of Zhongshuang 9, but no QTL has been described yet underlying this tolerance [200]. A cross between six cultivars differing in waterlogging tolerance revealed additive and non-additive effects pointing to several genes or alleles involved, with Zhongshuang 9 being the most potent cultivar [201]. An independent group used a double haploid population between two lines different from the ones mentioned above with high and low waterlogging and drought tolerance [202]. They identified at least 11 QTL for waterlogging tolerance, suggesting a complex regulation also in this species.

An attempt to associate waterlogging tolerance with physiological traits suggested the importance of the antioxidant system [196]. Other analyses observed a difference in nitrate metabolism between two Chinese cultivars under waterlogging [203]. Furthermore, a correlation of low ethanolic fermentation and higher waterlogging tolerance was described [204].

So far, there is little knowledge on the molecular response of rape to waterlogging or submergence, making it hard to find a basis to select candidate genes in the QTL regions. A first attempt to solve this problem was done on two Chinese cultivars with contrasting waterlogging tolerance, GH01 and Zhongshuang 9 [205,206]. The transcriptional response of roots to 12 h of waterlogging was studied by use of RNAseq, and many genes responded to the stress treatment, mainly similar in both genotypes. However, there were some differences between the two cultivars, possibly related to the plant hormone abscisic acid, but this has to be explored further. Unfortunately, these two lines have not yet been subjected to a full QTL analysis. Another transcriptomic study focused on shoot responses after 36 and 72 h of waterlogging, revealing downregulation of photosynthesis [207], as was also shown for soybean [186].

8.2. Can Relatives of Brassica napus Help to Enhance Its Flooding Tolerance?

The genus Brassica contains, among other species, the diploid species *B. rapa*, *B. nigra*, and *B. oleracea*, and the allotretraploid species *B. juncea*, *B. napus*, and *B. carinata*, which often contain multiple subspecies and cultivars (e.g., [208]). Little is known on waterlogging tolerance of other Brassica species, besides one study that described *B. juncea* and *B. carinata* as more waterlogging tolerant than rape [192]. A direct comparison of rape with diploid Brassica species under waterlogging would be most helpful.

So far, some other diploid Brassica species have been studied on their own, revealing also cultivar differences in flooding tolerance. An analysis with two populations of *B. rapa* with different waterlogging tolerance suggested the importance of carbohydrate supply to roots as a potential parameter for tolerance [209]. Second, *B. oleracea* was studied at the seed stage. As reported for other species above, seed germination of Brassica species is particularly strongly dependent on oxygen. A QTL analysis between two *B. oleracea* cultivars, a sensitive Chinese cultivar (A12DHd) and a more tolerant calabrese cultivar (GDDH33), revealed three QTLs that are associated with germination ability under low-oxygen concentrations [210], but associated genes have not yet been identified.

The relative high relatedness of rape to the well-studied model Brassicaceae *Arabidopsis thaliana* (L.) Heynh. might be another way to use existing knowledge in improving rape waterlogging tolerance. First ecotype screens on Arabidopsis have demonstrated differences in submergence tolerance [11, 42–44]. However, since Arabidopsis is also rather flooding-sensitive, the analysis of flooding-tolerant Brassicaceae, for example from the *Rorippa* genus [211,212], might be more suitable.

9. Conclusions

Over recent years, many studies have been published on flooding-tolerant cultivars of temperate crop species. In several cases, QTLs could be discovered, and certain tolerance-related traits were described. However, in most studies, the underlying mechanism(s) or the responsible gene(s) have not yet been identified. It is therefore of great importance to continue with well-designed QTL analyses in order to truly improve crop resistance to flooding. All studies so far have demonstrated that a QTL analysis is only promising when it is associated with a specific, well-defined tolerance trait. The most promising traits so far have been related to morphological adaptations that appear to be similar across genera, such as enhanced root porosity, a barrier against ROL, and the formation of adventitious roots. Metabolic adaptations could be related to the antioxidant system, and to primary metabolism, mainly to carbohydrate availability. However, metabolic traits are rather hard to compare in a large collection of cultivars. The knowledge on the overall crop response to the stress at the transcriptomic, proteomic, and metabolomic level will certainly help to understand the molecular mechanisms that provide the basis for underlying tolerance traits.

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Perspective

Good Riddance? Breaking Disease Susceptibility in the Era of New Breeding Technologies

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Abstract: Despite a high abundance and diversity of natural plant pathogens, plant disease susceptibility is rare. In agriculture however, disease epidemics often occur when virulent pathogens successfully overcome immunity of a single genotype grown in monoculture. Disease epidemics are partially controlled by chemical and genetic plant protection, but pathogen populations show a high potential to adapt to new cultivars or chemical control agents. Therefore, new strategies in breeding and biotechnology are required to obtain durable disease resistance. Generating and exploiting a genetic loss of susceptibility is one of the recent strategies. Better understanding of host susceptibility genes (*S*) and new breeding technologies now enable the targeted mutation of *S* genes for genetic plant protection. Here we summarize biological functions of susceptibility factors and both conventional and DNA nuclease-based technologies for the exploitation of *S* genes. We further discuss the potential trade-offs and whether the genetic loss of susceptibility can provide durable disease resistance.

Keywords: plant immunity; effector-triggered susceptibility; necrotrophic effector; biotroph; susceptibility gene; host reprogramming; pathogen nutrition; plant cell development; natural diversity; CRISPR

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1. Introduction

In crop production systems, plant diseases are controlled by standard field management practices (e.g., crop rotation, ploughing), usage of disease-resistant cultivars and pesticide applications. However, disease resistance and pesticide efficacy are often not durable because pathogen populations rapidly adapt to the selection pressure that is exerted by these disease control mechanisms. This and potentially harmful effects of pesticides on off-target organisms can render plant protection unsustainable, necessitating novel approaches to combat plant pathogens. In recent years, fundamental research on molecular plant-microbe interactions has revealed new insights on how plants defend themselves against pathogens and how pathogens subvert plant immunity. This knowledge and the development of new breeding technologies holds the potential for innovative approaches in genetic plant protection, which could complement the limitations of conventional technologies to provide greater resistance durability [1].

In plants, invading pathogens are challenged at several levels of plant-pathogen interactions [2]. Preformed defensive barriers together with pathogen-induced plant defense responses successfully restrict parasitic growth on resistant plants. Induced plant defenses have, however, led to the adaptation of pathogens to certain host species and the evolution of host-specific virulence strategies. This includes the secretion of proteinaceous and non-proteinaceous pathogenicity factors that support pathogen virulence. Since these so-called effector molecules are required to actively overcome host immune barriers, the term "effector-triggered susceptibility" (ETS) was coined [1]. Most reports on effector

activities show that effectors function as suppressors of plant immune receptor functions, signal transduction and defense reactions [3]. Hence, the suppression of plant defense appears to be pivotal for virulence. Effectors can either specifically modulate host immune processes or more broadly influence host physiology. The latter often contributes to the development of disease symptoms. Such effectors may even provoke strong symptoms when applied as pure substances to plants. In this case, they are considered as toxins that can act either host-specifically or host-nonspecifically. Some effectors influence plant development and some pathogens produce plant hormones or hormone analogs to manipulate host development or physiology.

Pathogen detection either takes place at the plant cell surface, where surface receptor complexes function, or in the host plant cytoplasm or nucleoplasm by intracellular receptor proteins or receptor complexes [1,4]. Plant immune receptors (so-called resistance proteins encoded by major disease resistance genes [*R*]) detect the presence of effector proteins in a race-cultivar specific manner as determined by monogenic inheritance in both the host and parasite. At the molecular level, this classical gene-for-gene model is described by the term "effector-triggered immunity" (ETI) [1]. A more basal, race-nonspecific type of immunity operates within the broader context of ETS and ETI and it is mediated by the detection of a broad spectrum of non-self or altered-self molecules. This is collectively summarized as pattern-triggered immunity (PTI) [4,5].

2. The Principle of Susceptibility Genes and How to Find Them

Host immune components are encoded by dominantly inherited genes, which show either major (qualitative) or minor (quantitative) effects on disease resistance. However, the observation that disease resistance can also be recessively inherited indicates that pathogens can also profit from dominantly inherited host functions or susceptibility factors [1,6,7]. The corresponding dominantly inherited genes are called susceptibility genes (*S*). Recessive *s* genes have been successfully used in conventional and marker assisted plant breeding for the improvement of disease resistance. Recessive *mlo* (*mildew locus o*), several virus resistance genes and ToxA-insensitive *tsn1* genes are prominent examples for this [8,9]. Here we discuss the mechanisms of disease susceptibility and how provoking and exploiting genetic loss of susceptibility can aid durable disease resistance.

Farmers and breeders have selected naturally occurring mutations of *s* genes for the improvement of crop health. Additionally, breeders and researchers searching for mutagenesis-induced resistance in crop and model plants have identified a broad variety of recessive disease resistances. High research effort over the last three decades enabled the identification of several of the corresponding mutations in *S* genes and first insights into the mechanisms of disease susceptibility.

The *mlo*-mediated powdery mildew resistance is perhaps the most prominent example of recessive plant disease resistance. It is of particular interest, because it is race-nonspecific and durable in the field. The *MLO*-gene was originally characterized in spring barley but it seems to function in all interactions in which *MLO S*-gene functions have been studied in detail [8]. Ethiopian highland farmers may have originally selected the barley *mlo-11* allele in old land races, collected during expeditions in the 1930s, and used later in European plant breeding in the 1970s [10]. However, the first description of *mlo* goes back to an X-ray induced powdery mildew mutant generated in the 1940s [11]. Across multiple plant species, many mutagenesis-derived loss-of-function *mlo* alleles exist and *MLO* null-mutants are generally resistant to powdery mildew assuming no genetic redundancy with other *MLO* family members exists. The exact biochemical function of the f protein is not understood, but it may act as a negative regulator of pathogen-triggered and spontaneous defense reactions, putting *mlo* mutants in a primed defensive status [8,12].

The model plant *Arabidopsis thaliana* has been instrumental for the identification of many more susceptibility factors. For instance, forward genetic screens for powdery mildew resistance (*pmr*) or gene expression studies of compatible interactions with diverse biotrophic pathogens, followed by reverse genetic approaches have identified several candidate *S* genes [6,13–15]. Additionally, educated guesses and translational approaches have proved similarly successful in discovering

S genes in crop plants [16,17]. In vivo protein-protein interaction screens are yet another suitable approach to identify susceptibility factors using effector proteins as bait [18,19]. Candidate susceptibility genes were also identified via host gene expression profiling, as it was shown with *Hyaloperonospora arabidopsidis*-infected *Arabidopsis thaliana* and *Phytophthora cinnamoni*-infected *Castanea* [20,21]. Once an *S* gene is identified, studying the physiological function of the susceptibility factor and genetic or physical interactions can identify susceptibility mechanisms or associated pathways and thereby new susceptibility factors [22,23].

3. Biological Functions of Susceptibility Genes

Considering the role of susceptibility genes in compatible plant-microbe interactions, the question arises as to what physiological function host susceptibility factors (or compatibility factors in terms of microbial symbiosis) may exert in healthy and microbe-attacked plants (Figure 1). Some plant susceptibility factors are regulators of host defense responses or cell death. Depending on whether the pathogen is a biotroph, hemibiotroph, or a necrotroph, it can be more or less sensitive to individual plant defense reactions or even profit from host cell-death. Biotrophs often profit from negative regulators of host defense reactions or cell death whereas necrotrophs can profit from host programmed cell-death. This might explain why certain host susceptibility factors show an ambivalent character and can turn into a resistance factor in interaction with another pathogen (see also chapter 5 for trade-offs below). Similarly, individual plant hormone pathways can positively or negatively influence plant-pathogen interactions, depending on the pathogen's lifestyle [24]. In other cases, susceptibility factors do not have reported functions in regulating plant defense. They could be involved in physiological reprogramming of the susceptible host to establish and maintain a compatible interaction. This is particularly well described for the interaction with biotrophic pathogens that show a tight parasitic symbiosis with their host plants and appear to depend on many host functions for disease development. An increasing amount of publications support that successful obligate biotrophs not only successfully inhibit plant immunity, but also heavily rely on and reorganize host cell physiology and development. In the next paragraphs we discuss some prominent examples of *S* gene functions. For more comprehensive overviews, we refer to other review articles [25–27].
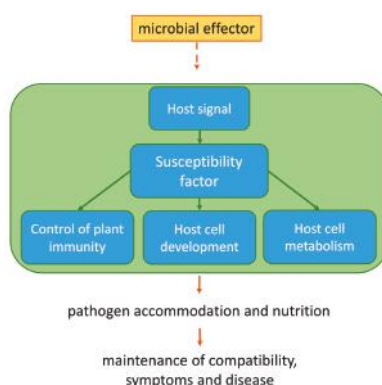
Figure 1. Plant physiological functions and microbial utilization of host susceptibility factors (S). Host S factors can have diverse physiological functions (highlighted by the green background box), which would also operate in pathogen-free plants. This can include control of immune responses or normal host cell development and metabolism. In healthy plants, S factors are regulated by host endogenous signals (e.g., hormones, peptides, second messengers, protein-protein interaction, and protein modification). Microbial pathogens can simply profit from S factor functions or they actively take advantage of S factors via virulence effectors. Effectors might directly act on S factors or on their physiological environment.

3.1. Host Cues for Recognition by the Pathogen

Upon first contact of a pathogen with the host aerial surface or rhizosphere, silent pathogen genes need to be activated e.g., for germination, directed growth, the development of infection structures, and for secretion of virulence factors. Gene activation requires recognition of host cues that trigger pathogen development. For example, epicuticular waxes and cutins of plants provide such cues for germination and formation of appressoria (the pathogen organ for surface adherence and cell wall penetration) by oomycetes, powdery mildew, anthracnose, and rust fungi. Correspondingly, plant mutants that show alterations in leaf wax composition can be less susceptible to fungal invasion [28–33]. Wild type forms of mutated genes that function in or interfere with biosynthesis of wax or cutin components can be considered as *S* genes. There are also reports about other types of chemical cues from the host (e.g., volatiles, flavonoids, acetosyringone, etc.), which are considered to be responsible for metabolic compatibility with adapted pathogens [34–36], and corresponding biosynthetic pathways may contain S factors. Additionally, plant surface hydrophobicity and topology trigger early pathogen development on susceptible plants [37,38].

3.2. Support of Pathogen Demands

Host-adapted necrotrophs can usually tolerate preformed and induced plant defensive barriers, but biotrophs, in particular, require additional support from the host because they lack saprophytic potential [39]. This host support means that, for example, the plant is involved in establishing pathogen feeding structures (haustoria or analogous feeding hyphae) in living host cells. Additionally, the host actively provides nutrients, e.g., by changes in carbohydrate metabolism, sugar transport, or carbohydrate source-sink transitions. Some obligate biotrophs have lost certain biosynthetic pathways, and hence, they might depend on host metabolite supply for primary or secondary metabolite biosynthesis. Thus, the pathogen requires plant components, whilst simultaneously suppressing the same plant's defense. In plant-virus interactions, host primary metabolism and basic cell functions are involved in susceptibility because they provide the building blocks and biochemical machinery for synthesis of the virus itself. For instance, several components of the plant translation machinery contribute to virus replication and they are S factors in virus diseases [40]. An example for a host protein that supports fungal infection is the ROP GTPase RACB of barley, which is required for the susceptibility to fungal growth into epidermal cells and the expansion of haustoria of the powdery mildew pathogen *Blumeria graminis* f.sp. *hordei*. The physiological function of RACB in a healthy plant lies in polar cell development of leaf and root epidermal cells. RACB supports the outgrowth of root hairs from trichoblasts and the ingrowth of haustoria in leaf epidermal cells [41,42]. Another example is SWEET proteins; SWEET sugar transporters transport sucrose out of plant cells for reallocation of sugars. SWEETS are S factors, because they can be overexpressed in pathogen interactions and function in providing nutrients for the pathogen [43]. In summary, host cellular processes support certain demands of pathogens that feed from live tissue and the components of these processes can be S factors.

3.3. Control of Plant Defense Responses

Many S genes encode negative regulators of plant defense responses. Consequently, corresponding homozygous loss-of-function-mutants show either instantaneous defense responses or stronger defense responses after pathogen contact, which can be considered as a genetic priming mechanism. Mutant screens provided several lesion mimic or constitutive defense gene expression mutants, and in many of these mutants, stress hormone signaling is imbalanced. Prominent examples are lesion-simulating disease 1 (*lsd1*) or constitutive expressor of PR genes (e.g., *cpr1* or *cpr5*). These mutants are usually less susceptible to biotrophic pathogens. In fewer cases, such mutants show a resistance to necrotrophs or broad-spectrum resistance [44]. Powdery mildew resistant *mlo* mutants show primed defense in young tissues and spontaneous defense in older tissues. However, in this particular case, it remains unclear

as to whether deregulated defense is decisive for disease resistance because double mutants in *mlo* and stress hormone pathways lose spontaneous defense yet they retain pathogen resistance [45]. Genetic studies have shown that secondary indole metabolism appears crucial for *mlo*-mediated resistance in Arabidopsis [46], and vesicle fusion involved in protein secretion appears to be generally crucial for *mlo*-mediated resistance [45,47].

4. Effector Targets

Plant pathogenic microbes benefit from a certain repertoire of secreted effector proteins that interact with host molecules aiming to create a more favorable environment for the microbe (Figure 1). Hence, any host protein that directly or indirectly supports the susceptibility to any given phytopathogen represents an attractive potential effector target. Our knowledge of microbial effector proteins and susceptibility factors as genuine targets of these effectors has increased significantly over the last years [3,26], and some examples are described below.

4.1. Hub Proteins

Proteins at the center of signaling networks constitute hubs that are by definition key players during plant development and hence represent potential effector targets. In a recent study, a protein-protein interaction network was generated based on *Arabidopsis thaliana* host proteins and virulence effector proteins of biotrophic (*Golovinomyces orontii* and *Hyaloperonospora arabidopsidis*) and hemibiotrophic (*Pseudomonas syringae*) phytopathogens. The authors showed that certain plant proteins are targeted simultaneously by effector proteins from bacterial, fungal, and oomycete pathogens, thereby demonstrating exemplary effector convergence on key targets [19]. Several of these concurrently targeted plant proteins have also been shown to be susceptibility factors. Mutants of the COP9 signalosome complex subunit 5A (CSN5A), for example, showed enhanced disease resistance against both biotrophic and hemibiotrophic pathogens, suggesting a role for CSN5A in facilitating pathogen sustenance in the host. In contrast, transcription factor TCP14, being the most effector-targeted protein, seems to act as a susceptibility factor only in hemibiotrophic interactions, similar to other TCPs. Likewise, APC8, a protein involved in cell-cycle phase transitions, is one of the five most targeted hub proteins and acts as susceptibility factor in hemibiotrophic interactions [19]. The C3HC4 RING finger protein HUB1 was found to be targeted by only one pathogen effector protein. However, its function as susceptibility factor was demonstrated in *hub1* mutant plants that showed enhanced disease resistance against biotrophic pathogens [19].

RIN4 (RPM1-INTERACTING PROTEIN 4) is another excellent and well-studied example of an effector-targeted susceptibility factor. This negative regulator of plant immune responses is also guarded by R proteins RPM1 (RESISTANCE TO PSEUDOMONAS SYRINGAE PV. MACULICOLA 1) and RPS2 (RESISTANT TO PSEUDOMONAS SYRINGAE 2), which are activated upon perception of the effectors AvrB-, AvrRpm1- or AvrRpt2-induced state modifications of RIN4 to subsequently trigger ETI. RIN4 nicely demonstrates that guarding of susceptibility factors by R-proteins sometimes is the plant's only efficient way for protection, as opposed to *S*-gene mutation or the entire elimination from the genetic background, which regarding RIN4 would have detrimental pleiotropic consequences [48,49].

4.2. Bacterial Effector Targets

Certain race-specific susceptibility genes are targeted by transcription activator-like effectors (TALEs) of phytopathogenic bacteria from the genus *Xanthomonas*. They are known to drive host gene expression in a sequence-specific manner, leading to enhanced disease symptoms [50]. For example, AvrXa7 and PthXo3 activate the expression of sugar transporter OsSWEET14 in rice cultivars by directly binding to the effector binding element (EBE), which is located in the promoter region of *OsSWEET14*, *OsSWEET11*, and *OsSWEET13*, like *OsSWEET14*, are likewise major susceptibility genes and targets of TAL effectors [51,52]. Intriguingly, host transcription factors seem to be an attractive target for TALEs,

as several other studies have now shown [53–55]. With the increasing amount of EBEs found in nature, the next years will continue to enrich the arsenal of TALE-targeted susceptibility genes.

4.3. Effector Targets of (Hemi)Biotrophic Pathogens

In contrast to bacteria, filamentous pathogens like fungi and oomycetes possess a plethora of effector proteins, indicating probable effector function redundancy. Biotrophs might, furthermore, require multiple effectors for manipulating host S factors, as suggested recently [56]. Barley MLO has not been described yet as a powdery mildew effector target, in contrast to its functional ortholog MLO2 from *Arabidopsis thaliana*, which is targeted by bacterial *Pseudomonas syringae* effector HopZ2 [8,57]. Likewise, the barley S factor ROP GTPase RACB was recently shown to interact with the ROP-interactive peptide 1 (ROPIP1) from *Blumeria graminis* f.sp. *hordei*. ROPIP1 is encoded on repetitive DNA, supports fungal penetration, and can provoke host cell microtubule disorganization. It may therefore represent an unconventional powdery mildew effector [58].

Hemibiotrophic pathogens require living host tissue during the initial stage of infection, suggesting that any host proteins that are involved in suppressing early resistance-associated cell death reactions might constitute susceptibility factors. For example, the effector Avr3a from the late blight pathogen *Phytophthora infestans* targets and stabilizes the E3 ubiquitin ligase CMPG1. CMPG1 degradation is required for INF1 elicitor-mediated cell death, which would, in turn, obstruct further pathogen spreading during the biotrophic phase of the infection [59]. A similar stabilization, followed by an enhanced infection, was observed with the putative potato K-homology (KH) RNA-binding protein KRBP1 that is targeted by *P. infestans* effector Pi04089 [60]. Targeting S factors is a well-exploited strategy that oomycetes like *P. infestans* follow, as additional studies have shown [61,62].

4.4. Effector Targets of Necrotrophic Pathogens

The pathogenicity of necrotrophs can also rely on secreted effectors that interact with host susceptibility factors to establish foliar necrosis and/or chlorosis in a cultivar-specific manner. For example, it was recently reported that some subunits of the membrane tethering exocyst complex from solanaceous plants seem to act as susceptibility factors for the grey mold pathogen *Botrytis cinerea* [63]. Profound knowledge of necrotrophic effector proteins was gained from studying effector proteins ToxA and ToxB from the tan spot pathogen *Pyrenophora tritici-repentis*, with ToxA being the best studied to date. The *ToxA* gene was acquired by horizontal gene transfer from the leaf blotch pathogen *Parastagonospora nodorum* [64]. ToxA itself interacts directly with a chloroplast-localised protein, ToxABP1. The presence of both, ToxA and ToxABP1, or the silencing of *ToxABP1* in the absence of ToxA leads to similar necrotic phenotypes, indicating ToxA altering ToxABP1 function [65,66]. ToxA genetically interacts with the product of the toxin sensitivity gene *Tsn1*, which encodes a protein that resembles canonical Resistance proteins [9,67]. Functional TSN1 is involved in triggering ToxA-dependent cell death, which favors necrotrophic pathogen growth, and is thus a major S factor with agronomic relevance for both tan spot and leaf blotch in wheat [68].

5. Trade-offs and Prediction of Durability

S gene-based resistance has been suggested to be more durable than the widely applied *R* gene-based resistance [25]. Indeed, there are many reasons why *R* gene resistances, even when combined, might not be durable [69]. Thus, loss-of-susceptibility could potentially be a way to create more durable resistance. However, little is known about general *S* gene durability with only a few examples of long term durability being reported: *Mlo* resistance in barley has been in use for over 35 years and eIF4E-mediated virus resistance in pepper for almost 60 [11,70]. It is often thought that a combination of (functional) redundancy and pleiotropic effects contribute to the durability of *S* genes. Yet, which factors specifically contribute to this durability remains unclear. However, the non-race-specific nature of *s* gene-mediated resistance (e.g., *mlo*-mediated powdery mildew resistance)

makes it less likely that population dynamics rapidly select for single races that are capable of circumventing the loss of susceptibility.

5.1. Broad Spectrum Resistance & Functionality

It has been hypothesized that *S* genes are more durable because of the central role they play in facilitating the initial infection stages. This could be tightly linked to biological functions and the physical environment of S factors. As described above, S factors can generally be considered as (indirect) negative regulators of immunity [71]. In *s* mutant genotypes, it is therefore possible that the whole plant is in a primed state or that certain defense-associated processes are more active, preventing the pathogen from establishing infection [13].

S factors are often intra-cellular and well-embedded in physiological pathways. It has been shown that certain *S* genes can be classified as protein hubs [19]. They are therefore unlikely to steer a single physiological process, which, when missing, can be easily circumvented by the pathogen. In the case of host *S* gene null mutants, pathogens would have to evolve new functions to overcome loss of susceptibility. This would be particularly relevant if an S factor serves an essential requirement for the pathogen. Such evolution of a new biological function can be considered to be slow or even impossible, according to complexity. In addition, the subcellular location and the presence of many possible guard and decoy proteins will impede even rapidly evolving effectors to reach and modify their intracellular target, as opposed to cell-surface receptors or apoplastic R proteins [72]. Thus, it is easier for a pathogen to lose or modify a single R-protein-recognized effector to tackle ETI than to overcome the loss of a host susceptibility gene.

5.2. Pleiotropy and Trade offs

S genes often have key physiological functions within the host. This implies a high chance of pleiotropic effects of *S* gene mutation. On the one hand, if the recessive *s* gene of interest displays deleterious phenotypes, even in a different genetic background, it would not be easily exploitable [26]. On the other hand, mutants that do not show pleiotropic effects are extremely valuable and they could provide sustainable solutions in resistance breeding. Interestingly, pleiotropic effects also complicate the assessment of both durability and the broadness of the resistance spectrum. For example, elF4E1 provides virus resistance in cultivated tomato (*S. lycopersicum*) and the wild species *S. habrochaites*, yet the null mutant in *S. habrochaites* show stronger and broader resistance than the *S. lycopersicum* mutant. The addition of an independent mutation in a related gene, *elF4E2*, did increase the strength and breadth of resistance, but lead to detrimental growth [73], although *elF4E2* mutants alone do not confer resistance. Also, *Mlo* genes occur as gene families and in *A. thaliana*, independent mutations of three orthologs are required for *mlo* resistance, each having their own major or minor effect [46]. A trade-off has been shown between *mlo* resistance to powdery mildew and increased susceptibility to *Magnaporthe grisea* [74] or the toxins of the necrotroph *Bipolaris sorokiniana* [75]. On the other side, reduced pleiotropic effects in barley have been reported in moderate *mlo* variants [76]. So far, no trade-offs have been detected in *tsn1* plants, but recent work by See et al. [67] suggested that the tan spot infection could develop in some wheat backgrounds, independent of the *tsn1* allele. Moreover, the strong day/night rhythm of *Tsn1* gene expression might indicate an additional function in wheat.

5.3. Decoys and Protection

Many *S* genes have allelic variants in the genome that may act as a "sponge" decoy [72], attracting effectors and triggering defense responses. In addition, the molecular inclusion of *S* gene domains in *R* genes has been reported, thus combining recessive resistance with active defense signaling. *Pi21* is a gene that encodes an HMA domain containing protein that suppresses the plant defense response in rice [77]. HMA domains are virulence targets for multiple pathogens [78]. Additionally, HMA domains have also been found as integrated domains in *R* genes. Effectors from the rice blast fungus *Magnaporthe oryzae* bind to the HMA domain of the R protein Pik and trigger defense responses [79].

Many more integrated domains have been described to date [80], yet the question remains how many of these are "orthologs" of true *s* genes.

5.4. S Gene Diversity

Durability of *S* genes could possibly contribute to the fact that different homologs and *s* alleles are present in different plant cultivars or ecotypes. Assuming lack of pleiotropic effects, it will be theoretically possible to maintain many different *s* alleles in a population. Yet, evidence seems inconclusive. Additional *elF4E1* alleles have been identified in wild tomato species, albeit with a limited resistance spectrum [81]. Conversely, in chili peppers (*Capsicum annuum*), many variants confer resistance and are thought to have originated from loci under positive selection. Nevertheless, it is striking that *elF4E2* shows no polymorphisms in chili pepper [73]. In barley, the viral S factor HvEIF4E also occurs in many diverse resistance-conferring haplotypes. For another unrelated virus *S* gene, *HvPDIL5-1*, many haplotypes are present in wild barley and landraces, yet none of these are actual virus resistance conferring alleles (*rym*-alleles) [82,83]. The fact that several barley cultivars with different resistance conferring alleles exist, does suggest that the *s* alleles arose by mutation during domestication in an area where the virus was also present [83]. Also, *Tsn1* shows large variety in spelt and other grasses, but in turn, it has hardly any polymorphisms in bread and durum wheat [9].

6. Exploitation of Susceptibility Genes

6.1. Breeding for Reduced Susceptibility/Loss of Susceptibility

In several cases, naturally occurring *s* genes have been identified in breeding material without actually knowing the nature of the corresponding dominant gene. In many other instances, random mutagenesis and selfing have produced disease resistant offspring with mutant *s* genes (Figure 2). Recessive resistance is best identified in inbreeding plant species or artificial double haploid plants. Cloning of *S* genes from crop plants has been successful in several cases and has sometimes facilitated the identification of related genes in other crop or model plant species. *MLO* is an *s* gene with many *mlo* resistance alleles being identified in diverse plant species, making it a "universal weapon" against powdery mildew [8]. Likewise, once *ToxA* and the *Tsn1* tan spot *S*-gene of wheat were identified, it became straightforward to eliminate the susceptibility from breeding material. Even the phenotypic identification of *Tsn1* genotypes became possible because ToxA, which is as a host-genotype specific toxin, could be directly applied to distinguish *Tsn1* from *tsn1* genotypes by differentially ToxA-provoked symptoms [67].

Another breeding strategy for loss of susceptibility starts from a known *S* gene and looks for natural or induced allelic diversity by TILLING (Targeting Induced Local Lesions In Genomes). TILLING has the advantage that it starts from mutagenesis of any desired genotype, followed by *S* gene re-sequencing, and can identify mutant s alleles even in complex genomes, such as that of hexaploid wheat [84]. The hexaploidy of bread wheat and the presence of three barley *Mlo* orthologues (*TaMlo-A1*, *TaMlo-B1* and *TaMlo-D1*) make the natural occurrence of *mlo* mutants including pathogen resistance quite unlikely for bread wheat. Using TILLING technology to select partial loss-of-function alleles of *TaMlo* however enhanced powdery mildew resistance in some lines without negative pleiotropic effects [84]. Naturally occurring variation of *S* genes can be identified by EcoTILLING: re-sequencing *S* genes in natural populations of crop progenitors or land races. EcoTILLING identified a new allele of *eIF4E* for melon necrotic spot virus resistance [85], and similar to our previous example of *HvPDIL5-1*, a TILLING approach further confirmed the identity of the mutated *S* gene in *rym1* genotypes [83].
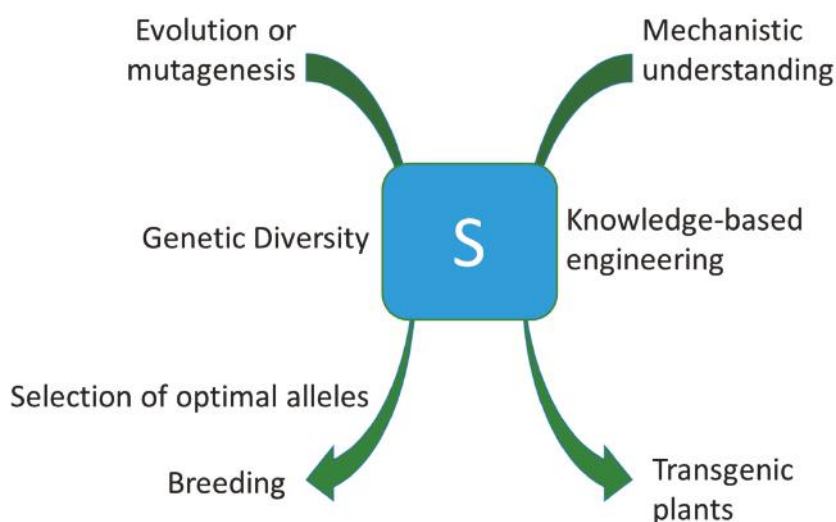
Figure 2. Plant susceptibility genes can be exploited for loss of susceptibility in breeding or biotechnology. Optimal mutant alleles (left side of the panel) that provide a balance between gain of resistance and potential trade-offs can be identified in natural plant populations by ECOTILLING or after random or targeted mutagenesis by Targeting Induced Local Lesions In Genomes (TILLING) or DNA-endonuclease mediated mutagenesis, respectively. Alternatively, knowledge-based (right side of the panel) approaches are enabled if a mechanistic understanding of *S* gene functions exists. This might, amongst others, include artificial effector decoys or silencing *S* genes on demand under control of pathogen-triggered promoters.

6.2. Gene Editing Possibilities for Targeted Exploitation of Susceptibility Genes

Besides targeted *S* gene-associated traditional breeding strategies, there are several additional opportunities for exploiting *S* gene function (Figure 2). In fundamental research, susceptibility factors can be used as bait to either find antagonists that are involved in resistance or downstream interactors that themselves act as susceptibility factors. If the molecular mode-of-action of an S factor has already been elucidated, one way to inhibit its function can be the application of a biocompatible chemical treatment. Alternatively, the ectopic expression of dominant negative *S* gene variants might outcompete and eventually eliminate the endogenous S factor function. Genome editing using sequence-specific nuclease mutagenesis technologies has gained much attention over the last ten years, as it provides researchers with constantly improving tools like zinc finger nucleases (ZFNs), TALE nucleases (TALENs), and clustered regularly interspaced short palindrome repeats (CRISPR)/CRISPR-associated systems (Cas) to create transgene-free genetically modified crops [86–88]. CRISPR/Cas9-targeted editing has been successfully applied on the citrus susceptibility gene *CsLOB1* and its promoter, resulting in resistance to *Xanthomonas citri* subsp. *citri* (Xcc), and also for creating novel alleles of rice *eIF4G*, which conferred resistance to *Rice tungro spherical virus* [89–91]. A strong disease resistance against powdery mildew has been achieved in tomato by transgene-free genetically CRISPR/Cas-mutated *slmlo1*, one of 16 *Mlo* genes in tomato [92], exemplifying again how powerful and versatile these novel genome editing approaches can be.

Furthermore, the EBE of *OsSWEET14* is targeted by TALEs AvrXa7, PthXo3, TalC and Tal5 from *Xanthomonas oryzae* pv. *oryzae*. TALEN-mediated genome editing of AvrXa7 and Tal5 EBEs conferred resistance to bacterial strains relying on the corresponding TALE [93]. Nature itself teaches us of how to benefit from the EBE-promoter repertoire. To counteract TALE activity, several R proteins

have recently been found in pepper and in rice that contain corresponding EBEs for *Xanthomonas* TALEs AvrBs3, AvrXa10, AvrXa23, and AvrXa27, displaying a promoter-trap strategy in a decoy-like manner to confer disease resistance [94–97]. Using TALENs or genome-based engineering, artificial EBE-promoter traps could be generated using EBEs of *S* gene promoter regions upstream of known *R* genes. Moreover, it is even feasible to combine different EBEs and *R* genes in a consecutive manner aiming for broad-spectrum resistance against a range of microbial pathogens [50]. Additionally, with the help of genome editing technology, broad resistance to different phytopathogenic fungi may be achieved by generating loss-of-function alleles of genes encoding HMA domain-containing proteins, like plant defense suppressor Pi21 [77,98].

6.3. Other Possibilities to Exploit S Factors

In the event that the constitutive knock-out or the silencing of susceptibility genes by genome editing is rendered impossible due to deleterious pleiotropic phenotypes, "silencing on demand" using pathogen-inducible promoters can be an alternative approach. In barley, the pathogen-inducible *Hv-Ger4c* promoter has been successfully used to control the expression of Ta-Lr34res, an ABC transporter that confers resistance against several fungal pathogens in wheat [99].

S genes can also be modified to give rise to artificial decoys that inform R proteins to trigger ETI. This neofunctionalization is of course only applicable for susceptibility factors that are effector targets. Targeting of the artificial decoy by the particular effector protein would consequentially lead to a dead end for this particular effector function. Artificial decoys based on susceptibility genes could eventually be even used to switch plant immunity between pathogen kingdoms, as it was recently shown for artificial R proteins. RPS5, which is an intracellular R protein from *Arabidopsis thaliana*, is normally activated upon the recognition of AVRPPHB SUSCEPTIBLE1 (PBS1) cleavage by *Pseudomonas syringae* effector AvrPphB, with PBS1 serving as a decoy. The AvrPphB cleavage site within PBS1 was substituted with cleavage sites for other pathogen protease effectors, e.g., protease effectors of Turnip mosaic virus, thereby conferring resistance to different pathogens [100].

7. Future Direction

We have discussed several methods and trade-offs for *S* gene exploitation (Figure 2). For the optimal exploitation of *S* genes, future research should focus on further unraveling the molecular mechanisms of *S* gene resistance. This is essential to identify novel susceptibility factors to increase our breeding capacities. Furthermore, intensive research is required to take full advantage of *S* gene exploitation by controlling and, in the best case, diminishing pleiotropic effects. Additionally, whole genome resequencing studies could reveal the diversity and variability of *S* genes in wild crop relatives and heirloom varieties. Combined with large scale protein-protein interaction studies, these findings can be put in a larger *S* gene defense signaling context. Such information will help to understand the durability of *s* gene resistance when compared to *R* gene resistance. One could also try to identify partial *S* gene mutants. Such genes might confer less, but still sufficient field resistance while suffering less from pleiotropic effects. Such genes might be found in natural populations, where they have been selected for, or they might be created by random or knowledge-based approaches.

These new findings can be used for modern breeding and genome editing technologies. In fact, transgenic and marker-assisted breeding have already been utilized for over several decades. More recently, new mutagenesis and gene editing approaches have also been shown to generate strong and functional *s* genes. Thus, the targeted exploitation of susceptibility factors forms a credible and potentially durable route for future resistance breeding.

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Perspective

Pattern Recognition Receptors—Versatile Genetic Tools for Engineering Broad-Spectrum Disease Resistance in Crops

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Abstract: Infestations of crop plants with pathogens pose a major threat to global food supply. Exploiting plant defense mechanisms to produce disease-resistant crop varieties is an important strategy to control plant diseases in modern plant breeding and can greatly reduce the application of agrochemicals. The discovery of different types of immune receptors and a detailed understanding of their activation and regulation mechanisms in the last decades has paved the way for the deployment of these central plant immune components for genetic plant disease management. This review will focus on a particular class of immune sensors, termed pattern recognition receptors (PRRs), that activate a defense program termed pattern-triggered immunity (PTI) and outline their potential to provide broad-spectrum and potentially durable disease resistance in various crop species—simply by providing plants with enhanced capacities to detect invaders and to rapidly launch their natural defense program.

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1. Introduction

Pathogens and pests of plants are a major problem in agricultural food production despite the application of plant protection chemicals [1]. Pre- and post-harvest diseases can cause significant losses in crop yield and impair crop quality. The emergence and global spreading of novel pathogens or pathogen races/strains capable of defeating existing, resistant crop cultivars, such as the wheat stem rust (*Puccinia graminis* f. sp. *tritici*) race Ug99 [2] or the kiwi fruit pathogen *Pseudomonas syringae* pv. *actinidae* [3], and the increasing resistance of many pathogen races/strains against available pesticides, illustrates the vulnerability of our current plant protection strategies and the looming risk of devastating disease outbreaks. Increasing the yield of high-quality plant products on the available arable farm land while reducing the amount of ecologically harmful agrochemicals, necessitates the development of future-oriented, sustainable agricultural production systems and effective but environment-friendly plant protection measures. In the last 20 years, the identification of a range of different molecular plant immune components has not only greatly advanced our mechanistic understanding of the plant immune system but also provided the conceptual framework to deploy these discoveries for genetic plant protection. Using selected examples, I will demonstrate in which ways PRRs and PTI can be deployed for disease resistance management in crop plants and illustrate future perspectives for molecular engineering of PRRs and PRR signaling.

2. Pattern-Triggered Immunity Forms a Robust Host Barrier to Invaders

Plants rely on genetically determined (innate) immunity to protect themselves from potentially harmful invaders such as pathogens, pests, and parasitic plants. Central to the plant immune system is a multi-layered surveillance system of various extra- and intracellular immune receptors that sense molecular features of the invader (non-self recognition) as well as perturbations of the cellular integrity provoked by the invader (altered-self recognition). Detection of such danger signals by plant immune sensors activates local and systemic defense mechanisms [4,5]. The capacity of a plant to detect invasion attempts early and to mount a defense response in time, i.e., before establishment and proliferation of the invader, largely depends on its repertoire of immune sensors capable of recognizing the invader.

2.1. Microbe-Associated Molecular Patterns (MAMPs)

Plants sense a variety of conserved microbial components with vital roles for microbial fitness as immune elicitors [4]. Because of their important functions, these components are usually conserved across microbial species and cannot readily be modulated to evade recognition without a fitness cost. Such immunogenic molecular structures are termed microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs). MAMPs are chemically diverse, e.g. proteins, polysaccharides, lipids or composite molecules. Classical examples of MAMPs are microbial cell wall structures, such as chitin (fungi), beta-glucans (oomycetes), lipopolysaccharide or peptidoglycan (bacteria), or microbial proteins, such as bacterial flagellin or elongation factor thermo-unstable (EF-Tu, part of the cellular protein translation machinery) [6]. In case of larger molecular structures, a defined partial structure (epitope or pattern) of these molecules is generally sufficient to activate plant immunity. One of the best-studied examples is the flg22 epitope, which corresponds to a stretch of 22 amino acids from the highly conserved N-terminus of flagellin, a region which is important for assembly of the flagellin monomers into of a functional flagellum and for bacterial motility. A synthetic flg22 peptide is sufficient to activate the same defense program as the natural flagellin protein [7]. Similarly, the peptide elf18, which corresponds to the conserved N-terminus of EF-Tu, is sufficient to trigger plant immunity [8]. The use of synthetic peptide MAMPs has greatly expedited functional studies of these proteinaceous elicitors.

The isolation and identification of MAMPs is often a challenging task. While it is relatively easy to enrich fractions of microbial extracts that trigger PTI-like defense responses in plants, the identification of the causal molecular motif is demanding and requires sophisticated biochemical and analytical skills [6,9]. This is largely due to the fact, that MAMPs are not necessarily very abundant microbial components. To detect invaders timely, host plants evolved highly sensitive immune receptors that detect MAMPs at very low concentrations (nanomolar range) [9]. Thus, although novel analytical techniques have expedited the identification of MAMPs, these are usually not routine methods applicable for high-throughput identification of MAMPs from microbial populations.

Studies on proteinaceous MAMPs are at the forefront of research because they can be produced in large amounts and high purity through recombinant expression systems or chemical synthesis, can be easily genetically modified, and are amenable to population-wide genetic studies. Once a protein motif has been identified as MAMP epitope in a microbial species/race/strain, the DNA sequence of the respective gene can be easily analyzed in silico in many species. Such bioinformatic studies in combination with functional assays have revealed that MAMP epitopes are not as strictly conserved as was initially assumed. Although the protein sequences of MAMPs are overall conserved to maintain their function, the MAMP epitopes were found to be more diversified, presumably due to the selective pressure exerted on microbes to evade host immune sensing through these epitopes [10]. flg22 epitopes from different bacterial species, for example, are sensed with different efficacy in different plant species and some flg22 motifs are not detected at all in some plant species [7,11–15]. The specific genetic fingerprints resulting from the opposing (purifying versus diversifying) selection pressure on MAMPs can, in turn, be exploited to identify putative MAMP epitopes *de novo* by means of bioinformatic genome surveys of microbial populations [16]. In a proof-of-concept study several candidate MAMP

epitopes identified by screening *P. syringae* and *Xanthomonas campestris* genome data were shown to elicit PTI in Arabidopsis. Hence, with the increasing availability of microbial genome data such in silico screenings will greatly speed up identification of proteinaceous MAMPs in the future and facilitate targeted screenings for MAMP epitopes in pathogen species of interest.

To date, numerous MAMPs from all microbial classes that are sensed by different plant species have been identified and the list is continuously growing (for a comprehensive summary see recent reviews [6,17,18]). In addition, immunogenic molecular patterns have also been enriched from nematodes, insects, and parasitic plants [6,18,19]. Taken together, this demonstrates the central role of MAMP sensing for plant defense against different types of invaders.

2.2. Damage-Associated Molecular Patterns (DAMPs)

Invaders are not only sensed by the plant immune system via their own molecular components (exogeneous elicitors) but usually also provoke the release of plant-derived signals characteristic of infection, called endogenous elicitors or damage-associated molecular patterns (DAMPs) [4]. Typical examples of DAMPs are fragments of cell wall components generated during attack by microbial cell wall-degrading enzymes, e.g., oligogalacturonides (OGs, derived from pectin) [20] or cutin monomers [21], and intracellular plant components released into the extracellular space upon cell lysis, such as extracellular ATP (eATP) [22], extracellular NAD (eNAD) [23], or intracellular proteins (e.g., Arabidopsis HMGB3) [24]. Like MAMPs, DAMPs are sensed by cell surface-resident PRRs and activate typical PTI signaling and defense responses [4].

In addition, plants release various endogenous peptide hormones into the apoplast upon wounding or pathogen attack. Because these peptides trigger typical PTI defense responses via PRR-like receptors, they are also classified as DAMPs. The systemin peptide, for instance, is produced upon wounding in tomato and contributes to plant defense against herbivorous insects [25]. Two peptide families from Arabidopsis, termed AtPEPs and AtPIPs, that are produced upon biotic stress and wounding, apparently play an important role in the amplification of PTI [26–28]. Peptides of the PEP family are found in various monocot and dicot plant species [29,30], suggesting that amplification of PTI by endogenous peptide hormones is a common strategy in plant immunity.

2.3. Pattern Recognition Receptors (PRRs)

MAMPs and DAMPs are detected by immune receptors localized at the host cell surface, called PRRs (Figure 1) [4]. PRRs are typically single-span transmembrane or membrane-anchored proteins with structurally diverse extracellular domains, such as leucine-rich repeat (LRR), Lysin-motif (LysM) or lectin domains, that bind MAMP/DAMP epitopes with high specificity and sensitivity [18]. Receptor-like kinase (RLK)-type PRRs possess a cytosolic kinase domain, which usually functions in intracellular signal transduction (Figure 1). Receptor-like protein (RLP)-type PRRs that lack active signaling domains and RLK-type PRRs with non-functional kinase domains require signaling-competent protein partners [18,31]. In general, however, PRRs (RLP-type as well as RLK-type) do not function alone but are part of multi-protein complexes where they engage with co-receptors, signaling partners and regulatory proteins that fine-tune PRR activation/deactivation for appropriate signaling output (Figure 1) [31]. Among the best-studied PRRs to date are the flg22 receptor FLS2 and the elf18 receptor EFR, both of which are LRR-type RLKs [8,32]. Many other LRR-type RLKs and RLPs also detect proteinaceous MAMPs, e.g., RLP23 senses the nlp20 epitope of necrosis- and ethylene-inducing peptide 1-like proteins (NLPs) which show a remarkably broad distribution in bacteria, fungi as well as oomycetes [33,34]. Whereas binding of a MAMP or DAMP to its respective PRR is highly specific, other receptor complex components are commonly shared by several PRRs (Figure 1) [31]. Various LRR-type PRRs (e.g., FLS2 and EFR) interact with the LRR-RLK BAK1 in a ligand-dependent manner [35]. LRR-RLP-type PRRs constitutively associate with the signaling adapter LRR-RLK SOBIR and also recruit BAK1 upon ligand binding [36]. The LysM-RLK CERK1 similarly interacts with different LysM-type PRRs [31].

Individual plant species apparently harbor numerous PRRs that sense different classes of invaders in a partially redundant manner. In this way, each invader is sensed by several PRRs. Arabidopsis, for instance, senses at least seven MAMPs from Pseudomonas bacteria through distinct PRRs [37]. Presumably, the diversity and functional redundancy of PRRs is central to the remarkable robustness of the PTI system. Some PRRs are broadly distributed across the plant kingdom (evolutionary old, e.g., FLS2) whereas others are specific to individual plant families or species (evolutionary young, e.g., EFR) [4]. As a consequence, different plant species have different but partially overlapping sets of PRRs. Just as invaders continuously evolve to evade MAMP sensing, plants continuously adapt their PRRs and evolve new PRRs that facilitate detection of novel MAMPs or of other epitopes within a MAMP. Tomato, for instance, has a second flagellin receptor, FLS3, that detects an epitope (termed flgII-28) distinct from flg22 (sensed by FLS2) [38]. Similarly, rice can sense another EF-Tu epitope (EFa50) distinct from elf18 which is sensed in *Brassicaceae* [39]. Thus, the genetic diversity of plants provides a rich source of PRRs not only for a multitude of different MAMPs from various kinds of invaders but also for distinct MAMP epitopes.
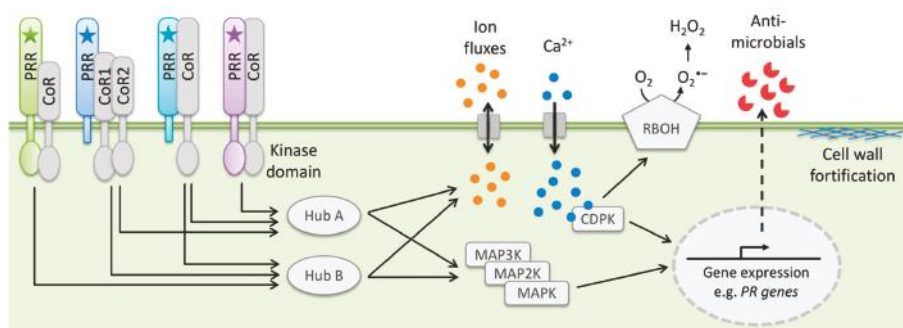
Figure 1. Scheme of typical MAMP and DAMP-activated immune responses in plant cells. MAMPs and DAMPs (star symbols) are sensed by specific host PRRs localized at the cell surface. Signaling pathways downstream of PRRs converge on common signaling components, for instance co-receptors (CoR) or signaling HUBS. PTI responses include for example fluxes of various ions—including the secondary messenger Ca^{2+}—across the plasma membrane, production of ROS by RBOH-type oxidases, activation of calcium-dependent and mitogen-activated protein kinases (CDPKs and MAPKs), gene expression changes, production of antimicrobial compounds, and fortification of the plant cell wall.

2.4. Pattern-Triggered Immunity (PTI)

Detection of a MAMP or DAMP by its respective PRR is highly specific. However, downstream of PRRs, signaling pathways converge on common signaling hubs, often already at the receptor complex level, because certain co-receptors (e.g., BAK1, SOBIR, and CERK1) can act in multiple receptor complexes (Figure 1) [4,5,31]. Therefore, PRRs generally activate a quite stereotypic defense program referred to as PTI. Typical PTI signaling and defense responses are, for example, the depolarization of the plasma membrane, an increase in the cytosolic concentration of the secondary messenger Ca^{2+}, activation of different protein kinases, production of reactive oxygen species (ROS), induction of defense-related genes, cell wall fortifications, and production of antimicrobial enzymes and secondary metabolites as well as defense-related plant hormones (Figure 1) [4,31]. PTI is not restricted to the site of infection but establishes in a systemic manner, resulting in an increased resistance of distal, uninfected parts of the plant to secondary infection with the same or unrelated pathogens [4,40]. Plant immune components including PRRs are systemically upregulated upon elicitor sensing, which further enables the plant to respond faster and stronger to a subsequent pathogen attack [4].

Although PTI is a temperate immune response that usually does not culminate in a hypersensitive response (HR, a programmed cell death reaction), it nevertheless constitutes a robust host barrier

effective against commensals and non-adapted pathogens and maintains a basal level of immunity during infection with adapted pathogens [5]. The vital role of PTI is illustrated by the following observations: (i) Loss of individual PRRs renders plants more susceptible to infection with microbes harboring the respective MAMP [5,41,42]; (ii) Treatment of plants with MAMPs to activate PTI (which depends on the presence of a functional, matching PRR) results in enhanced resistance to subsequent local or systemic infection [5,40–42]; (iii) For successful host colonization pathogens need to overcome PTI by evading MAMP sensing or by releasing effectors that subvert PTI through interfering with MAMP/DAMP detection or downstream signaling and defense responses (adapted pathogens). If the effector repertoire of the pathogen sufficiently suppresses host immunity, the pathogen can establish itself in the host and cause disease (effector-triggered susceptibility, ETS) [5,43].

MAMPs are not specific to pathogens. Accordingly, commensal and beneficial microbes are also sensed by PRRs unless they have adapted their MAMP epitopes to evade detection [44,45]. Indeed, it is now evident that PTI restricts not only the growth of pathogenic microbes but also controls colonization of plant tissues with commensal and beneficial microbes [44–47]. Presumably, the diversity and functional redundancy of PRRs in combination with signal amplification through feedback-induction of genes encoding PRRs and PTI signaling components and diverse plant hormones is key to the apparent robustness of the PTI system. Indeed, breaching of host immunity against natural commensal bacteria requires inactivation of multiple PRRs simultaneously or of co-receptors shared by several PRRs [46]. In conclusion, the PRR class of immune sensors and PTI form a robust protective barrier against various types of plant colonizers and are central to plant health.

3. Prospects of Deploying PTI for Broad-Spectrum Disease Resistance Engineering in Crops

It has been known for decades that microbial and plant-derived elicitors (now usually called MAMPs and DAMPs) trigger defense responses in diverse plant species [48–51]. However, only with the molecular identification of PRRs it was possible to unravel the perception and cellular signaling mechanisms in sufficient detail to eventually exploit this central layer of the plant immune system for genetic plant protection in a purposive manner.

3.1. Pattern- versus Effector-Triggered Immunity

Because of the conserved nature and broad occurrence of MAMPs in different microbes, sensing of MAMPs to activate PTI enables the host to detect and efficiently control a wide range of microbes [4]. Adapted pathogens employ effectors to dampen PTI and to modulate host cell metabolism for their own needs. Plants, in turn, evolved immune sensors, called resistance (R) proteins that detect microbial effectors directly or indirectly by monitoring the effector targets, thus turning effectors into avirulence factors. This results in effector-triggered immunity (ETI), which includes overall similar defense responses like PTI but usually develops faster and in a stronger fashion and is mostly accompanied by an HR [5]. Since effector genes are typically specific to certain pathogen races, while *R* genes are limited to certain plant cultivars, this form of resistance is called race-specific or gene-for-gene resistance. In a co-evolutionary arms-race with their hosts, pathogens may be able to overcome *R*-gene mediated ETI relatively easy [5,52]. Mostly, recognized effectors are not essential and/or functional redundant allowing microbes to evade recognition by diversifying or even losing them. Alternatively, microbes may produce novel effectors that e.g. suppress the same or other PTI components or R protein signaling [5,52]. To date, plant resistance breeding widely relies on race-specific resistance mechanisms, largely because effectors and *R* genes typically result in complete or near-complete resistance. The selective pressure exerted on pathogen populations by ETI, however, may result in rapid resistance breaching in the field by the appearance of novel pathogen races/strains [52]. PTI, by contrast, enables defense against a broad spectrum of pathogens and presumably is more durable because of the evolutionary constraints on MAMP modulation and the quantitative resistance conferred by PRRs which reduces the selective pressure on the pathogen.

3.2. PRR Transfer Between Plants Species

One feature makes PRRs particularly attractive targets for genetic engineering of plant immunity: signaling networks downstream of PRRs are sufficiently conserved (Figure 1), not only within but also between plant families and even between monocot and dicot classes, to facilitate functional transfer of PRRs between them [53–55]. Apparently, PRRs can plug into the existing signaling network by engaging with conserved interacting signaling partners (e.g., SOBIR, BAK1 or CERK1) (Figure 2) [54,55]. Because PRRs as the ligand binding components of PTI receptor complexes determine the epitope specificity, introducing a PRR with a novel epitope specificity can confer recognition of this epitope onto previously insensitive plant species. In this way, plants can be equipped with additional MAMP sensing capacities to enhance disease resistance to pathogen classes harboring the respective MAMPs (Figure 2). These can either be PRRs for MAMPs not yet sensed by the recipient plant or for additional epitopes within a given MAMP (e.g., flgII-28/FLS3). A breakthrough study utilizing the *Brassicaceae*-specific EFR receptor has proven the general feasibility of this strategy [53], which was subsequently shown for multiple PRRs in various plant species (summarized in [6,56]). EFR-transgenic tomato (*Solanum lycopersicum*) and *Nicotiana benthamiana* plants gained immune responsiveness to the elf18 epitope and were more resistant to several major bacterial pathogens carrying this epitope including *Ralstonia solanacearum*, *P. syringae*, *Xanthomonas perforans*, and *Agrobacterium tumefaciens* [53]. EFR was subsequently also successfully transferred into monocots, such as wheat (*Triticum aestivum*) and rice (*Oryza sativa*) [55,57]. Similarly, transfer of the rice PRR XA21, which detects the RaxX protein from *Xanthomonas oryzae* pv. *oryzae*, to banana (*Musa* sp.) and sweet orange (*Citrus sinensis*) confers increased resistance against different *Xanthomonas* spp. [58,59] and expression of the PRR ELR from a wild potato species renders cultivated potato (*Solanum tuberosum*) more resistant to the oomycete pathogen *Phytophthora infestans* [60]. Taken together, these results demonstrate that addition of only a single PRR can already significantly enhance resistance of plants to adapted pathogens. Given that these pathogens are equipped with an arsenal of effectors to dampen PTI, this is quite astonishing and suggests that increased pathogen sensing provides a substantial advantage to the plant, possibly because of a stronger and/or faster onset of defense reactions.
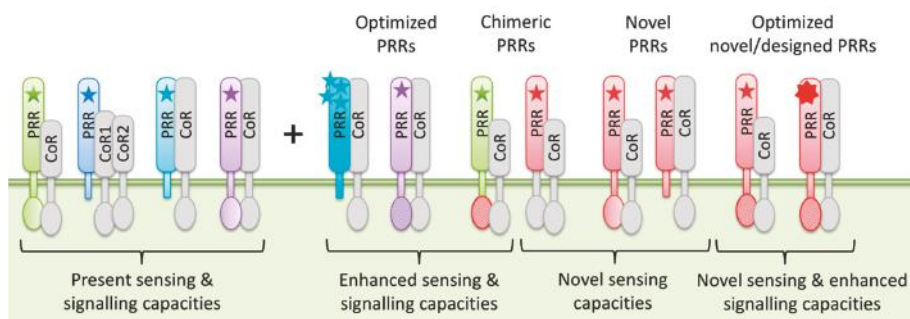
Figure 2. Schematic representation of potential PRR transfer and engineering options. PRR-mediated sensing of MAMPs/DAMPs (star symbols) and PRR signaling capacities can be enhanced by optimization of endogenous PRRs or introduction of novel, chimeric or even "designer" PRRs. This equips plants with additional MAMP/DAMP sensing and improved signaling capacities and results in enhanced PTI against a broad spectrum of invaders.

Often, the isolation of MAMPs is the bottleneck in identifying PRRs [6,9]. However, it is not necessary to structurally identify a MAMP but sufficient to enrich it to a degree that facilitates genetic screenings for the respective PRR [61–63]. Furthermore, the identification of various PRRs for chemically diverse types of MAMPs has revealed that plant PRRs are typically RLKs or RLPs. Thus, these protein families can now also be systematically tested for a putative role in plant immunity,

for instance through screening for altered resistance against the pathogen of interest in loss- or gain-of-function mutants or natural accessions, or through heterologous expression in suitable model plants (e.g., *Arabidopsis thaliana* or *N. benthamiana*) [64–69].

A major concern of introducing novel PRRs into plant species is that this might negatively affect beneficial interactions of these plant species with their natural microbiota and/or symbiotic interactions, such as legume-rhizobia or mycorrhizal interactions. Transgenic Medicago plants expressing EFR from Arabidopsis, however, are apparently not defective in symbiosis and are more resistant to the root pathogen *R. solanacearum*. Although the EFR transgenic plants showed a delay in nodule formation the final extent of nodulation and nitrogen fixation of the EFR transgenics was comparable to the wild-type plants [70]. Potentially, adapted symbionts have evolved efficient strategies to evade or suppress host PTI at various levels.

While to date mostly strong, constitutive promoters (e.g., viral promoters such as the 35S promoter of cauliflower mosaic virus, plant ubiquitin or actin promoters) are used to drive heterologous PRR expression in plants because of their universal functionality in diverse plant species and different plant tissues, this can result in unwanted side effects on general plant performance. Overexpression of RLKs, for instance, may trigger their activation in the absence of ligand and lead to growth defects because of constitutive activation of PTI or interference with developmental signaling [71]. This can be overcome by using plant promoters from e.g. endogenous PRR genes that drive PRR expression in plant tissues preferentially targeted by the pathogen of interest (e.g., root) or at sites of pathogen entry (e.g. stomata guard cells) and are strongly induced above the basal level upon infection [72,73].

The growing interest in PTI in recent years already led to the identification of numerous PRRs from different model and wild plant species and there is more to come (summarized in [6,17]). Natural diversity provides plant breeders with a versatile genetic tool box for crop improvement. Relevant PRR genes can be introduced, for instance, from wild relatives through classical breeding strategies. However, this is usually a lengthy process, bears the risk of co-segregation of unfavorable traits (linkage drag), and is not applicable to all crop species (e.g., banana, which is sterile) [56,74]. Alternatively, modern genetic engineering tools facilitate direct transfer of PRR genes across plant families beyond the constraints of sexual compatibility with the advantage that PRR genes can be quickly introduced into elite crop varieties as single traits (Figure 2). Additionally, this allows to utilize virtually any plant species as source of PRR genes. In conclusion, PRR transfer has great potential for conferring broad-spectrum and potentially durable resistance traits onto crop plants.

3.3. PRR Engineering

During co-evolution with host plants, pathogens modulate MAMP epitopes to evade host immunity whereas plants adapt their PRRs [16]. Hence, there is a natural diversity of PRRs that recognize slightly different variants of a given MAMP epitope. Ecotype collections of plant species harboring a particular PRR are thus a rich source of PRR variants with enhanced sensing capacities for these epitope variants (Figure 2) [75,76]. Additionally, in vitro evolution of PRRs can be applied to produce PRRs with altered ligand specificities [77]. With the increasing availability of PRR ectodomain structures and computational modelling tools ligand binding sites of PRRs can be modified in a directed manner to perceive a desired epitope variant or may eventually even be designed *de novo* (Figure 2). Potentially, ligand binding sites can be engineered at the native gene locus through CRISPR/Cas-mediated genome editing.

Some PRRs may not be able to integrate optimally with the endogenous signaling adapters upon transfer in more distantly related plant species. In such cases, full signaling competence can be restored by exchanging the transmembrane and/or intracellular signaling domain with a related PRR from the recipient species (Figure 2) [54,62]. Such chimeric PRRs have been shown to be fully functional and can be further exploited to combine different ligand binding specificities with different downstream signaling capacities (Figure 2). Some PRRs naturally induce a stronger immune response including an HR, such as the rice RLKs XA21 [78]. Sensing of chitin fragments by the rice PRR CEBiP,

by contrast, does not result in HR [79]. A chimeric receptor combining the chitin-sensing ectodomain from CEBiP with the intracellular signaling domain of Xa21 activates HR upon chitin sensing and enhances resistance to the rice pathogen *Magnaporthae oryzae*, a pathogen that does not naturally activate Xa21 signaling [80].

3.4. Exploiting DAMP Signalling

Transfer and engineering of PRRs sensing DAMPs are also possible given that the respective DAMP is produced in the recipient species during infection with the pathogen of interest. Expression of the Arabidopsis eATP receptor DORN1 (also known as P2K1) in potato, for instance, enhances resistance to *P. infestans* [22,81]. Alternatively, plants can be engineered to produce the desired DAMP upon infection with diverse pathogens, for instance, by introducing a microbial enzyme producing the DAMP under control of a pathogen-responsive promoter. In this way, any pathogen that induces transcription of the transgene will trigger PTI independent of the ability to produce this DAMP itself. A promising example is the production of elicitor-active OG fragments through the balanced action of a microbial polygalacturonase (PG) and a plant PG inhibitor. Pathogen-inducible expression of this OG factory in Arabidopsis increased resistance to *Botrytis cinerea*, *Pectobacterium carotovorum* and *P. syringae* infections [82]. Such pathogen-responsive *in situ* production of DAMP signals in combination with providing the respective PRR could be a generally applicable strategy to boost PTI in any plant species and to confer resistance to a wide range of pathogens.

4. Conclusions

Most PRRs known to date have been identified from model species such as Arabidopsis, rice and recently also increasingly from tomato and potato [6,17]. These rather limited studies already illustrate the enormous resource of plant immune sensors available in different plant species that just await discovery. With novel genetic and computational tools at hand we can now exploit this natural genetic tool box for providing plants with expanded capacities to sense any kind of undesired invaders and strengthen their natural defense to resist them. Moreover, a mechanistic understanding of PRR function enables us to accelerate PRR evolution artificially, to modify PRRs in a targeted manner or even design novel PRRs.

Although PTI is generally more difficult for pathogens to overcome than ETI, pathogens will, albeit presumably on a longer time scale, eventually succeed. Hence, the deployment of single PRRs still poses the risk that pathogen populations eventually adapt their MAMP epitopes or their effector repertoire, particularly if the selection pressure on pathogen populations is constantly high. To deploy PRR engineering in a durable manner, combination of several PRR and R genes (stacking or pyramiding) appears to be a promising strategy as pathogens are unlikely to overcome several immune receptors at the same time. Possibly, resistant varieties equipped with different PRR/R gene combinations can be used in alternation to lower the selective pressure of the individual components on pathogen populations. If utilized in a thoughtful manner, these genetic tools have great power for developing truly durable and sustainable plant disease management practices.

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Review

Functional Metabolomics—A Useful Tool to Characterize Stress-Induced Metabolome Alterations Opening New Avenues towards Tailoring Food Crop Quality

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Abstract: The breeding of stress-tolerant cultivated plants that would allow for a reduction in harvest losses and undesirable decrease in quality attributes requires a new quality of knowledge on molecular markers associated with relevant agronomic traits, on quantitative metabolic responses of plants to stress challenges, and on the mechanisms controlling the biosynthesis of these molecules. By combining metabolomics with genomics, transcriptomics and proteomics datasets a more comprehensive knowledge of the composition of crop plants used for food or animal feed is possible. In order to optimize crop trait developments, to enhance crop yields and quality, as well as to guarantee nutritional and health factors that provide the possibility to create functional food or feedstuffs, knowledge about the plants' metabolome is crucial. Next to classical metabolomics studies, this review focuses on several metabolomics-based working techniques, such as sensomics, lipidomics, hormonomics and phytometabolomics, which were used to characterize metabolome alterations during abiotic and biotic stress in order to find resistant food crops with a preferred quality or at least to produce functional food crops.

Keywords: plant stress; abiotic stress; biotic stress; metabolomics; phytometabolomics; sensomics; phytohormonics; liquid chromatography-mass spectrometry (LC-MS/MS); nuclear magnetic resonance spectroscopy (NMR); targeted metabolomics; untargeted metabolomics; functional food

1. Importance of Metabolomics for Agricultural Research

In the environment, plants are often exposed to an enormous number of biotic and abiotic stress factors, such as pathogen and insect infestation as well as extreme temperatures, drought, salinity, pollutants, heavy metals or nutritional deficiencies. That leads to harvest or quality losses, such as the formation of a pronounced bitter off-flavor and sometimes causes toxicological problems as well as huge global economic losses [1]. The breeding of stress-tolerant cultivated plants that would allow for a reduction in harvest losses and undesirable decrease in quality attributes requires a new quality of knowledge on molecular markers associated with relevant agronomic traits, on quantitative metabolic responses of plants on stress challenges, and on the mechanisms controlling the biosynthesis of these molecules [2]. Thereby, knowledge of the biologically active metabolites, especially secondary metabolites as well as metabolic networks of primary and secondary metabolites affected by plant stress conditions, is essential.

The metabolome of plants represents the complete set of low-molecular weight metabolites (such as primary metabolites including all necessary metabolic intermediates, hormones and other signaling molecules, as well as secondary metabolites) in a given organism, a biological cell, tissue,

or organ at a certain point in time and development. For a long time, primary and also secondary metabolites were simply considered as one of the end-products of gene expression and protein activity. Nowadays, it is increasingly accepted that low-molecular weight molecules modulate macromolecular processes through, e.g., feedback inhibition and by signaling phytohormons [3]. Therefore, Dixon et al. [3] conclude that, metabolomic studies are "intended to provide an integrated view of the functional status of an organism". But monitoring the whole metabolome with exclusively one technique is not possible at the moment [4]. Although for a single plant like *Arabidosis thaliana* no more than 5000 metabolites have been assumed, the plant kingdom is reported to contain between 200,000 and 1,000,000 metabolites, all of them having different structural features and polarities [5–9]. This wide structural diversity with different chemical properties is a challenging task during sample extraction and analysis [4]. In addition, the knowledge about functional, secondary metabolites, on having an antifungal, toxic, off-flavor activity or promising human health-promoting activities, is not yet sufficient.
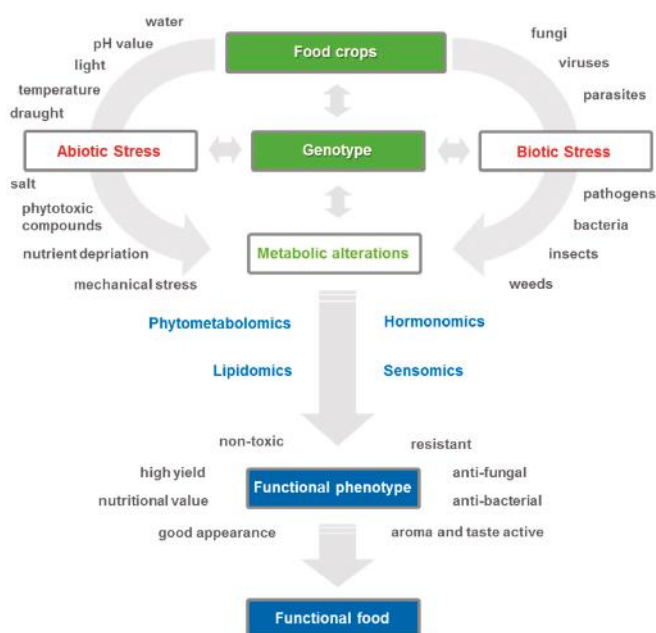
Figure 1. Functional metabolomics—a useful tool to discover metabolome alterations during abiotic and biotic stress to find the perfect functional pheno-/genotype to produce functional food.

Therefore, an important aspect of the use of crop plants for human consumption is the characterization and determination of their nutritional value as well as their individual flavor-active, health-promoting, non-toxic, functional constituents. The aim of crop plant research should be to find an optimal functional pheno-/genotype as a golden standard with high resistance and best possible quality criteria for future breeding experiments (cf. Figure 1). Analytical marker compounds can help to support molecular breeding efforts to obtain phenotype knowledge-based plant populations, no matter which analysis method is used, bulk segregant analysis, mapping by sequencing or quantitative trait loci (QTL) analysis [10,11]. In sum, metabolomics, especially the characterization of functional relevant metabolites, also called functional metabolomics, can be used as a tool for functional genomics [2,9,12,13]. For example, integration of metabolomic data with genomics and transcriptomics, especially that obtained from transcriptome coexpression network analysis, helped to decode several functions of genes and to gain a deep insight into the biological processes of the model

plant *Arabaidopsis* [2,9]. Although more studies are desirable, at the moment only manuscripts are available in which secondary, functional metabolites of non-model plants are characterized [2,9,12,13]. Next to studies using potatoes, strawberries, carrots or *Brassica napus* [9,14–18], Rambla et al. [18] performed a QTL analysis using a cross between *Solanum lycopersicum* and a red-fruited wild tomato species *Solanum pimpinellifolium* to relate 102 QTLs to 39 corresponding volatile organic compounds, which partly contribute to the typical flavor impression of tomatoes. In addition, by means of genome-wide association studies 323 associations among 143 single nucleotide polymorphisms and 89 metabolites were identified, including non-volatile, functional, secondary metabolites, which can be used for new human drug production [19].

Next to the genetic code, environmental stress especially influences the metabolome composition of plants. Stress conditions are sensed by the plant, which then activates a network of signaling pathways, including the participation of phytohormons, and leads to changes, on the one hand, in the primary metabolism and, on the other hand, in the up-regulation of phytochemicals [20].

For example, during fungi infestations, antifungal phytochemicals, especially metabolic pathways leading to isoprenoids, phenylpropanoids, alkaloids, fatty acids, and polyketides are up-regulated [21]. Many phytoalexins, which partly have been described as flavor-active, nutritional components and as a source for development of health-promoting food products, have been well documented in crop plants in the field of plant defense [21]. In contrast, primary metabolism is documented to be mainly influenced by abiotic stress challenges [5,20–22]. For instance, in *Arabidopsis* leaves, next to the amino acid profiles, oligosaccharides, γ-amino butyrate and the metabolites from the tricarboxylic acid cycle are influenced during drought conditions [23].

Analysis of those changes in the molecular composition of plant material by means of mass spectrometry (MS) or nuclear magnetic resonance spectroscopy (NMR) based techniques is called metabolomics, which can profile the impact of time, stress, nutritional status and environmental perturbation on hundreds of metabolites simultaneously. It is an essential technology for functional genomics and systems biology which can visualize and answer questions about biological systems [3,24]. Kushalappa and Gunnaiah [25] already predicted that while in the past, genetic tools were mainly used for crop improvement on yield, flagged as the green revolution, in the next few decades functional genomics, which allows the identification of genes that enhance crop yields without losses in nutritional value while minimizing loss due to stress, will be the main topic. Metabolomics is especially helpful to discover metabolites up-regulated during stress conditions [2]. These metabolites then can be tested for their biological functions. Thereby, the combination and understanding of information received from genomics, transcriptomics, proteomics and metabolomics studies is a complementary tool for functional genomics and systems biology investigations. Although nowadays, next to the human genome [26], the genomes of several plants such as *Arabidopsis thaliana* [27], rice [28,29], tomato [30], and barley [31] have been decoded by means of automated nucleotide sequencing, knowledge about the regulation of gene expression, about the action of these genetic products, and especially about the metabolic networks resulting from catalytic proteins, is rather fragmentary [3].

Besides aiming to avoid quality or yield losses induced by abiotic or biotic stress conditions, there are exciting new strategies of using plant stress as a way of producing phytoalexin-enriched functional foods [32] or to enhance the flavor quality of crop plants by stress or elicitor challenges to produce new functional foodstuffs with high quality [33]. Dixon et al. [3] additionally suggest that the long-term goal should be the application of new knowledge of functional metabolites for "rational custom-designed breeding by classical methods as well the application of genetic engineering techniques to improve and develop new aromatic plants". Therefore, publicly open databases, containing all spectroscopic, spectrometric and bioactivity data as well as the biosynthetic pathways of worthy metabolites are necessary in the future [3].

Targeted as well as non-targeted metabolomic-based working techniques, such as phytometabolomics, sensomics, lipidomics or hormonomics can help to unravel metabolic pathways,

signal transmissions as well as metabolites with different bioactivity which can be simultaneously up- or down-regulated by stress conditions. After decoding marker compounds by means of these non-targeted approaches, marker metabolites can be isolated from the plant material and characterized by means of 1/2D-NMR or MS based structure identification techniques as well as by biological assays (e.g., using antifungal susceptibility test systems). Although the last-named structure identification techniques and the testing of the metabolite's bioactivity do not belong to the metabolomics approach, its application is mandatory to understand biological processes.

2. Analytical Techniques Used to Characterize Stress-Induced Metabolome Alterations in Plants

Enormous technological progress has been achieved since the early days of plant metabolome characterization. Thin-layer chromatography (TLC) together with gas chromatography coupled to a flame ionization detector (GC-FID) have so far been applied to characterize primary and secondary metabolites in plant materials. Nowadays, besides NMR, high-throughput, sensitive techniques using gas chromatography, liquid chromatography, ultra-high performance liquid chromatography/time-of-flight as well as capillary electrophoresis coupled to mass spectrometry (GC-MS, LC-MS/MS, UHPLC-TOF-MS or CE-MS), together with bioinformatics tools, are state-of the art working techniques [3,13]. With the development of mass spectrometry imaging approaches, including matrix-assisted laser desorption (MALDI) or desorption electrospray (DESI) ionization procedures coupled to high resolution mass spectrometers, it is possible to perform metabolomics analysis in situ, as well [34]. Although NMR-based techniques are used in plant metabolomics, it is less common in plant-stress metabolomics. However, new working techniques including NMR-technology, such as differential off-line LC-NMR (DOLC-NMR), show promise for characterizing metabolites up- or down-regulated during biotic or abiotic stress influences on plants [35].

Although the term plant metabolomics is defined as the identification and quantification of all low-molecular weight substances in an organism, at a defined point of harvest time and development stage and in a given organ, tissue or cell type, there is no single work-up protocol or standard technique available to detect all metabolites using only one platform. Therefore, several analytical separation and detection techniques are usually combined to visualize abiotic and biotic stress induced metabolome alterations of different plants induced by abiotic and biotic stress [4].

In the last four decades, different methods have been established for the targeted and non-targeted characterization of plant metabolites (cf. the following reviews: Shualev et al. [20]; Urano et al. [22]; Obata and Fernie, [5]; Arbona et al. [4]; Kumar et al. [2]). Nowadays, we differentiate between the non-targeted, sometimes also called untargeted, and the targeted metabolomics approach. While the non-targeted approach represents a comprehensive analysis of all measurable compounds, including unknown metabolites, targeted metabolomics is the measurement of defined groups of chemically characterized and biochemically annotated substances [36]. For both, targeted and non-targeted analyses GC-MS, UHPLC-TOF-MS, as well as LC-MS/MS are the most common techniques to characterize stress-induced metabolome alterations in plants [5].

In particular, primary metabolites, such as carbohydrates, amino acids or fatty acids and all volatile compounds are mainly characterized by means of targeted GC-MS with or without derivatization [37]. Due to the fact that standard compounds are commercially available and spectrometric data is published in several public libraries, such as NIST (National Institute of Standards and Technology), the identification and exact quantification of primary metabolites by means of GC-MS analysis is straightforward. In contrast, structure identification of secondary metabolites exclusively by means of MS data is rather challenging [4,38]. Metabolomics approaches mainly identify secondary non-volatile compounds by means of non-targeted UHPLC-TOF-MS techniques. In contrast to GC-MS, LC-MS libraries for structure identification are less developed, as instrument-type dependent mass spectra and MS fragmentation patterns, as well as retention time shifts depending on used LC columns complicate the comparison of structure identification results using different instruments [39]. In the

future, a worldwide concept of raw data processing, extensive mass spectral libraries, and powerful database management systems that can store and provide both raw and meta data is desirable.

At the moment, biological active compounds are unequivocally identified by means of MS and 1/2D NMR experiments after their isolation by means of preparative HPLC techniques. But the MS spectra obtained has mostly not been shared with the metabolomics community due to the absence of a standardized data-handling concept. Once identified, sensitive quantitation methods using stable isotope dilution assays (SIDA) are carried out by means of LC-MS/MS or GC-MS techniques. Therefore, nowadays, determination of the exact concentration of the isotopically labelled internal standards can best be achieved by means of quantitative NMR spectroscopy (q NMR) [40].

Although the aforementioned analytical metabolomics techniques nowadays are reliable and very sensitive, they still have their limitations regarding small sample volumes or low quantities of metabolites (partly in the nanomolar range). This is especially so when metabolomics is used to answer specific questions in the field of functional genomics or systems biology to get a comprehensive understanding of plant growth, development, defense or productivity, and knowledge about metabolic pathways and networks at cellular levels is required [9,12,41–43]. In addition to single cell-cell genomics, transcriptomics or proteomics, several groups developed plant single-cell and single-cell type metabolomics during the last decade [43–46]. While using single-cell metabolomics, the metabolome of one individual cell, such as a single epidermal cell, is analyzed, while in single-cell-type metabolomics the metabolome of a population of cells, such as guard cells or trichomes, is characterized [43]. Whereas in studies using single animal cells, more than 300 compounds could, nowadays, be visualized by means of capillary electrophoresis-electrospray ionization-time-of-flight-mass spectrometry (CE-ESI-TOF-MS) methods. The amounts of metabolites characterized in smaller plant-based single-cells is still much lower [42,43]. To gain a better understanding of biological systems and characterize metabolic pathways in single cells or in situ, special attention should, in the future, be given to the development of more sensitive MS apparatuses. This is especially the case in the development of more sensitive visualizing MS techniques, such as MALDI or DESI imaging experiments, and in the improvement of extraction protocols [13,43]. Newly developed derivatisation strategies are also promising strategies to improve the MS sensitivities in future. Next to limitations in measuring low sample amounts, data interpretation sometimes is challenging.

A major challenge for combining all omics-techniques is the integration of detailed metabolomics data into transcriptomic and proteomic profiling data sets [3,9,12,13,20]. Although Arbona et al. [4] only recently reviewed the role of metabolomics as a tool to investigate abiotic plant stress in the context of systems biology, and how metabolomics can be integrated into other omics data sets, there is still a lack of knowledge. Furthermore, Urano et al. [22] summarized arguments that holistic omics analysis and their data interpretation are essential to identify the broad function of metabolite regulatory networks during the responses to plant stress. Therefore, bioinformatics knowledge and tools should be used more often in the future to combine the information from both research fields. Although databases and pathway viewers (e.g., an overview is given by Bino et al. [12] or de Souoza et al. [47]) have been further developed in recent years, inter alia Sumner et al. [13] also emphasized that standardized databases integrating metabolomics with other global-omics data are required.

3. Phytometabolomics—From Plant Stress to Metabolic Response

Plants continuously encounter various biotic and abiotic environmental stresses during their growth and development phases. While biotic stress is caused by pathogens, parasites, predators, and other competing organisms, abiotic stress arises from inappropriate levels of physical components in the environment, such as temperature or water extremes [21]. Both stress factors lead likewise to yield and quality losses in plant crops used for human nutrition. The aim is, on the one hand, to avoid those immense quality and yield losses to ensure feeding the world's growing population and on the other hand, to develop new functional foods and to reduce insecticides and pesticides used in

agriculture. Therefore, knowledge of molecular networks and pathway activation of plants during their stress response is essential.

Only recently Mithöfer and Boland [48] asked the question "Do you speak chemistry?". They highlighted that plants always respond by emitting chemical compounds to signal their environment. Moreover, they pointed out that plants use metabolites as chemical sensing and communication systems. On the one hand, signaling molecules and phytoalexins are formed, on the other hand it is suggested that plants "cry for help" by producing secondary metabolites as indirect defense system. Those cry-for-help compounds, usually belonging to the group of volatile organic compounds, attract predators or herbivores helping the plant to get rid of other stress triggers [48,49]. Therefore, it is important to notice that stress-specific as well as common metabolites, so-called generalist and specialist, can either be formed or their amounts up-regulated [5].

The understanding of metabolic stress pathways is just possible if comparative metabolite characterization by means of metabolomics analysis of stressed and non-stressed plants followed by structure identification experiments is performed. In the following three sub-sections an initial overview of the literature about biotic and abiotic stress studies is undertaken.

3.1. From Abiotic Plant Stress to Metabolic Response

It is well expected that climatic changes due to the global warming will lead to massive abiotic stress factors influencing the metabolome spectra of all plants in the future. Next to water stress, including soil flooding and drought stress, salt stress, temperature stress, light stress, sulfur and phosphorus stress, oxidative stress, as well as heavy metal stress will play important roles for food crop producers. To understand and avoid quality and yield losses induced by abiotic stress factors, several metabolomics studies have been carried out (cf. the following reviews: Shulaev et al. [20]; Kaplan et al. [21]; Urano et al. [22]; Obata and Fernie [5]; Arbona et al. [4]).

Among them, Arbona et al. [4] already summarized in their review "Metabolomics as a tool to investigate abiotic stress tolerance in plants" that the plants' metabolic responses to abiotic stress can mainly be found in the primary metabolism. Next to metabolic responses in mono- and disaccharides, sugar alcohols, amino acids, especially proline, polyamines and members of the tricarboxylic acid cycle were found to be influenced by abiotic factors. Thereby, the carbohydrate metabolism in particular is directly influenced by stress conditions, because during stress instead of glucose, plants use fructanes and starch as an energy source [50].

In their review, Obata and Fernie [5] compared several metabolic fingerprints of *Arabidopsis* leaves influenced by dehydration, salt, heat and cold, high light and sulfur limitation, ultraviolet (UV) light quality change, low nitrogen amounts, and potassium limitation with each other. Thereby, they highlighted that specific compounds and compound classes are generally accumulated during abiotic stress factors (except of light treatment), such as sucrose, raffinose, proline, other branched chain amino acids or γ-aminobutyric acid (GABA). However, the amounts of such up-regulated "generalists" varies between stress factors. They concluded, that the stress-specific plant response is the result of an inhibition or activation of a defined metabolic pathway. In particular, enzymes activities are known to be influenced by temperature or ion concentrations in plants. By contrast, other metabolites are accumulated only under specific conditions, such as trehalose.

Next to the carbohydrates, the lipid composition of plants is additionally influenced by abiotic stress factors. Lipids in plants are a crucial and diverse class of biomolecules that forms the so called plant lipidome. Lipidomics is defined as the identification and quantification of all lipids within a biological system at a defined stage of development [51]. Within the field of omics-analysis lipidomics represents a sub-unit of targeted and non-targeted metabolomics approaches, mainly using MS- and NMR-based techniques. The development of lipidomics and its increased promise in systems biology over the past two decades has been the subject of several reviews (e.g., Blanksby and Mitchell [34]). Changes in the lipidome, induced by modifications of its biosynthesis, regulation, adaption, remodeling, function, role, and interaction, as well as membrane lipid remodeling are

relevant responses from plant cells to counteract biotic and abiotic stress challenges [51]. In particular, the oxidation of polyunsaturated fatty acids is one of the most fundamental reactions in lipid chemistry, which for example leads to off-flavor (perceived rancidity) or to phytohormon precursor formations [52]. Different groups of lipids, such as fatty acids, phosphatidic acids, inositol phosphates, diacylglycerols, oxylipins, shingolipids, and *N*-acetylethanolamine are involved in signaling systems during stress conditions as well [53–58]. Those compounds are shown to be directly synthesized after abiotic stress influences by a wide range of enzymes, such as fatty acid amide hydrolases, phospholipases, acyl hydrolases, diacylglycero kinases or phytoshingosine kinases [58]. In addition, it is known that in response to abiotic stress factors, lipids migrate into cell walls to repair damage through membrane remodeling [58–60]. Moreover, a combination of transcriptomics and lipidomic profiling showed that cold stress induces the prokaryotic pathway and suppresses the eukaryotic pathway for glycerolipid biosynthesis [61]. Next to cold stress, light and temperature stress, as well as nutrient starvation as typical abiotic stress factors were studied by means of lipidomic approaches in *Arabidopsis* (for details cf. the following review: Tenenboim et al. [51]). Only recently, lipidomics studies led to the clarification of the role of leaf lipids in thyme plant response to drought stress [58].

In the future, more stress-related lipidomic studies are expected, due to the fact that in nutritional research lipid profiling also plays a crucial role [51]. The food industry, for example, shows great interest in the use of lipidomics to characterize defined crop plant genotypes. As a replacement of palm kernel fat by a natural alternative, new plant genotypes having the same triglyceride pattern are needed. Furthermore, although most genes annotated as lipid-related are functionally characterized, their corresponding metabolites are not assigned. That would be an interesting field of research in the future, as well.

Next to numerous papers and reviews which highlight metabolome alterations induced by one single stress factor, Karin Köhl's review [62] gives a very nice overview of the metabolites influenced by combined abiotic stress effects in crops. Due to the fact that plants often have to deal with more than one stress factor, further metabolomics studies in crops which take several stress factors into account would be useful in the future [62].

In sum, although integrated omics data of model plants such as *Arabidopsis* have markedly increased our knowledge of understanding the mechanisms involved in plants' response to various abiotic stress factors [22,63,64], knowledge about secondary metabolome alterations in abiotic stressed food crops is rather fragmentary. Only few studies indicate abiotic stress influences the amounts of phenolic compounds, glucosinolates, carotinoides terpene derivatives and phytohormons in plants [4,51]. Urano et al. [22] emphasize again and again that phytohormon levels, especially those of abscisic acid, vary a lot in different plant compartments. Next to knowledge gaps in the stress influence on the secondary metabolome, more studies are needed to characterize stress combinations as well [5].

3.2. From Biotic Stress Metabolomics to Metabolic Response

During biotic stress exposure, plants use qualitative and quantitative measures to resist pathogen attacks [65]. While, in the past, the qualitative resistance, based on monogenetic inheritance, has been successfully used to elite cultivars to improve resistance, the quantitative resistance, which is presumed to be durable, non-race-specific, and effective against a large bouquet of pathogens, is mainly unknown [25]. However, a huge number of quantitative trait loci (QTLs) associated with qualitative resistance against pathogen-associated molecular patterns (PAMs), found for example in soy beans (*Glycine max*), and with quantitative resistance, found for blast in barley (*Hordeum vulgare*), or powdery mildew in wheat, have already been identified for biotic stress challenges [25,66–70]. However, both the mechanisms of resistance and the metabolites up-regulated during plant stress controlled by the QTLs are mainly unexplored [66]. In particular, targeted metabolomics approaches enable the possibility to monitor marker compounds up-regulated during plant stress. They can then be isolated, identified and biologically characterized, for example by application of an antifungal test.

Non-targeted metabolomics in comparison with bioinformatics and database research can also give an idea of which metabolic pathways are activated during a plant's stress response. Moreover, targeted analysis of known bioactive, e.g., anti-microbial or anti-fungal, compounds can help to identify QTLs, by using targeted metabolomics techniques for phenotyping experiments in breeding crossing lines. Thereby, alongside breeding improvement, metabolomics helps in the understanding of biological functions and the study of host–pathogen interactions [25].

It is already known from plant–pathogen interaction studies that signaling molecules, such as ethylene, salicylic acid, jasmonic acid or inositol, as well as antifungal/antimicrobial phytochemicals and cell wall compartments are formed or their concentrations up-regulated during plant stress [25]. Numerous studies already indicate that a complex network of secondary metabolites is influenced by biotic stress challenges. Next to phenylpropanoids, including flavonoids [34,50,51,71], alkaloids [72,73], terpenoids [33], and fatty acids [74] are also known to be up-regulated [25]. In addition, lipids act under pathogen or herbivore attack as mechanical barriers. The plants' wax layers form the first line of defense against pathogens and herbivores [51,75]. Additionally, some lipids act as signaling molecules, namely oxylipins and jasmonates, which participate in immune response cascades. Phytoalexins, a group of fatty acid degradation products, have antimicrobial and antifungal activities [51]. In addition, only recently Caroline Gutjahr's lab could show that fatty acids, like carbohydrates, are also transported from the plant host to fungi [76].

Moreover, as plants produce a diverse array of more than 100,000 low molecular weight natural products [32], countless studies are available using targeted quantifications of phytoalaxins in plants.

In 2009, Boue et al. [32] published a forward-looking paper called "phytoalexin-enriched functional foods" in which they proposed to use stress conditions to enhance the amounts of phytoalexins in food plants to produce functional foods. Several phytoalaxins are known for their health beneficial properties, including antioxidant activities, anti-inflammatory activities, cholesterol-lowering abilities, and anticancer activities [32]. In particular, flavonoids, which are ubiquitous in many food plants, have been linked to important health-promoting activities. For example, consumption of legumes, especially soy, have been linked to the reduction of cancer risks and coronary heart diseases simply due to its high flavonoid content [32]. Therefore, besides resistance and yield stability, functional food ingredients from the phytoalexin family could also be a possible target for plant breeders. In addition, mild stress conditions could possibly help to enhance the amounts of bioactive secondary metabolites, thereby producing functional foods [32].

Next to aspects in nutrition, diet and health, food and environmental safety can especially be monitored by means of metabolomics [3]. Toxic compounds can, for example, be formed as a stress response by plants, such as furanocoumarins in celery and glycoalcaloides in potatoes, or by fungi contamination, which can produce human toxic mycotoxins [77]. Therefore, versatile, sensitive, reliable and fast-targeted LC-MS/MS and GC-MS methods using stable isotope dilution techniques have been developed in the past to monitor food quality [78,79].

In sum, in the future more versatile and sensitive multiple-targeted MS quantification methods to evaluate food and crop quality are required.

3.3. Functional Phytometabolomics—Characterization Approach of Plant Stress Metabolites

In conclusion, the science-driven breeding of stress-tolerant cultivated plants that would allow for a reduction in harvest losses and undesirable decrease in quality attributes requires a new quality of knowledge on molecular markers associated with relevant agronomic traits, on quantitative metabolic stress responses of plants, and the mechanisms controlling their biosynthesis. The field of "functional phytometabolomics", using targeted and non-targeted MS or NMR techniques to quantitatively assess key metabolome alterations in plant-derived crops and foods induced by biotic stress challenges as well as abiotic stress conditions, is, therefore, a promising field of research.

In the phytometabolomics approach, metabolites up-regulated during stress challenges are visualized by means of working techniques used in the field of metabolomics. Markers previously

not published in literature could e.g., be visualized by retention time and mass to charge ratio in UHPLC-TOF-MS analysis. They are isolated in purities higher than 98% from the plant material using medium pressure liquid chromatography (MPLC) and preparative HPLC techniques and are identified by means of LC-MS, LC-MS/MS, UHPLC-TOF-MS and 1/2D NMR experiments. Testing of different biological activities (anti-fungal, anti-bacterial, anti-oxidant activities etc.) of those compounds allows initial insights into their biological functions. To translate the knowledge on how stress-resistant traits master their successful defense against stress conditions into breeding programs, genotype-specific metabolome alterations have to be characterized. Subsequently, the gene clusters controlling the biosynthetic pathways of key stress metabolites have to be identified by means of genome-wide association and QTL mapping studies. Just to highlight two examples: Firstly, Matsuda et al. [19] characterized several bioactive flavon glycosides, which can be used for new human drug discovery in rice by means of a metabolom genome-wide association study. Thereby, they highlighted "that one plant species produces more diverse phytochemicals than previously expected, and plants still contain many useful compounds for human applications". Secondly, only recently Rambla et al. [18] used a QTL analysis, to show that 102 QTLs correspond to 39 different volatile organic compounds, including flavor active key metabolites, in tomatoes. This research will help to navigate breeding programs and to optimize post-harvest treatment of plant-derived food products from producer to consumer/processor towards the production of high-quality food products.

4. Phytohormone Profiling by Means of Plant Hormonomics

Phytohormones are a class of low molecular weight, structurally diverse, but highly bioactive compounds in plants. They act as chemical messengers, triggering and controlling physiological processes during plant growth and development (e.g., cell elongation, regulation of apical dominance, vascular differentiation, fruit development, latal and adventitious root formation) as well as in response to abiotic and biotic stress conditions [2,80,81]. Next to ethylene, auxins, cytokinins, brassinosteroids, gibberellins, jasmonates, salicylates, polyamines, abscisates and signal peptides, strigolactones are part of the phytohormone family [81,82]. During stress exposure those phytohormone classes interact with each other by means of synergistic or antagonistic cross-talks, resulting in each other's biosynthesis or up-regulation response [83]). In the past, several studies provided evidence that plant hormones are necessary for plants to adapt during stress conditions, especially, during abiotic stress factors by mediating a wide range of adaptive responses [83]. They play a key role in the plant's intricate signal networks, often immediately altering gene expression by inducing or preventing the degradation of transcriptional regulators via the ubiquitin–proteasome system [83,84]. Thanks to Kumar et al. [2], metabolic engineering of phytohormons can be used to improve quality and stress tolerance of crops. Although the analysis of plant hormones, such as auxins, especially their quantification by means of SIDA-LC-MS/MS revealed significant insights in their tissue- and cell-type-specific analysis, their distribution profiles in plant organs, tissues, and cells still remains elusive [81]. A wide variety of targeted GC-MS and LC-MS/MS methods have been published dealing with the quantification of exact amounts of single members of the phytohormone family (e.g., cf.: Porifiro et al. [85]; Novák et al. [81]). However, in the last two decades, only one method was described to characterize those physiologically important molecules in their network interactions in one single run [86]. Only recently, Ondřej Novák and his team developed a new, versatile and sensitive targeted UHPLC-MS/MS method, which enables the simultaneous quantification of 101 phytohormone-related metabolites (phytohormones and their precursors) in less than 20 mg plant material [86]. This allows the characterization of the majority of known phytohormones as well as their biosynthetic precursors, that regulate diverse processes in plants by intricate signaling networks. With this newly developed, targeted metabolomics approach, so called "plant hormonomics", Šimura et al. [86] were able to detect 45 and quantify a total of 43 endogenous compounds out of the 101 phytohormones in both root and shoot samples, in salt-stressed and non-stressed, 12-day-old *Arabidopsis thaliana* seedlings. Subsequent multivariate statistical analysis cross-compared with data obtained from transcriptomic studies enabled the identification of the

main phytohormones involved in the adaption of *Arabidopsis thaliana* to salt stress. The multivariate statistical analysis revealed that 23 of the quantified 43 metabolites significantly differed between the salt-stressed and non-stressed roots. In contrast, the shoots and the roots differed in the concentrations of 15 compounds. Those hormone profiles obtained were cross-compared with transciptomic data. Well in line with findings from Rhy and Cho [87], who found that jasmoic acid and abscisic acid promote salt tolerance in *Arabidopsis*, Šimura et al. [86] observed increased levels of abscisic acid, its oxidation products phaseic acid, dihydrophaseic acid, and jasmonic acid in salt-stressed root samples. In addition, different responses of gibberillinic acid derivatives, especially its active form GA4, and its transcriptomic data, were found by Šimura et al. [86]. In sum, this newly developed multiple parallel analysis of phytohormones, called "plant hormonomics" enables the real-time profiling of hormone networks of large collections of phytohormones, their precursors, transport forms and degradation products in single stressed or non-stressed samples [86,88].

Although, Šimura et al. [86] were already able to quantify the main phytohormones in less than 20 mg of salt stressed *Arabidopsis* samples, special derivatisation reactions of the analytes prior to their analysis will conceivably lead to an increase in sensitivity and selectivity during MS analysis in the future [85,88–90]. For example, the response/sensitivity during LC-ESI-MS/MS analysis of metabolites only present in very low concentrations in plants, like indole-3-acetic acid, a member of the auxin family, can be tremendously increased up to 200-fold after methylation [89]. In addition, several aldehyde trapping derivatisation reagents are known to enhance the sensitivity during ESI-MS analysis of biological mixtures [91]. In future, it could be an extremely important and advantageous step in hormonomics to analyze a combination of derivatised and non-derivatised phytohormone classes, simultaneously in one method.

5. Sensomics—A Phenotyping Tool to Characterize Crops Flavor Impression

In general, more than 83% of consumers say that flavor influences their decision the most when purchasing any kind of food products or beverages [92]. Therefore, it is no surprise that, although aided by visual inspection, the final recognition and quality evaluation of food crops made by consumers are mainly mediated by its flavor perception. Human flavor perception is induced by the interaction of volatile odor-active and non-volatile taste-active molecules with ~390 odorant receptor proteins located in the *regio olfactoria* in the nose and ~40 taste receptor proteins on the tongue [93]. To meet consumers' demand for continuously available, fresh foods with a premium quality, the "flavor blueprint" of a golden standard, which is a combinatorial code of the entire set of odor- and taste-active metabolites in their natural concentrations, has to be known [94]. To decode all flavor-active molecules of a crop, the so-called sensometabolome, several high-end working techniques, including combinations of state-of-the-art metabolomics and chromatography approaches in combination with human sensory science experiments, such as aroma or taste dilution analysis, have been developed in the past. This so called molecular sensory science or sensomics approach has especially been shaped by Peter Schieberle's and Thomas Hofmann's working groups during the last three decades [93,94]. Today, it is well accepted that the presence of certain structural elements, so-called olfactophores and gustophores, as well as specific concentrations exceeding the sensory thresholds are important prerequisites of low molecular weight metabolites to become flavor-active [94]. Although a total of more than 10,000 volatiles occur in crop plants, the use of the so-called sensomics approach gave evidence that the typical aroma impression of a crop-based foodstuff is caused by a limited number of aroma-active volatiles [93]. In conclusion, only a surprisingly small group of so called "key odorants in food" contribute to the typical aroma profile of a crop plant. For example, only two compounds, namely ethyl (2S)-2-methylbutanoate and 1-(ethylsulfanyl)ethane-1-thiol, are necessary to explain the typical aroma impression of durian fruits [95], and to mimic the typical aroma impression of mangos only eight compounds are required [96]. While only a small number of aroma-active compounds contribute to the key odorants in food and interact with a huge number of odorant receptors, several thousands of non-volatile taste-active compounds are already known. In particular, the application of taste dilution

analysis followed by dose/activity considerations led to the discovery of many bitter, sweet, umami, pungent, astringent, salty, or sour sensing molecules in several plants, such as carrots [97,98], cocoa [99], asparagus [100–102], pepper [103], red currants [104], tea [105], stevia [106] or spinach [107] in the past. It is nowadays well accepted that not only a single flavor-impact molecule, but a combinatorial code of multiple odor- and taste-active key compounds, each in its specific concentration, reflect the chemosensory phenotype and trigger the typical flavor profile of food products. In particular, for flavor improvement of crops, the analysis of aroma- and taste-active compounds presents a major challenge for flavor improvement of crops [94]. It has been shown in the past, that the biosynthesis of several key odorants in food and tastants is controlled by genes, the expression of which is altered or even induced by biotic or abiotic stress challenges. But the primary target of crop production then was field performance, yield, and storage characteristics, while ignoring quality traits, such as the flavor code [33,108].

On the one hand, induced by several biotic and abiotic stress factors during growth in the field as well as during post-harvest storage, the attractive sensory quality of miscellaneous crop plants is hindered by sporadic off-flavors, which is often the reason for consumer complaints and therefore a major problem for plant processors. On the other hand, mild stress factors can lead to an increase in in the concentration of flavor-active constituents and hence to a more intensive desirable aroma or taste impressions [33,108].

Moreover, abiotic factors, such as mechanical stress, are reported to increase the amounts of the key bitter tastants, members of the C17-polycetylenes, present in native carrots (*Daucus carota* L.). The increase causes a perceived bitter off-flavor, occurring especially often during the production process for infant diet carrot products [97,98,109–115]. In addition, a decrease in flavor quality accompanied by an increase in bitter taste has also been reported in raw hazelnuts (*Corylus avellana* L.) upon biotic stress challenges, such as upon infection by bugs, belonging to the hemipteran family, like *Gonocerus acuteangulatus* and *Coreus marginatus* [116].

Besides the non-volatile, taste-active metabolites, aroma-active compounds are also known to be influenced by individual stress factors [33]. In particular, studies dealing with tea (*Camellia sinensis*), which is enjoyed as freshly brewed green, black, oolong, or decaffeinated tea infusion, and its quality changes induced by stress are available in literature. For example, the tea green leafhopper (*Empoasca* (*Matsumurasca*) *onukii* Matsuda) attacks, at pre-harvest stages, can decisively influence the unique aroma quality of tea leaves as a result of the upregulation of the linalool synthase (CsLIS1 and CsLIS2) causing higher concentrations of the key odorant, linalool [33,117]. Besides linalool, the formation of key odorants, such as (*E*)-nerolidol, can also be influenced by a combination of low-temperature stress and mechanical damage [33,118]. Therefore, Wüst [33] concluded that the tea-related findings illustrate how the use of the stress response of plants within the sensometabolome can lead to an improvement of flavor of agricultural products. In addition, he pointed out, that the plants' contact with stress elicitors, such as methyl jasmonate instead of the actual biotic or abiotic stress factors could also lead to "stress induced" flavor improvement [33,118].

In order to gain a more comprehensive knowledge on the chemical mechanisms involved in quality changes of cultivated crop plants in response to biotic or abiotic stress challenges or to improve the flavor quality by the application of moderate, well controlled stress, numerous volatile, aroma-active as well as non-volatile taste-active key metabolites, the so-called sensometabolites, of stressed and non-stressed plant genotypes should be comparatively characterized by means of a fast and robust high-throughput GC-MS systems with high peak separation capacity and sensitivity, such as comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GC $\times$ GC/TOF-MS) or UPLC-TOF-MS metabolic profiling analysis in the future. This strategy aims at reducing the flavor deficiencies in modern commercial varieties as a "green" alternative to genetic engineering. The workflow for a successful implementation of this approach—from the identification of key odorants by molecular science techniques to the investigation of mechanisms controlling their biosynthesis—is complex and calls for interdisciplinary research [33,108].

6. Conclusions

Plants continuously encounter various biotic and abiotic environmental stresses during their growth and development phases, which leads likewise to yield and quality losses. To avoid those previously listed economical losses, to reduce insecticides and pesticides used in agriculture, to ensure feeding the world's growing population, and to develop new functional foods knowledge about molecular networks and the pathway activation of plants during their stress response is essential.

This review has emphasized the importance of metabolomics-based working techniques to discover metabolome alterations during abiotic and biotic stress conditions. Therefore, metabolomics is a promising tool for knowledge-based targeted breeding programs. It also shows that due to missing databases and non-standardized LC-MS conditions, basic metabolomics, lipidomics and phytohormonics strategies, without isolation and unequivocal structure identification experiments, are sometimes insufficient. Therefore, techniques using biological and molecular structural characterizations of marker metabolites in combination with metabolomics techniques, such as phytometabolomics or sensomics approaches, are useful solutions to produce high-quality phytoalexin-enriched functional foods in the future.

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Plant Protection by Benzoxazinoids—Recent Insights into Biosynthesis and Function

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Abstract: Benzoxazinoids (BXs) are secondary metabolites present in many Poaceae including the major crops maize, wheat, and rye. In contrast to other potentially toxic secondary metabolites, BXs have not been targets of counter selection during breeding and the effect of BXs on insects, microbes, and neighbouring plants has been recognised. A broad knowledge about the mode of action and metabolisation in target organisms including herbivorous insects, aphids, and plants has been gathered in the last decades. BX biosynthesis has been elucidated on a molecular level in crop cereals. Recent advances, mainly made by investigations in maize, uncovered a significant diversity in the composition of BXs within one species. The pattern can be specific for single plant lines and dynamic changes triggered by biotic and abiotic stresses were observed. Single BXs might be toxic, repelling, attractive, and even growth-promoting for insects, depending on the particular species. BXs delivered into the soil influence plant and microbial communities. Furthermore, BXs can possibly be used as signalling molecules within the plant. In this review we intend to give an overview of the current data on the biosynthesis, structure, and function of BXs, beyond their characterisation as mere phytotoxins.

1. Introduction

A unique feature of plants is the biosynthesis of secondary metabolites, also termed specialised metabolites [1]. The number of secondary metabolites in the plant kingdom is estimated at about 200,000 [2]. Although not essential for the survival of the individual plant, secondary metabolites are vital for "communication" within the bio-system including attraction, repelling, and defence reactions. The concept underlying the functionality of secondary metabolites is diversity and reactivity. Families of secondary metabolites can occur in certain plant taxa and can then be used for taxonomy [3], but some metabolites also have scattered appearance. Secondary metabolites are often unstable and react with other compounds, thereby generating toxicity, which qualifies them as natural defence compounds. The multitude of compounds active in defence limits the emergence of generalist deleterious insects and pathogenic microbes.

During the breeding of crop plants, the inherent toxicity has led to the reduction of secondary metabolites. Well known examples of counter selection are given by the quinolizidine alkaloids present in lupines and alkaloids in tubers of potatoes (see [3] for review). Benzoxazinoids (BXs) are well-known secondary metabolites found in cereal crops and, in contrast to the cases of counter selection, benzoxazinoids have been targets of positive selection in breeding for maize tolerance to insects since the 1970s [4–6]. The untargeted reactivity implies autotoxicity that has to be circumvented by the producing plant. In the so-called two-component defence systems, reactivity is reduced

by chemical modification, mostly glycosylation, and simultaneously a reactivating enzyme, e.g., a glycosidase, is provided. The stabilised metabolite (component one) and activating enzyme (component two) are physically separated in different organelles or tissues but meet in case of cell damage, thereby liberating the toxin. Examples are alkaloid glucosides, benzoxazinoid glucosides, cyanogenic glucosides, glucosinolates, iridoid glucosides, and salicinoids (see [7] for review).

Advances in chemical analysis and well-developed genetic resources, especially in maize, have revealed distinct functions for different BXs in defence. Furthermore, in planta signalling is a matter of debate [8]. Within maize populations, diversity in the quality and quantity of different BXs has been revealed. Recent reviews summarise the role of BXs on plant-plant allelopathy [9], deal with the interaction between BXs and insects [10], and describe the BX structure diversity and function in maize [8]. Here we aim to give an overview on the present knowledge of the biosynthesis, distribution, and biological function of different BXs. The available data on BX-mediated interactions are summarised in Table 1, providing a guide through the literature.

2. Structure of Benzoxazinoids and Chemical Properties

BXs comprise two classes, benzoxazolinones (1,3-benzoxazol-2-one, e.g., 6-methoxy-2-benzoxazolinone (MBOA) in Figure 1) and benzoxazinones (1,4-benzoxazin-3-one, e.g., 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA), 2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one (HDM$_2$BOA) in Figure 1), with several subclasses each. In plants, N-hydroxyl benzoxazinones are predominant. Decoration by hydroxyl- and methoxy- groups at positions C-7 and C-8 and the additional methylation of the N-hydroxyl are the most common modifications found for benzoxazinones (Figure 1). While the 2-OH glucosides (GDIBOA, GDIMBOA, etc.) are quite stable [11], the aglucons have significant reactivity. Both the N-OH function and the cyclic hemiacetal unit are largely essential for the reactivity. The hemiacetal undergoes an oxo-cyclo-tautomerisation. In the oxo form, the aldehyde group is free to react with the epsilon-NH$_2$ group of the N-alpha-acetyl-Lys. N-alpha-acetyl-L-lysine is a lysine analogue used as a model substrate to demonstrate the targeting of Lys residues and general nucleophilic residues in proteins. [12]. The resistance of maize towards the herbicide atrazine is attributed to nucleophilic attack by DIMBOA [13,14].

Comparing the major benzoxazinoids DIBOA and DIMBOA, the latter is definitely more reactive. The 7-MeO group of DIMBOA facilitates N-O bond heterolysis as a donor [15] and the dehydration of DIMBOA [16]. In this way, a multicentered cationic electrophile and a reactive formyl donor toward -NH$_2$, -OH, and -SH groups are generated. Reactivity towards thiols potentially interferes with enzyme function if the cysteine residues in proteins are affected; furthermore, glutathione (GSH) can be targeted. The resulting formation of stable thioether conjugates leads to the rapid depletion of GSH levels [17]. In addition, the heterocycle of the benzoxazinone can assume ring-opened conformation after oxo-cyclo-tautomerisation and spontaneous transformation into the respective benzoxazolinone takes place. Since the half-life time of HDMBOA in watery solution is 10 times shorter than that of DIMBOA [18] and the generation of the reactive MBOA is accordingly more rapid, GHDMBOA might be the fastest activated BX for defence.

Among the defined molecular interactions of benzoxazinones that disturb cell function are the inhibition of alpha-chymotrypsin [19], cholinesterase [20], and plasma membrane H$^+$-ATPase [21]. Furthermore, BXs intercalate with nucleic acids [15] and might cause mutations. However, BXs are not super toxins; effective defence requires concentrations in the millimolar range and acute mortality is not caused.

3. Distribution of Benzoxazinoids

Characteristically, benzoxazinoids are found in wild and cultivated Poaceae [22,23]. Besides that, BXs have been detected in distant orders of eudicots, in the family Ranunculales, and in the Lamiales families Acanthaceae, Plantaginaceae, and Lamiaceae. While just one species each of the families

Ranunculaceae (*Consolida orientalis* Schrödinger), Plantaginaceae (*Scoparia dulcis* L.), Lamiaceae (*Lamium galeobdolon* (L.) L.), and Calceolariaceae (*Calceolaria thyrsiflora* Graham) synthesise BXs, several Acanthaceae species do so [22,23]. Within the family Poaceae, BX presence has been shown for the perennial bunchgrass *Chrysopogon zizanioides* (L.) Roberty as well as the aggressive perennial grass *Agropyron repens* (L.) P.Beauv. Most importantly, BXs are found in the crops *Zea mays* L. and the wild progenitor Teosinte, *Triticum aestivum* L., and diploid wheat species, *Secale cereale* L., as well as some wild *Hordeum* species (*Hordeum roshevitzii* Bowden, *Hordeum brachyantherum* Phil., *Hordeum flexuosum* Steud., *Hordeum lechleri* Steud.).
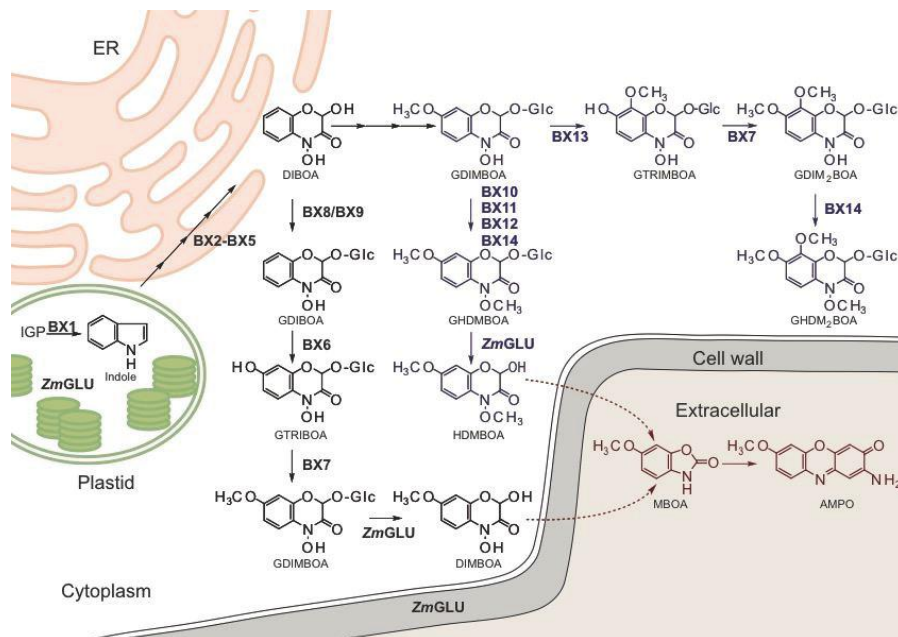
Figure 1. Biosynthesis of benzoxazinoids (BXs) as determined for maize. Compounds and enzymes of the core pathway are depicted in black. Depending on developmental stages and biotic interactions, modifications of BXs are induced. The resulting compounds and the employed enzymes of the inducible branches are coloured in blue. The enzymes locate to the plastid (BX1), to the endoplasmic reticulum membrane (BX2 to BX5), and to the cytosol (BX8 and BX9, BX6, BX7, and BX10 to BX14). The glucosides might be stored in the vacuole and hence are protected against the specific glucosidase, which is found in the plastid and associated with the cell wall. When the cell is disrupted, the glucosides come in contact with the glucosidase and the aglucons are generated. 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one (HDMBOA) are unstable and the degradation product 6-methoxy-2-benzoxazolinone (MBOA) is spontaneously formed in a watery solution (red colour). Enzymatic transformation by microbes, especially in the soil, yields further catabolites, e.g., stable amino-phenoxazinones such as 2-amino-7-methoxy-phenoxazin-3-one (AMPO). Indole-3-glycerol phosphate (IGP).

In cereal crops, (G)DIMBOA (e.g., maize, wheat) and (G)DIBOA (wild *Hordeum* species) are prevalent in unstressed plants. A distinction in the dominating BXs is found in rye; in aboveground tissue (G)DIBOA dominates, while (G)DIMBOA is prevalent in rye roots. The highest concentrations of BXs are found in seedlings of the family Poaceae.

Maize can be considered as model system for BX analysis in the crop cereals. DIMBOA is detectable shortly after the onset of maize germination and the highest concentrations are found 4 to 11 days after imbibition (10 to 30 mM in the shoot, 0.5–15 mM in the root, [24]). The maximal concentration and the persistence differ for different maize lines. Effective concentrations for defence beyond the seedling stage are restricted to exceptional lines in the Nested Association Mapping (NAM, [25]) diversity panel [24]. In mature maize plants, however, the concentration and modification of BXs can be changed by microbial and herbivore attack (see below). BXs have not been detected in maize seed. By contrast, kernels of wheat and rye contain BXs [26]. While in rye the largest part is located in the bran, wheat also has significant amounts of BXs in the germ [27]. The BXs in seeds are mostly DIBOA- and 2-hydroxy-1,4-benzoxazin-3-one (HBOA)-diglycosides and concentrations reach up to 0.15 nmol in dry matter. Diglycosides are unusual benzoxazinoids and might function as special storage metabolites.

(G)DIBOA is the main BX in dicots, others, e.g., DIMBOA (in *Aphelandra* sp.), are only present in traces [22,23,28]. Peak amounts are not restricted to the seedling stage. On the contrary, juvenile stages of *C. orientalis* have the lowest concentrations. Interestingly, flowers proved to have high BX concentrations while the root might have levels below detection limits [28]. Whether the differences in BX patterns of dicots and monocots reflect different defence strategies is unknown. Data for dicots are rare but antifeeding activity of BXs towards the larvae of the moth *Pseudaletia impuncta* (Guenée 1852) has been shown for *Acanthus mollis* L. [29].

4. Biosynthesis

BX biosynthetic pathway elucidation was largely done in maize and the biosynthetic steps and *Bx*-gene functions were defined in maize. The biosynthesis of the core BXs GDIBOA and GDIMBOA was discovered in maize [30–32] and subsequently in wheat and in part in rye [33–39]. The pathway branches off from tryptophan biosynthesis (Figure 1) by the signature enzyme BX1, which is a homologue of the alpha subunit of tryptophan synthase (TSA). BX1 and TSA share the substrate, indole-3-glycerol phosphate, and the product, indole [30,40]. In contrast to TSA, which requires allosteric activation by the beta-subunit of the tryptophan synthase (TSB) in the hetero-dimeric tryptophan synthase (TS) complex [41], BX1 is active as a monomer and indole is released. In tryptophan biosynthesis, indole remains in the tunnel connecting TSA and TSB and is further metabolised. Free indole delivered by BX1 is converted by four consecutive hydroxylation reactions catalysed by the cytochrome P450 enzymes BX2 to BX5 into the benzoxazinoid DIBOA [30,42]. DIBOA is stabilised by glucosylation by either of the two UDP-glucosyltransferases (UGT) BX8 and BX9 [31]. Hydroxylation of GDIBOA is by the 2-oxoglutarate dependent dioxygenase (ODD) BX6. Methylation of the resulting 2,4,7-trihydroxy-8-methoxy-1,4-benzoxazin-3-one-O-glucoside (GTRIMBOA) by the O-methyltransferase (OMT) BX7 completes GDIMBOA biosynthesis [32].

The ratio and kind of modification of the basic BXs depends on developmental stages, abiotic and biotic stress, and genotypes (see [23] for review). The levels at the seedling stage are quite stable, while mature plants respond to different challenges with changes in BX amounts and patterns (e.g., [43]). In planta GDIMBOA can be hydroxylated by the OMTs BX10, BX11, and BX12 to yield GHDMBOA (Figure 1, [44]). While the expression of *Bx12* is constitutive, *Bx10* and *Bx11* are induced by herbivory (*Spodoptera exigua* (Hübner 1808), [45]). BX13, an ODD homologous to BX6, and a further OMT, BX14, are required for the synthesis of 2,4-dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one-O-glucoside (GDIM$_2$BOA) and GHDM$_2$BOA (Figure 1, [46]). BX14 and BX10 to BX12 share the in vitro function as GDIMBOA methyltransferases to yield GHDMBOA, but BX14 is distinguished by its activity towards DIM$_2$BOA and is required for

the biosynthesis of GHDM$_2$BOA [46]. Enzymes of BX biosynthesis locate to different intracellular compartments. BX1 is found in the stroma of the plastid [47], BX2 to BX5 are anchored in the endoplasmic reticulum membrane, the ODDs (BX6, BX13) and the OMTs (BX7, BX10, BX11, BX12, BX14) are soluble cytosolic enzymes. BX-glucosides are stored in the vacuole.

The core genes *Bx1* to *Bx5* and *Bx8* form a biosynthetic cluster in maize and locate within 264 kb on the short arm of chromosome 4. A distant enhancer element for *Bx1* expression is situated in the middle of the cluster [24,48]. The first intermediate unique to BX biosynthesis is indolin-2-one, generated by BX2. Phylogenetic data suggest the co-evolution of *Bx1* and *Bx2* to be an essential driver in the establishment of the pathway. Indeed, both genes are extremely tightly linked in maize (separated by 2.5 kb, [30]) and the linkage is preserved in wheat and rye while the second part of the cluster, *Bx3* to *Bx5*, *Bx8*, is found on different chromosomes [36,37]. The essential impact of BX1 for benzoxazinoid biosynthesis has been shown by transgenic overexpression in maize [24] and by QTL (quantitative trait locus) analysis [49]. *Bx6* and *Bx7* are also found on the short arm of chromosome 4, but are separated from the core cluster by several centimorgans. The genes involved in the decoration of GDIMBOA are not linked to the core gene set. *Bx10* to *Bx12* most probably represent gene duplications and the paralogues are located within 200 kb on chromosome 1 [44]. *Bx13* and *Bx14* are both found on chromosome 2 but are distant from each other.

Benzoxazinoid biosynthesis in maize was the first example of a biosynthetic cluster in plants. Since then, they have also been discovered for secondary metabolism in several plant species (reviewed in [50]). Clustering was even used as a criterion to elucidate pathways [51]. However, many biosynthetic pathways are dispersed in the genome [52]. Whether clustering provides an evolutionary advantage for pathway establishment by preserving superior allelic combinations in the coupling phase, or facilitates coordinated regulation through the domains of modified chromatin is a matter of debate. The local separation of the core gene set and the genes of modification might be underlying the diversity of BXs patterns found in maize [44,46,53]. It is suggested that the glucosylated benzoxazinoids are stored in the vacuole. The BX-glucoside specific beta-glucosidases were detected in the plastid and connected to the cell wall [54–57]. The disintegration of cells joins stored glucosidase and substrate and delivers the defence compound.

Recently, signalling components involved in induced BX changes have been discovered. It has been shown that *Zm*PEP1 and *Zm*PEP3, members of the maize elicitor peptide family, were rapidly induced by either fungal infection or oral secretions of the *Spodoptera exigua* larvae [58,59]. In both cases, jasmonate and ethylene biosynthesis and a broad spectrum of defence reactions is induced, including the expression of *Bx*-genes. Whether the observed changes in gene expression and BX patterns are direct or indirect consequences of *Zm*PEP activity is unclear. A significant function might be attributed to the jasmonate (JA) signalling cascade since JA application and JA synthesis inhibition enhanced and constrained, respectively, the concentration of GHDMBOA [60,61].

As mentioned before, most data about BX biosynthesis were gained through analyses of maize. The respective genes in other Poaceae species were found based on homology searches and all genes identified so far are orthologues, hence the BX biosynthetic pathway is monophyletic in Poaceae. The knowledge about the pathway in dicots is scarce. It has been shown that, like in monocots, indole is generated by a homologue of TSA and further metabolised to yield BXs. However, the *Bx1* genes of the family Poaceae and the dicots *A. squarrosa*, *C. orientalis*, and *L. galeobdolon* are results of individual convergent evolution [28]. Similarly, the UGT gene *CoBx8* and the specific glucosidases *CoGlu* and *LgGlu1* from *C. orientalis* and *L. galeobdolon* are the result of repeated evolution [62,63]. It cannot be excluded that a loss of genes and secondary recruitment took place, but most likely BX biosynthesis evolved several times independently.

5. Biological Interaction

Different techniques were applied to study the biological impact of BXs. Pure substances were applied in solution (e.g., in Sections 5.1–5.3 and 5.5), added to artificial diets, or applied through

the painting of leaves (Section 5.4). Concentrations in the millimolar range might face insects and microbes attacking seedlings. In older plants, such a high concentration can result locally from induced biosynthesis [58,59]. Another line of investigation was to analyse plant lines or cultivars producing BXs at different levels for the effect on target organisms (Sections 5.3 and 5.4). A summary of compound- and species-specific interactions is given in Table 1.

5.1. Human Health Clinical Studies, Detrimental and Health-Promoting Effects

Maize seed and silage tissue are lacking significant amounts of BXs, but due to the presence of BX in seeds of wheat and rye, BX can enter the human food chain. BXs have been found in conventionally baked bread and water-soaked or -boiled pearled rye and rye flakes [27]. Furthermore, BXs have been detected in wheat and rye beer [64]. The health-promoting effects of whole grain wheat and rye products have been associated with the presence of BXs. Anti-allergenic and anti-inflammatory effects have been demonstrated, and it was reported that BXs are appetite-suppressing in humans and may reduce cellular glucose uptake (see [65] for review). Hamilton described DIMBOA in 1962 as a "corn sweet substance" [66], as for human taste buds it is about 400 times sweeter than sucrose [8]. This might account for the reduction of calorie uptake by humans consuming BX-supplemented diets. Additionally, BXs might influence the microbial community of the digestive system positively and their health-promoting effects might be the result of differential sensitivities of microbes.

On the other hand, detrimental properties of BXs are well known. In vitro mutagenicity of DIMBOA in the Ames test has been described [67]. More recently BXs were described as potent polyploidy-inducing agents in human-derived cell lines (HepG2 and HeLa), which has raised concern for their potential adverse health effects [68,69]. However, due to the ability of potent BXs to induce cell death, applications in cancer treatment are considered [70].

5.2. Interactions with Microbes

Early after the detection of BXs in cereal crops, studies were performed to evaluate the antimicrobial potential of the compounds. The microbe targeted was *Pantoea stewartii* (Smith 1898) Mergaert et al. 1993, (*Xanthomonas stewartii* (Smith 1898) Dowson 1939, [71]), a causal agent of Stewart's wilt. Similarly, a bacteriostatic effect of DIMBOA was determined for *Erwinia* spp. and *Agrobacterium tumefaciens* (Smith & Townsend 1907) Conn 1942; growth restriction by MBOA was less pronounced [72,73]. In addition to the restriction of bacteria proliferation, BXs have the capacity to influence microbial communities and are like other plant metabolites implicated in mutualistic interactions (see [74] for review). *Pseudomonas putida* Trevisan, 1889 KT2440, a competitive coloniser of the maize rhizosphere with plant-beneficial traits, is attracted by DIMBOA [75]. Root colonisation by the strain was shown to prime the emission of stress-inducible aromatic and terpenoid volatiles from shoots and the expression of the JA-inducible serine proteinase inhibitor SerPIN. SerPIN priming was shown to require the presence of BX. The underlying mechanism is at present unknown [76].

Local changes of BXs are caused by phytopathogenic fungi (*Bipolaris maydis* (Y. Nisik. & C. Miyake) Shoemaker, *Curvularia lunata* R.R. Nelson & Haasis, and *Alternaria alternata* (Fr.) Keissl. (Fr.)) and influence the germination of conidia negatively [43]. A significant accumulation of HDMBOA and thereafter of MBOA was induced by the inoculation of the fungi. The most significant antifungal potential was attributed to MBOA. For cereals, interaction with endophytic *Fusarium* species is of special interest due to yield loss and contamination with fungal toxins. BXs were first described as anti-*Fusarium* factors in rye seedlings (benzoxazolinones, [77]; benzoxazinones, [11]). The impact of benzoxazinones and the benzoxazolinones MBOA and 1,3-benzoxazol-2-one (BOA) arising as degradation products on *Fusarium* infection has been verified repeatedly (e.g., [78]) It has been shown that the biosynthesis of toxic trichothecenes by *Fusarium graminearum* Schwabe is almost completely abolished by DIMBOA in wheat. The effect is attributed to the suppression of the fungal genes of the trichothecenes biosynthetic pathway [79]. However, *Fusarium* spp. gained tolerance to BXs multiple times [80]. Tolerance was achieved by the inducible production of modifying enzymes (FDB1, FDB2,

Fusarium verticillioides (Sacc.) Nirenberg, *Fusarium pseudograminearum* Aoki and O'Donnell, [81,82]). The *F. verticillioides* gene FUG1 is required for successful maize kernel colonisation. FUG1 shows features of transcription factors and activates the biosynthesis of fumonisins, a class of mycotoxins. At the same time, FUG1 is required for BX tolerance, though the specific tolerance mechanism is unknown [83]. The presence of BX-tolerant fungi has a major impact on the microbial population structure. The microbial community proportion with low BOA tolerance was significantly reduced (up to 35-fold lower frequency in leaf isolates) in maize BX producer lines [84]. Given the positive correlation between BX tolerance and mycotoxin biosynthesis, the selective advantage of tolerant *Fusarium* strains when facing high BX concentrations might increase toxin levels of BX-producing cereals. Hence, the presence of BXs might be advantageous or deleterious for the reduction of *Fusarium* infection and mycotoxin contamination.

BXs were also analysed for antimicrobial properties against the model organisms for human pathogens *Escherichia coli* (Migula 1895) Castellani & Chalmers 1919, *Staphylococcus aureus* Rosenbach, 1884, and *Candida albicans* (C.P.Robin) Berkhout, as well as the yeast *Saccharomyces cerevisiae* Meyen *ex* E.C. Hansen [85–87]. Antimicrobial activity required concentrations above the millimolar range.

5.3. Benzoxazinoids in the Soil: Allelopathy and the Chelating of Metals

BXs are found in the soil when BX-producing plants are grown and initially BXs received much attention as allelopathic substances in plant-plant interactions (see [9] for review). The release of BXs follows two modes, root exudation by the vital plant and the shedding of dead tissue. The level of root exudation varies for different species and within species for lines and cultivars. The presence of BXs in the apoplast has been demonstrated in maize [88], but the mechanism of root exudation is unknown. We assessed BXs exudation in young maize seedlings using a system that allowed exudate collection without damaging the root tissue (Figure 2) [89]. Staining revealed the presence of BXs above the root cap (Figure 2A). Collecting exudates separately from the root cap border cells as opposed to the whole root confirmed that the root cap border cells do not play a major role in BXs exudation (Figure 2B). This was somewhat unexpected since the root cap is known to be involved in the exudation of other defence compounds, such as pisatin in pea [90]. Preliminary data using specific inhibitors suggested that exudation takes place passively rather than by primary or secondary active membrane transporters such as the ATP-binding cassette (ABC) transporters and MATE (multidrug and toxic compound extrusion) transporters (Figure 2C,D) [89].

In maize benzoxazinoid content is high in seedling roots, in young adventitious roots, and in crown roots [91,92]. In agricultural practice, the effect of BXs is exploited by mulching and the allelopathic potential has been extensively studied in rye. Experiments with the mulching of rye demonstrated a substantial reduction of weeds, e.g., a reduction of *Chenopodium album* L. greater than 90% was detected [93]. BX levels between 0.5 and 5 kg/ha were reached with field-grown rye. The effects of intact plants on weed control proved to be cultivar-specific and correlated with BX content [94]. Hence, it was postulated that BXs might be used as lead structures for the design of herbicides. Benzoxazinone aglucons are unstable in watery solutions [95], e.g., the half-life values of DIMBOA are between 7 h and 20 h depending on pH. The benzoxazolinones BOA and MBOA are quite stable under sterile conditions but are converted via aminophenol as an intermediate by microbes to yield either of the catabolite classes aminophenoxazinone, malonamic acids, or acetamides within days [9,96]. Aminophenoxazinones (2-amino-phenoxazin-3-one (APO), 2-amino-7-methoxy-phenoxazin-3-one (AMPO), Figure 1) are persistent in the soil with half-life values of several months [97]. The precursors DIBOA and DIMBOA and (more substantially) the degradation products APO and AMPO have been detected in the roots and shoots of target plants [98]. Hence, the allelopathic potential in the soil depends on both the BXs and, maybe even to a higher extent, on the catabolites. The selectivity in plant communities is given by differences in the uptake and metabolisation of the chemicals. Generally, cereals are more tolerant than dicots, although even maize cells are affected by DIMBOA [99]. Different detoxification pathways are established in different plant species [9,100]. Considerable changes in the transcriptome of *Arabidopsis*

thaliana (L.) Heynh. seedlings by exposure to BOA might reflect the activation of detoxification reactions. The functional categories 'cell rescue' and 'defence' were overrepresented after BOA treatment [100]. One common mechanism to reduce toxicity is the glucosylation of intermediates. Indeed, the transgenic expression of the respective UGTs BX8 or BX9 conferred DI(M)BOA tolerance to *Arabidopsis* [31]. The major detoxification product of BOA in Poales was identified as glucoside carbamate and the biosynthetic steps were defined recently in maize roots [101,102]. Thereby, it has been shown that BX9 is also effective in BOA metabolism.
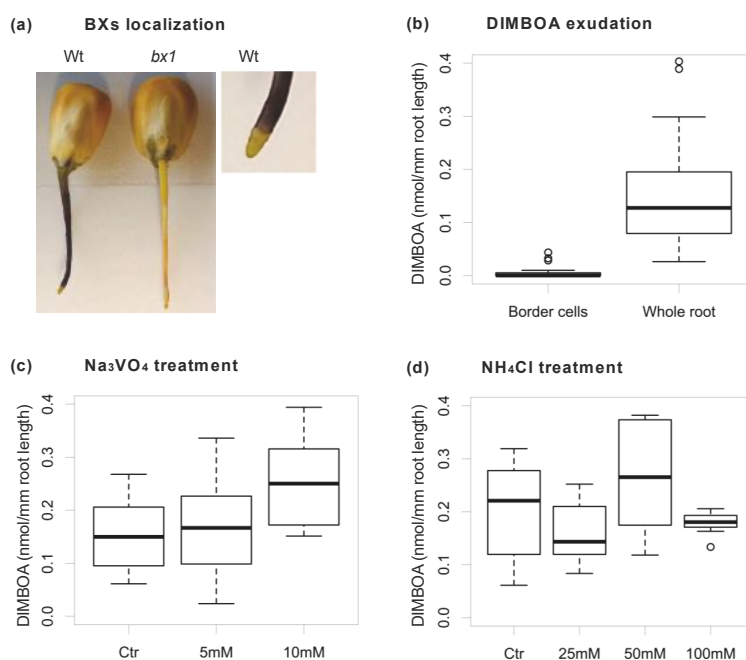
Figure 2. BXs exudation in young maize seedlings. (**a**) Detection of BXs by staining with $FeCl_3$. BXs form a blue-coloured complex with Fe (III). The benzoxazinless (*bx1*) mutant root does not show any staining while the wildtype (WT) root is stained, except for the root cap, suggesting that the root cap is not involved in BXs biosynthesis and exudation. (**b**) DIMBOA amounts in exudate of the root cap border cells compared to the whole root ($n = 16$). Border cell exudate was collected by dipping the root tip in 100 μl of water, while whole root exudate was collected by washing the root with 100 μl of water. Only very low amounts of DIMBOA were detected in the border cell exudate. (**c**) Effect of Na_3VO_4 (inhibitor of ATP-binding cassette (ABC)-type transporters) on exudation. No significant inhibition of exudation was observed after treatment with 5 mM or 10 mM Na_3VO_4 ($6 < n < 9$). (**d**) Effect of NH_4Cl (inhibitor of multidrug and toxic compound extrusion (MATE)-type transporters) on exudation. No significant inhibition of exudation was observed after treatment with 25 mM, 50 mM, or 100 mM NH_4Cl ($6 < n < 7$) [89].

The impact of BXs and their metabolites is not restricted to distinct plant species, but rather it is quite general. As a corollary, universal cell structures are suggested targets. Proposed modes of action include induced H_2O_2 production leading to lignin accumulation, increased cell wall rigidity leading to reduced growth, and interaction with auxin signalling [9]. Intriguingly, Venturelli et al. [103] detected for *Arabidopsis* that AMP and AMPO directly inhibit histone deacetylases. The physiological effects of the metabolites might result from chromatin modifications that subsequently disturb gene expression patterns and will affect plant performance.

BXs have metal-chelating properties toward trivalent metal ions. (G)DI(M)BOA forms complexes with Fe (III) and can promote iron uptake as phytosiderophores. It has been shown that BX amounts in and outside of the root increase with higher iron concentrations in the media [104]. Thus, iron-complex formation with BXs will be adjusted to iron availability. Since the uptake of iron-complexes by the root is driven by the plant's demand [105], excess amounts of Fe (III) could be preserved in built-on BX-iron complex storages. Similarly, aluminium tolerance conferred by chelating complexes of DIMBOA and Al (III) has been proposed [106]. However, recent studies using defined maize lines and BX-free mutants could not detect a significant correlation between BXs and Al (III) sensitivity [8,107,108].

5.4. Insects

The effects of BXs on insect performance are on different levels. They span from repelling to attracting, including developmental stage-specific retardation but also growth promotion, direct toxicity, and metabolic costs due to detoxification. Differences for distinct species might vary with respect to developmental stage at the insect side and age at the plant side.

5.4.1. Chewing Insects

Most data on the impact of BXs on insects have been gained from maize. The resistance of plants towards insects can be implemented by anti-feeding activity and interference with the larval metabolism, e.g., the inhibition of digestive enzymes. A correlation between BX concentration and the control of the European corn borer (*Ostrinia nubilalis* (Hübner, 1796)) was recognised early on [109].

Table 1. Impact of different BX families on target organisms and their effective concentrations.

Target Organism	Benzoxazinoids Core Benzoxazinones	Induced BXs *	BX Metabolites **
Chewing insects			
Leaf-feeders [59]	induce increase in GHDMBOA		
Generalists			
Ostrinia nubilalis (Hübner, 1796) [110–113]	DIBOA, DIMBOA	DIM₂BOA	MBOA
Ostrinia furnacalis (Guenée, 1854) [114,115]	DIMBOA	HDMBOA	MBOA
Ostrinia latipennis (Warren, 1892) [114]			
Ostrinia scapulalis(Walker, 1859) [115]			
Spodoptera littoralis (Boisduval, 1833) [113,116]	DIMBOA	HDMBOA deterring	
Spodoptera exigua (Hübner 1808) [117]	DIMBOA		
Spodoptera eridania (Stoll, 1781) [118]	#		
Sesamia nonagrioides (Lefèbvre, 1827) [119]	DIMBOA		
Diatraea grandiosella (Dyar, 1911) [120]			MBOA
Specialists			
Spodoptera frugiperda (J. E. Smith, 1797) [10,113,116]	DIMBOA	HDMBOA deterring	MBOA
Rootworms			
Generalists			
Diabrotica balteata (LeConte, 1865) [91]	DIMBOA	DIM₂BOA	HMBOA
D.undecimpunctata howardi (Barber, 1947) [121]	#		
Specialists			
Diabrotica virgifera virgifera (LeConte, 1858) [122,123] [91] [121]	DIBOA, DIMBOA	DIM₂BOA	
Delia coarctata (Fallén, 1825) [124]	DIMBOA		MBOA
Aphids			
Metopolophium dirhodum (Walker, 1849) [125–127]	DIMBOA	GHDMBOA	MBOA
Schizaphis graminum (Rondani, 1852) [125,128,129]	DIMBOA		
Sitobion avena (Fabricius, 1775) [130–133]	#		
Rhopalosiphum padi (Linnaeus, 1758) [88]	#		

Table 1. *Cont.*

Target Organism	Ref	Benzoxazinoids		BX Metabolites **
		Core Benzoxazinones	Induced BXs *	
		DIMBOA	GDMHBOA	?
Rhopalosiphum maidis (Fitch, 1856)	[134,135]			
Fungi	[58]		induce increase in GHDMBOA	
Helminthosporium turcicum Pass.	[136,137]	DIMBOA		
Cephalosporium maydis Samra, Sabet & Hing.	[138]			MBOA
Puccinia graminis Pers.	[139,140]			MBOA
Bipolaris maydis (Y. Nisik. & C. Miyake) Shoemaker	[43]			MBOA
Curvularia lunata R.R. Nelson & Haasis	[43]			MBOA
Alternaria alternata (Fr.) Keissl. (Fr.)	[43]			MBOA
Colletotrichum graminicola D.J. Politis	[141]			
Ustilago zeae (Link) Unger	[23]	increased fungal toxin production		
Fusarium verticillioides (Sacc.) Nirenberg	[23]	increased fungal toxin production		
Gibberella zeae (Schwein.) Petch	[83]	reduced fungal toxin production		
Plants				
Dicots				
Lepidium sativum L. (Brassicales) ****	[142]	DIBOA, DIMBOA		APO
Lepidium sativum L. (Brassicales) ****	[143]	DIBOA		BOA
Raphanus sativus L. (Brassicales) ****	[144]			BOA
Solanum lycopersicum L. (Solanales) ****	[142]	DIBOA, DIMBOA		BOA, MBOA, APO
Cucumis melo L. (Cucurbitales) ****	[145]	DIBOA		BOA
Cucumis sativus L. (Cucurbitales) ****	[145]	##		
Cucurbita pepo L.(Cucurbitales) ****	[145]	##		
Lactuca sativa L. (Asterales) ****	[145]	DIBOA		BOA
Vicia faba L. (Fabales) ****	[21]	DIBOA		
Vigna radiata (L.) R.Wilczek (Fabales) ****	[146]	DIBOA		BOA
Portulaca oleraceae L.(Caryophylales)	[147]	##		
Arabidopsis thaliana (L.) Heyhn (Brassicales)	[31]	DIBOA, DIMBOA		
Abutilon theophrasti Medik. (Malvales)	[145]	##		
Amaranthus palmeri S. Watson (Caryophyllales)	[145]	DIBOA		BOA
Amaranthus retroflexus L. (Caryophyllales)	[145]	##		
Chenopodium album L. (Caryophyllales)	[93,148]	##		
Ipomoea hederacea Jacq. (Solanales)	[145]	##		BOA

Table 1. *Cont.*

Target Organism		Benzoxazinoids Core Benzoxazinones	Induced BXs *	BX Metabolites **
Ipomoea lacunosa L. (Solanales)	[145]	##		
Sesbania exaltata (Raf.) Rydb. (Fabales)	[145]	##		
Senna obtusifolia (L.) H.S.Irwin & Barneby (Fabales)	[145]	##		
Rumex acetosa L. (Rumex acetosa)	[149]			BOA
Sida spinosa L. (Malvales)	[145]	##		
Monocots				
Allium cepa L. (Aspargales) ****	[142]	DIBOA, DIMBOA		MBOA, APO
Zea mays L. (Poales) ****	[145]	DIBOA		BOA
Triticum aestivum L. (Poales) ****	[142]	DIBOA, DIMBOA		APO
Avena sativa L. (Poales) ****	[21]	DIMBOA		
Avena fatua L. (Poales)	[95,150]	DIBOA, DIMBOA		BOA, MBOA, APO
Lolium rigidum Gaudin (Poales)	[95,150]	DIBOA, DIMBOA		BOA, MBOA, APO
Lolium perenne L. (Poales)	[95,150]			BOA
Dactylis glomerata L. (Poales)	[149]			BOA
Digitaria sanguinalis (L.) Scop. (Poales)	[145]	##		
Echinochloa crus-galli (L.) P.Beauv. (Poales)	[143,151]	DIBOA, DIMBOA		APO, BOA
Eleusine indica (L.) Gaertn. (Poales)	[145]	DIBOA		BOA
Panicum miliaceum L. (Poales)	[152]	DIBOA		BOA

* *also present in distinct developmental stages,* ** *including benzoxazolinones,* *** *affecting either root length, shoot length, or germination,* **** *crop plants,* # *assay using plant lines with different BX concentrations,* ## *assays using plant extracts or mulching,* ? *BX species not defined.* Abbreviations: APO 2-amino- phenoxazin-3-one;BOA 1,3-benzoxazol-2-one; DIBOA 2,4-dihydroxy-1,4-benzoxazin-3-one; DIMBOA 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; DIM2BOA 2,4- dihydroxy-7,8-dimethoxy-1,4-benzoxazin-3-one; HDMBOA 2-hydroxy-4,7-dimethoxy-1,4-benzoxazin-3-one; HDM2BOA 2-hydroxy-4,7,8-trimethoxy-1,4-benzoxazin-3-one; HMBOA 2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one; MBOA 6-Methoxy-2-benzoxazolinone. G indicates the presence of the O-glucoside

Minimal concentration for negative effect

< 1mM	1-2 mM	>2 mM- 10 mM	> 10 mM/ not affected	Concentration/ spec. BX not defined
Positive effect	Attraction	Repelling		

Since the BX levels are higher in seedlings and juvenile plants, mainly the first brood of the insect is affected; the control of following generations is less effective. However, fighting herbivores by pure quantity is only one strategy and is probably not conceivable for the mature plant due to high metabolic costs. Recently it has been shown that local events, including the induced biosynthesis and modification of BXs, are essential defence weapons even for juvenile plants (see [8,153] for review).

Like in the case of microbial infection, peptides can be involved in the induction of BXs (*Zm*Pep3, [59]). A major role in defence is attributed to the conversion of (G)DIMBOA to (G)HDMBOA [154]. A significant increase of (G)HDMBOA and to lesser extent (G)HDM$_2$BOA has been shown after *O. nubilalis*, *Ostrinia furnacalis* (Guenée, 1854), *Spodoptera frugiperda* (J. E. Smith, 1797), *S. exigua*, and *Spodoptera littoralis* (Boisduval, 1833)caterpillar herbivory [45,116,154–156]). Induction takes place on gene level. Within hours, the genes *Bx10* and *Bx11* that are required for the O-methylation of GDIMBOA are upregulated, followed by an increase of core *Bx*-gene transcription [45,156]. The activation of enzymes and transport of (G)HDMBOA might also contribute to fast local defence response. HDMBOA levels are associated with increased resistance and can be the effect of direct toxicity to the larvae or a result of the aforementioned faster conversion to MBOA compared to DIMBOA.

5.4.2. Aphids

Sucking insects such as aphids have both a direct and indirect influence on plant performance. Direct by the loss of energy-rich metabolites, and indirect since the transmission of viruses is often associated with sucking. The signals the plant experiences due to aphid infestation are different from those caused by the feeding of caterpillars. Preferentially, the path followed by the aphid's stylet to reach the phloem is intercellular and hence plant cell damage is minor. By contrast to findings with caterpillars, no changes in *Bx*-gene transcript levels were displayed in experiments with, e.g., *Rhopalosiphum padi* (Linnaeus, 1758) [157].

The performance of aphids is negatively affected by BXs in wheat and wild *Hordeum* for *Sitobion avenae* (Fabricius, 1794), *R. padi*, and *Schizaphis graminum* (Rondani, 1852) [23]. Repelling in choice tests and growth effects with artificial diets are documented. Toxicity and anti-feeding symptoms in the aphids were found to be similar to starvation [126,158]. Recently it has been shown that the effect of BXs on aphids can be more complex than mere toxicity. The aphid's contact with BXs in the first phase of infestation is limited to some punctured mesophyll cells. It is proposed that the minimal perception of BXs experienced there extends the time to reach the phloem and has a negative effect on feeding efficiency. Since aphids feeding on maize excrete substantial amounts of BXs in the honeydew [159], uptake from the phloem sap takes place and the aphids have also to deal with the reactive compound. Species-specific differences in metabolisation and excretion might account for varying susceptibilities.

In maize and wheat for *Rhopalosiphum maidis* (Fitch, 1856) (corn leaf aphid) no clear correlation between BX level and aphid performance was detected. An elaborate study combining metabolomics and genetics identified an alternative BX-based resistance mechanism by elucidating the cause of natural variation in *R. maidis* resistance [44]. Resistance is conferred by the mutation of *Bx12*, the constitutively expressed GDIMBOA O-methyltransferase. Hence, without inducing stimulus as conferred by chewing insects, GHDMBOA is missing in the plant. This finding appears contradictory since GHDMBOA is more toxic to the aphids in an artificial diet compared to GDIMBOA. However, it has been shown that DIMBOA is the required signal for the induction of callose, while HDBMOA does not affect callose concentration [88]. Since callose deposition can hinder the access to the phloem by blocking sieve elements and constricting intercellular paths, aphid performance (*R. maidis* and probably *R. padi*) can be efficiently restricted by high DIMBOA levels. Hence, the activity of the core BX biosynthesis [53,134] and prohibition of further metabolisation [44] increases resistance. The restriction of caterpillar damage conferred by HDMBOA and the reduction of aphid infestation depending on DIMBOA-induced callose deposition are conflicting concepts. The search for resistance to *R. maidis* with the background of *S. exigua*-mediated BX induction identified a QTL that coincides with the

O-methyltransferase cluster *Bx10* to *Bx12* [45]. The differences found in the gene expression and the resulting different GHDMBOA/GDIMBOA levels induced by caterpillar feeding affect aphid control and demonstrate that there is a trade-off between aphid and caterpillar resistance. Another factor to be considered the presence of further modifications of the BX structure by hydroxylation and N-OH methylation to yield $GDIM_2BOA$ and $GHDM_2BOA$ (Figure 1, [46]). Both metabolites were shown to confer aphid control but do not contribute to resistance against chewing insects.

As mentioned before, BX10 to BX12 are equally functional in vitro as GDIMBOA O-methyltransferases to yield GHDMBOA (Figure, [46]). However, in maize the three genes displayed different expression levels. Only *Bx12* is expressed constitutively, while transcript levels of *Bx10* and *Bx11* were significant only upon caterpillar damage [45]. Some maize lines carry a transposon insertion in *Bx12* that inactivates the gene. Hence, GHDMBOA biosynthesis is restricted to caterpillar-damaged tissue in these lines. This mutant allele is present in most temperate maize lines of the 276 inbred lines of the Goodman Panel [160]. In the maize progenitor teosinte population, the allele is rare [161]. Since the threats by insects differ in tropical (chewing insects) and temperate regions (aphids), the selection of the appropriate efficient BX—GDIMBOA in case of aphids—might have been a consequence of the domestication process [8,44–46,161].

5.4.3. Specialist Insects

Specialist insects can cope with defence metabolites and use the compounds to locate their favourite plant species. Moreover, young metabolic rich tissue is often characterised by high levels of the metabolite that is used as a cue by herbivorous insects, e.g., wheat seedling exudates attract the specialist herbivore wheat bulb fly *(Delia coarctata* (Fallén, 1825)). The reaction is MBOA dose-dependent; the response to DIMBOA is less pronounced [124]. Similarly, nutritious crown roots of maize are located by the corn rootworm (*Diabrotica virgifera* (LeConte, 1858)) by the content of BXs [91]. Moreover, *D. virgifera* employs BXs for its own defence [162]. MBOA and HDMBOA are used in different strategies by the insect. MBOA is *N*-glucosylated and released. The stabilised metabolite repels young nematodes that would otherwise attack the larvae. GHDMBOA is sequestered into the insect body and activated upon nematode attack. Nematodes and their symbiotic bacteria are killed by the generated high local MBOA concentrations.

Insects are exposed to the ad hoc delivery of BX aglucons following plant cell damage. Obviously instantaneous stabilisation will reduce deleterious effects. Indeed, glucosylation by UGTs in insect intestines was detected and the efficiency was correlated with the performance of the caterpillars in the presence of BXs (see [10] for review). The caterpillars excrete glucosides of DIMBOA, 2-Hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA), and MBOA. Interestingly the stereochemistry of the plant-derived glucoside (*2R*) and the insect product (*2S*) is different, and hence the plant BX glucosidase is not functional in re-activating the defence compound. Other modifications, e.g., hydroxylations, might also contribute to detoxification. The formation of GMBOA-carbamate has been revealed for *Mythimna separate* (Walker, 1865) [163]; however, the steric conformation differs from the respective carbamate produced by plants.

5.5. BXs as Signalling Components

Glucosinolates (GS) in the family Brassicaceae share features with BXs: their biosynthesis is tightly linked to tryptophan and hence auxin biosynthesis, GS are expressed constitutively at developmental stages but the pattern can be changed by biotic and abiotic stress, and both play an essential role in callose deposition [88,164]. Although a strong piece of evidence is lacking, it can be speculated that both specialised metabolites are recruited in parallel to serve beyond defence as signalling molecules as well. A further analogy could be the impact on flowering time. In a mapping approach with maize landraces, *Bx12* was identified as a locus affecting female and male flowering [165]. Similarly, GS biosynthesis has been found to influence flowering time [166]. As mentioned before, *Bx12* was proposed as selection target in domestication during the move from tropical to temperate regions due

to changes in classes of challenging insects. However, the geographical spread was also obviously connected with changes in the length of day, which influences flowering time. The impact of different BX patterns on either of the two traits is not clear at present.

The background for the speculatively supposed signalling function is found in the relation to the phytohormone auxin. BXs have been described as antagonists of auxin binding and auxin-induced growth [167,168] and were proposed to function contradictory as co-auxins [169]. The light-induced curvature of coleoptiles was attributed to blue light-induced BX accumulation and H_2O_2 released by DIMBOA production on the illuminated side [170,171]. However, the almost BX-free introgression line of the *bx1* reference mutant [13,30] in the maize line B73 does not show any morphological or physiological phenotype as a seedling or adult plant, under laboratory conditions, in the greenhouse, and in the field compared to B73, apart from the lack of BX [92]. This would not be expected if BX had a significant role in the auxin signalling network.

6. Conclusions

In defined experimental setups, the effect of BXs after damage by insects and microbes is well established. Delivered into the soil, BXs serve mutualistic interactions with geobionts. In recent years, it has become obvious that biologically active BXs constitute a compound family, comprising biosynthetic derivatives and intermediates of catabolism including microbial modifications. The amount and composition of BXs was shown to differ within species for lines and developmental stages. Furthermore, biotic and abiotic stresses influence the BX pattern. The data were mainly derived from maize, and it has yet to be evaluated how far the structural diversity and expression patterns are the same in other plants, e.g., wheat. Single BX species might be used as attractants or repellents and can be toxic. In maize, natural variation exists that might allow tailoring BXs to fit to specific requirements. However, it has to be considered that single BXs might have conflicting effects in different biotic interactions. Since the maize genes of the biosynthetic pathway are known, molecular markers can assist in selection. Mutants can clarify the impact of a given compound for plant protection.

Selectivity in the control of weeds, insects, and microbes is mediated by the different abilities of the organisms to detoxify BXs. At least for plants and insects, glycosylation seems to be the essential step to reduce deleterious effects. The long-time experience with BXs in plant protection is positive. No general breakage of resistance (e.g., by *O. nubilalis*) was observed. This might be due to the fact that diverse BXs are present that are not super toxins, i.e., killing at the lowest concentration, but that do have moderate effects on different levels in the lifecycles of the target organisms.

Although much information has been gained in recent years, much research has yet to be done to clarify the role of BXs in nature.

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Article

Arabidopsis thaliana Immunity-Related Compounds Modulate Disease Susceptibility in Barley

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Abstract: Plants are exposed to numerous pathogens and fend off many of these with different phytohormone signalling pathways. Much is known about defence signalling in the dicotyledonous model plant *Arabidopsis thaliana*, but it is unclear to which extent knowledge from model systems can be transferred to monocotyledonous plants, including cereal crops. Here, we investigated the defence-inducing potential of *Arabidopsis* resistance-inducing compounds in the cereal crop barley. Salicylic acid (SA), folic acid (Fol), and azelaic acid (AzA), each inducing defence against (hemi-)biotrophic pathogens in *Arabidopsis*, were applied to barley leaves and the treated and systemic leaves were subsequently inoculated with *Xanthomonas translucens* pv. *cerealis* (*Xtc*), *Blumeria graminis* f. sp. *hordei* (powdery mildew, *Bgh*), or *Pyrenophora teres*. Fol and SA reduced *Bgh* propagation locally and/or systemically, whereas Fol enhanced *Xtc* growth in barley. AzA reduced *Bgh* propagation systemically and enhanced *Xtc* growth locally. Neither SA, Fol, nor AzA influenced lesion sizes caused by the necrotrophic fungus *P. teres*, suggesting that the tested compounds exclusively affected growth of (hemi-)biotrophic pathogens in barley. In addition to SA, Fol and AzA might thus act as resistance-inducing compounds in barley against *Bgh*, although adverse effects on the growth of pathogenic bacteria, such as *Xtc*, are possible.

Keywords: systemic acquired resistance; barley; salicylic acid; folic acid; azelaic acid; *Blumeria graminis* f. sp. *hordei*

1. Introduction

Plants are constantly challenged with a plethora of pathogens, including herbivorous insects, fungi, oomycetes, bacteria and viruses [1]. To cope with these attacks, plants have evolved an effective immune system of pre-formed and inducible defence mechanisms. The former include morphological barriers, for instance the plant cell wall, cuticle, phytoanticipins, and antimicrobial proteins. Inducible defence mechanisms include processes like cell wall reinforcement by lignin and callose or the synthesis of phytoalexins and defence-related proteins or enzymes [1–4].

After the first contact between plant and pathogen, conserved structures such as bacterial flagellin or fungal chitin—so-called pathogen-associated molecular patterns (PAMPs)—encounter pattern recognition receptors on the cell surface. This triggers a first immune response termed PAMP-triggered immunity (PTI) [2,4,5]. During long-term evolution of plant–pathogen interactions, some pathogens developed effector proteins to suppress PTI. In response to this development, plants evolved resistance (*R*) genes, which encode proteins that directly or indirectly recognise effectors, and on recognition elicit so called effector-triggered immunity (ETI) [2–4,6].

Both PTI and ETI are associated with the induction of various cellular responses. These include the synthesis of antimicrobial compounds, the generation of reactive oxygen species, the activation

of mitogen-activated protein kinases, transcriptional reprogramming, and the accumulation of phytohormones such as salicylic acid (SA) [2–4,7,8]. SA is known to affect the redox status of plants, thereby leading to transcriptional reprogramming and enhanced transcription of *PATHOGENESIS-RELATED* (*PR*) genes, which are thought to promote resistance [7–11]. Furthermore, SA not only helps the plant to defend itself against a present infection, it is also involved in the induction of a process protecting the plant in case of future pathogen challenge [4,6,8,12]. This response is termed systemic acquired resistance (SAR) and is usually triggered by a local, foliar infection. It elicits long-lasting, broad-spectrum resistance in systemic plant tissues (reviewed in [6–9,12]). Many signals and genes involved in SAR have been discovered in *Arabidopsis thaliana*. During SAR, SA levels in the infected and systemic tissues rise, and transcripts of *PR* as well as other defence-related genes accumulate. This establishes a status of heightened alert; the plant is "primed" to respond more quickly to a secondary infection [13,14]. SAR is most effective against pathogens fended off via SA-dependent responses [3,4,8].

During SAR, long-distance signals are generated in the infected leaves and travel to the systemic leaves, presumably via the phloem [12]. Recent evidence suggests that signal transmission also occurs via volatile compounds, in particular monoterpenes, which appear to be transmitted via the air [15]. In *Arabidopsis*, candidate long-distance SAR signals further include methyl salicylate [16], glycerol-3-phosphate (G3P) [17,18], the C_9 dicarboxylic acid azelaic acid (AzA) [19], the diterpene dehydroabietinal [20], N-hydroxy-pipecolic acid [21,22], and the lipid-transfer proteins DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1) and DIR1-like [23,24] (reviewed in [12,25,26]). Some of these compounds accumulate in the phloem after pathogen attack, among them SA, AzA, G3P, and pipecolic acid [19,27–29]. AzA and G3P were proposed to be symplastically loaded onto the phloem via plasmodesmata while SA appeared to be transported via an apoplastic route [29]. Only small proportions of SA and AzA are transported to the systemic tissue, and the majority of accumulation in systemic leaves thus appears to come from de novo synthesis [30–32]. Importantly, biosynthesis of the SAR-related compounds SA and pipecolic acid in systemic leaves is crucial for SAR establishment, questioning the biological relevance of the systemic mobility of these signals [18,33,34].

Most of the mobile metabolites induce SAR when exogenously applied to *Arabidopsis* plants. Local application of AzA, for example, induces a SAR-like state in the treated plants, protecting the systemic tissue against a subsequent infection with the hemi-biotrophic bacterium *Pseudomonas syringae* [19]. Additional SAR-inducing compounds include folate precursors or folic acid (Fol), which induces SA-mediated immunity in *Arabidopsis*, both locally and systemically, and in pepper (*Capsicum annuum*) [35,36].

In monocotyledonous plants, less is known about SAR and the signalling mechanisms involved. Key players in SA signalling, including NPR1 (NONEXPRESSOR OF PR GENES 1), the master regulator of SA signalling [7,9,10], several *PR* genes and SA-associated transcription factors are conserved between dicots and monocots, (reviewed in [37,38]). Most studies in this field focus on agronomically important plant species such as banana (*Musa acuminata*) [39], wheat (*Triticum aestivum*) [40–42], maize (*Zea mays*) [43], and rice (*Oryza sativa*) [38], in which important roles for SA signalling have been uncovered in resistance against pathogens including *Fusarium oxysporum*, *Puccinia striiformis*, *Xanthomonas oryzae* pv. *oryzae* and *Magnaporthe oryzae*. Treatments with the SA analogue benzothiadiazole (BTH) induce resistance in maize [44], wheat [45], and barley [46–48].

SA-associated immune responses in barley (*Hordeum vulgare*) often are studied in interaction with *Blumeria graminis* f. sp. *hordei* (*Bgh*), commonly named powdery mildew, an obligate biotrophic fungal pathogen that thrives on living host cells [49]. Since it causes reduced yield and serves as a model to study other mildews and obligate biotrophic pathogens [50], *Bgh* appeared on the list of the top 10 fungal pathogens by Dean et al. in 2012 [51]. In contrast to biotrophic pathogens of rice, *Bgh* inoculation does not result in SA accumulation in infected barley leaves [52–54]. Nevertheless, SA soil drench treatment of barley seedlings had a weak effect on *Bgh* infectivity [46,55], whereas soil drench treatment with BTH strongly enhanced barley resistance to *Bgh* [46].

Recently, Dey et al. [47] showed that in barley systemic resistance to the hemi-biotrophic bacterium *Xanthomonas translucens* pv. *cerealis* (*Xtc*) can be triggered by prior infiltration of a single barley leaf with the hemi-biotrophic bacterium *Pseudomonas syringae* pv. *japonica*. Unlike SAR in *Arabidopsis*, systemic immunity in barley was neither associated with SA nor with NPR1. In addition, local infiltration of SA or its functional analogue BTH did not induce systemic resistance to *Xtc*. Rather, local methyl jasmonate (MeJA) and abscisic acid (ABA) treatments, which in *Arabidopsis* induce systemic susceptibility to *P. syringae* [56] or antagonize SAR [57], respectively, triggered systemic resistance in barley to *Xtc* [47].

In plant immunity, cross talk between phytohormones is important and is believed to help achieve the best possible (defence) outcome. In phytohormone cross talk, an interaction is never defined by a single hormone, but rather by a complex network of interdependent positive and negative interactions [58–60]. The result of these interactions leads to responses in the plant, which allow it to appropriately respond to an invading pathogen. Pathogens of different lifestyles have different demands on their hosts and, therefore, must be combatted using different mechanisms; while biotrophs feed on living cells, necrotrophs acquire their nutrients from degraded and dead tissue. Several phytohormones are involved in plant defence responses against pathogens. The traditional three main players are SA, jasmonic acid (JA) and ethylene (ET), but recent evidence hints at the additional contribution of other hormones [58–60]. Mostly synergistic interactions are reported for JA and ET, which mainly promote defence against necrotrophic pathogens and insects [3,59–61]. SA and JA signalling pathways are interdependent; there is substantial cross talk between the two, comprising synergistic as well as antagonistic interactions, depending on the defence situation [3,59,62]. Biotrophic pathogens are mostly opposed using SA signalling, whereas necrotrophic pathogens are combatted using JA/ET-dependent pathways [3,59,60,62]. Because SA–JA cross talk is often antagonistic, SA-induced resistance against biotrophs can enhance susceptibility against necrotrophs and *vice versa* [60,63]. In *Arabidopsis*, for example, SA and Fol induce resistance against hemi-biotrophic bacteria and at the same time enhance susceptibility to the necrotrophic fungal pathogen *Alternaria brassicicola* [35,63]. Here, we query if such antagonistic cross talk between responses to biotrophic and necrotrophic pathogens also occurs in barley by using a barley pathogen from the necrotrophic side of the spectrum, the fungus *Pyrenophora teres*. *P. teres* is the causal agent of net and spot form net blotch, a major disease in many barley-growing areas, which can lead to severe yield loss, underlining the fungus' economic importance [64].

We want to study the possible effects of *Arabidopsis* SAR-associated compounds on barley defence responses. Therefore, we will investigate the influence of different chemical compounds, including SA, on the propagation of barley-specific pathogens. We will employ pathogens of different lifestyles, namely the hemi-biotrophic bacterium *Xtc*, the biotrophic fungus *Bgh* and the necrotrophic fungus *P. teres*. In addition to SA, we will include Fol, which induces an SA-dependent defence response in *Arabidopsis* [35]. Also, we will include AzA, a SAR signal, which in *Arabidopsis* primes SA accumulation, inducing a faster and stronger response after pathogen attack compared to that in unprimed plants [19]. The data shed light upon the possible transferability of signalling intermediates related to SAR from *Arabidopsis* to barley. As we detected differences and similarities in the responses of these plants interacting with (hemi-)biotrophic and necrotrophic pathogens, the data reinforce the necessity of studying metabolite-induced resistance in cereal crops.

2. Materials and Methods

2.1. Plants and Growth Conditions

Barley (*Hordeum vulgare* L. cultivar 'Golden Promise' (GP)) seeds were sterilized in 1.2% sodium hypochlorite for 3 min with 25 inversions per minute. Subsequently, seeds were rinsed 3 times with water for 10 min with 25 inversions per minute and then sown (Einheitserde classic CL-T, Bayerische Gärtnereigenossenschaft). Plants for *Xtc* infections were either grown in a greenhouse with additional

lights HQI-TS 400W/D (Osram) using a day-night cycle of 12 h with 24 °C during the day and 20 °C during the night or in a climate chamber with 16 h light and 8 h darkness at a temperature of 20 °C (day)/16 °C (night). Chamber-grown plants inoculated with *Xtc* were transferred to a climate chamber with 14 h light and 10 h darkness at a temperature of 29 °C (day)/19 °C (night). Plants for *Bgh* infections were grown in climate chambers with 16 h light and 8 h darkness at a temperature of 20 °C (day)/16 °C (night). Plants for *P. teres* infections were grown in the green house as described above.

2.2. Chemicals and Treatments

Stock solutions of each chemical compound were freshly prepared for each experiment. SA (Roth, Karlsruhe, Germany) was dissolved at 4 M in 100% methanol (MeOH; Merck, Darmstadt, Germany), Fol (Roth, Karlsruhe, Germany) was dissolved at 1 M and AzA (Sigma-Aldrich, St. Louis, MO, USA) at 2 M in 50% MeOH. For plant treatments, the substances were diluted to 1 mM SA [35,45], 50 or 500 µM Fol [35], and 1 mM AzA [19,31] in 10 mM $MgCl_2$ (Roth, Karlsruhe, Germany). 0.025% MeOH in 10 mM $MgCl_2$ served as the mock treatment. To monitor systemically induced resistance, the first true leaves of 3-week-old barley plants (6 plants per treatment) were infiltrated with the different compounds or the mock solution using a needleless syringe. Five days after the primary treatment, the second leaves of the treated plants were infected with either *Xtc*, *Bgh*, or *P. teres* (see below). To monitor local induced resistance against *Xtc*, 4-week-old barley plants were sprayed with 1 mM SA, 500 µM Fol, 1 mM Aza, or 0.05% MeOH (as mock treatment) in 0.01% Tween-20 (Calbiochem, San Diego, CA, USA). To monitor local induced resistance against *Bgh*, 1 mM SA, 500 µM Fol, and 1 mM AzA in 10 mM $MgCl_2$ were syringe-infiltrated into the first true leaves of 3-week-old barley plants. 0.025% MeOH in 10 mM $MgCl_2$ served as the mock treatment. To monitor local induced resistance against *P. teres*, the second true leaves of 3-week-old barley plants were syringe-infiltrated with the compounds. The treated leaves were inoculated with *Xtc* 1 day after treatment, with *Bgh* 5 days after treatment, or with *P. teres* 1 day after treatment.

2.3. Xanthomonas Translucens pv. Cerealis (Xtc) Infection

Xanthomonas translucens pv. *cerealis* (*Xtc*) strain LMG7393 was obtained from the Laboratory of Microbiology UGent (LMG) collection of the Belgian Coordinated Collections of Microorganisms. For infection, *Xtc* was grown on LMG medium (15 g/L tryptone, 5 g/L soya peptone, 5 g/L NaCl, and 18 g/L agar-agar (Roth, Karlsruhe, Germany); pH adjusted to 7.3) over night at 28 °C. Bacteria were subsequently resuspended in 1 mL 10 mM $MgCl_2$ (Roth, Karlsruhe, Germany). The concentration of the bacterial suspension was adjusted to ~10^5 colony forming units (cfu)/mL in 10 mM $MgCl_2$ using a photometer (assuming the formula: OD_{600} of 0.2 equals ~10^8 cfu/mL). Leaves of 3–5 barley plants were subsequently inoculated with the resulting *Xtc* suspension by infiltration using a needleless syringe. The infected plants were covered with a plastic hood and kept in the green house for 4 days. The resulting *in planta Xtc* titres were determined as previously described [47,65].

2.4. Blumeria Graminis f. sp. Hordei (Bgh) Infection

Blumeria graminis f. sp. *hordei* (*Bgh*) Swiss field isolate CH4.8 was obtained from Dr. Patrick Schweizer (Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung, Gatersleben, Germany). *Bgh* propagation and inoculation was performed essentially as described in [66]. In short, a pot containing 12 10-day-old seedlings was infected with *Bgh* one week prior to each experiment. Six hours prior to the start of an experiment, these plants were shaken in order to remove old conidia and provide a uniform inoculum for the experiment [67]. Compound- or mock-treated plants were subsequently inoculated with spores from the prepared *Bgh*-infected plants in an inoculation tower [66] at an inoculation density of ~30 spores/mm^2. The inoculated plants were placed back in the climate chamber for 6 days. Subsequently, 4 leaf discs (6 mm) were cut out of the distal halves of each first (local/compound-treated) and second (systemic) true leaf of the treated plants. The discs were incubated with 5 µM DAF-FM-DA (4-amino-5-methylamino-2′,7′-difluorofluorescein

diacetate; Sigma-Aldrich, St. Louis, MO, USA or Santa Cruz Biotechnology, Dallas, TX, USA) in MES buffer (2-(N-morpholino)ethanesulfonic acid, 50 mM MES-KOH pH 5.7 (Roth, Karlsruhe, Germany), 1 mM $CaCl_2$ (Merck, Darmstadt, Germany), 0.25 mM KOH) for 45 min in the dark and then vacuum-infiltrated. Subsequently, the leaf discs were placed in light (45 V lamp) for 1 h and 45 min and afterwards distributed on 96-well plates (1 leaf disc per well, 20–24 discs from 6 plants per treatment) with the wells filled evenly to the rim with 1% phytoagar. Fluorescence of appropriate z-stacks was visualized using the 5× objective of an inverse spinning disc confocal microscope (Zeiss Axio Observer. Z1, Zeiss, Oberkochen, Germany). Chlorophyll was excited using a laser with 561 nm and detected using a bandpass 629/62 filter; DAF-FM DA was excited using a laser with 488 nm and detected using a bandpass 525/50 filter [68]. The 96-well plates were transferred to the microscope with a KiNDx Robot (Model KX-300-660-SSU, Peak Analysis and Automation, Inc., Farnborough, UK; assembled and set up by Analytik Jena, Jena, Germany). The robot and the visualization as well as evaluation were controlled by the softwares Microscope AppStudio (Analytik Jena, Jena, Germany) and ZEN2 (Zeiss, Oberkochen, Germany). Fluorescence intensities were normalized to those of uninfected barley plants (background fluorescence) of the same age.

2.5. P. Teres Infection

A field isolate of *Pyrenophora teres* was donated by Günther Bahnweg (Helmholtz Zentrum München, Neuherberg, Germany) and grown on oat plates (10 g rolled oats (Alnatura, Germany), 7.5 g agar-agar (Roth, Karlsruhe, Germany), 500 mL H_2O) for ~1 week at room temperature in the dark and then transferred to light for at least 2 weeks. Two mL of infection solution for fungi (0.85 g KH_2PO_4 (Merck, Darmstadt, Germany), 0.1 g glucose (Roth, Karlsruhe, Germany), and 1 μL Tween 20 in 100 mL H_2O, pH 6.0) were pipetted onto the *P. teres* plates and spores were scratched off the agar using an inoculation loop. The spore suspension was subsequently pipetted into a 5 mL tube and vortexed. After determining the spore concentration under a binocular, the spore suspension was diluted to 65–110 spores per μL. Infections were performed on 6 cm-long segments of a leaf, at a distance of 1.5 centimetres from the leaf base. Five 3 μL droplets of spore suspension were pipetted alternatingly on each side of the leaf midrib. The drops were left to dry for ~1 h and the plants (6 plants per treatment) were then covered with a plastic hood. Necrotic lesions caused by *P. teres* were measured 4 days after infection using the ImageJ macro PIDIQ [69]. The macro was modified to measure brown necrotic lesions caused by *P. teres*. These modifications were restricted to the values used for colour characterisation; the values for lesion measurements were as follows: hue 0–52, saturation 150–255, brightness 0–150.

2.6. Statistics

Results of biologically independent replicate experiments with two groups were tested together for homogeneity of variance using the *F*-test in Microsoft Excel. Depending on the outcome of the *F*-test, a two-sided *t*-test either for equal (homoscedastic) or unequal variances (heteroscedastic) was conducted in Microsoft Excel. Results of biologically independent replicate experiments with more than two groups were analysed in GraphPad Prism 7 for Windows (version 7.04). Data with only positive values were log_2 transformed and data with negative and positive values were transformed according to the formula: $Y = log_2[Y + 1 − min(Y)]$, where min(Y) denotes the lowest measured value within the experiment. Subsequently, the data were analysed for outliers using Grubbs' test with $\alpha = 0.05$. The D'Agostino–Pearson normality test was performed with $\alpha = 0.01$ in order to test for normal distribution. If necessary the Grubbs' outlier test was repeated to assure normal data distribution (a maximum of 2 Grubbs' outlier tests were performed per data set). The data were subsequently analysed using one-way analysis of variance (ANOVA) with the Geisser-Greenhouse correction ($p < 0.05$) and a subsequent Dunnett's post hoc test with $\alpha = 0.05$.

3. Results

3.1. Folic Acid and Azelaic Acid Enhance the Susceptibility of Barley to Xtc

Exogenous SA or BTH application enhances the resistance of barley to the biotrophic fungus *Bgh* [46], but not to the hemi-biotrophic bacterium *Xtc* [47]. Here, we examined *Xtc* growth in barley after application of Fol and AzA, which each trigger SA-mediated resistance and/or SAR against the hemi-biotrophic bacterium *P. syringae* pv. *tomato* in *A. thaliana* [19,35]. In 3-week-old barley plants, the first true leaves were syringe-infiltrated with 50 or 500 μM Fol, 1 mM AzA, or the corresponding 0.025% MeOH mock control. Five days later, the second true leaves of the treated plants were syringe-infiltrated with *Xtc* and the resulting *in planta Xtc* titres determined at 4 days post-inoculation (dpi). Similar to SA, Fol and AzA did not reduce *Xtc* growth in the systemic tissue of barley (Figure 1). Whereas AzA application did not influence *Xtc* growth in the systemic tissue (Figure 1b), growth of the systemic *Xtc* inoculum was enhanced by a local Fol application (Figure 1a), suggesting the induction of enhanced susceptibility in barley against *Xtc* by Fol.
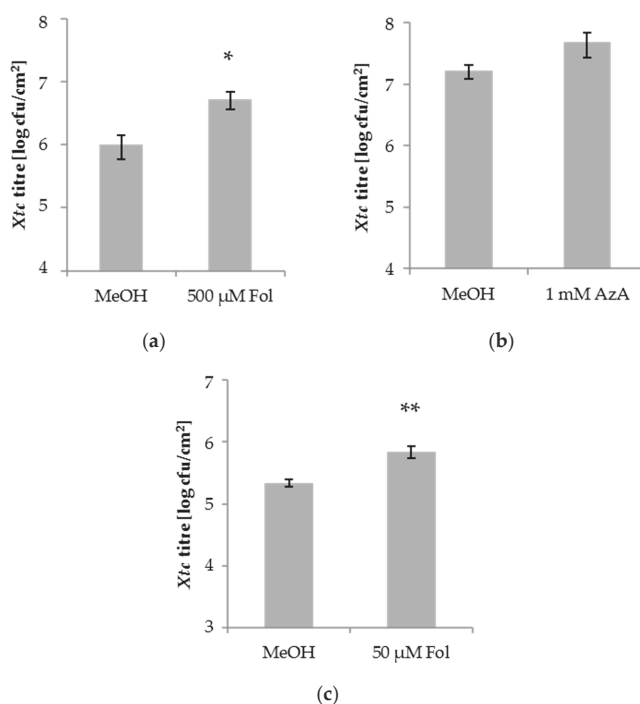
Figure 1. *Xanthomonas translucens* pv. *cerealis* (*Xtc*) titres in the systemic leaves of barley after local application of folic acid (Fol) or azelaic acid (AzA). Barley cultivar Golden Promise (GP) plants were infiltrated in the first true leaf with 0.025% methanol (MeOH) as control, 500 μM Fol (**a**), 1 mM AzA (**b**), or 50 μM Fol (**c**) in 10 mM MgCl$_2$ as indicated below the panels. Five days later, the second true leaves of the plants were inoculated with *Xtc* by syringe infiltration. The resulting *Xtc* titres in leaf 2 at 4 dpi are shown. Bars represent the average of 18–36 replicates from 5 ((**a**): 4× greenhouse, 1× growth chamber; (**b**): 3× greenhouse, 2× growth chamber) to 9 ((**c**): 8× greenhouse, 1× growth chamber) biologically independent experiments ± standard error; replicates were as follows: (**a**) MeOH: 19 (4 + 4 + 3 + 4 + 4), Fol: 18 (4 + 4 + 3 + 4 + 3), (**b**) MeOH: 19 (4 + 3 + 3 + 4 + 5), AzA: 20 (4 + 4 + 4 + 4 + 4), (**c**) MeOH: 34 (4 + 3 + 4 + 4 + 3 + 4 + 4 + 4 + 4), Fol: 36 (4 + 4 + 4 + 4 + 4 + 4 + 4 + 4 + 4). Asterisks above bars indicate statistically significant differences from the control treatments (*t*-test; * $p < 0.05$, ** $p < 0.005$).

Fol enhances *A. thaliana* resistance to *P. syringae* when applied at concentrations of 50 to 100 μM [35]. If we applied 50 μM Fol to the first leaves of 3-week-old barley plants, the growth of subsequently applied *Xtc* bacteria in the systemic leaves was enhanced in three out of 10 experiments and a strong tendency in the same direction was observed in a further two experiments. If we analyse all experiments together, the data suggest that application of 50 μM Fol can, similarly to 500 μM Fol, enhance barley systemic susceptibility to *Xtc* (Figure 1c), albeit perhaps less robustly.

We next tested the local barley responses to the compounds used above. To this end, 1 mM SA, 500 μM Fol, 1 mM AzA, or the appropriate mock control were applied onto the leaves of 4-week-old barley plants by spray treatment. One day later, treated leaves were syringe-infiltrated with *Xtc* and the resulting *in planta Xtc* titres determined at 4 dpi. SA and Fol appeared to moderately elevate local *Xtc* titres, with a significant trend observed only after Fol treatment. AzA application caused an increase of the *Xtc* titre (Figure 2). Thus, in addition to systemic susceptibility, Fol might also moderately enhance the local susceptibility of barley to the hemi-biotrophic bacterium *Xtc*. The effect appeared more robust in the systemic compared to local treated tissues (Figure 1a,c and Figure 2). AzA enhanced local, but not systemic susceptibility to *Xtc*.
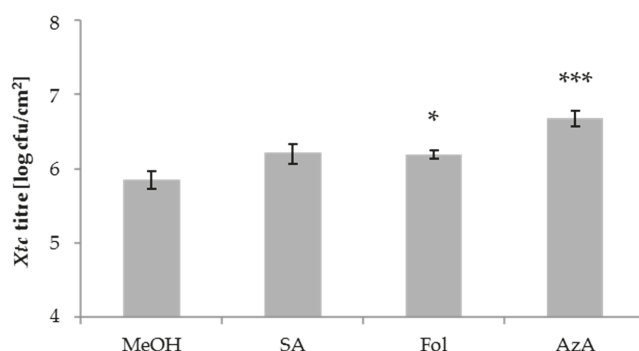
Figure 2. *Xtc* titres in salicylic acid (SA)-, Fol- and AzA-treated barley plants. Barley cultivar GP plants were sprayed with 0.05% MeOH as control, 1 mM SA, 500 μM Fol, or 1 mM AzA in 0.01% Tween-20 as indicated below the panel. One day later, the second true leaves of the plants were inoculated with *Xtc* by syringe infiltration. The resulting *Xtc* titres in leaf 2 at 4 dpi are shown. Bars represent the average of 8–9 replicates from 3 biologically independent greenhouse experiments ± standard error, replicates were as follows: MeOH: 9 (3 + 3 + 3), SA: 9 (3 + 3 + 3), Fol: 8 (3 + 3 + 2), AzA: 8 (3 + 3 + 2); asterisks above bars indicate statistically significant differences from the mock control treatment (one-way ANOVA and post hoc Dunnett's test, * $p < 0.05$, *** $p < 0.0005$).

3.2. Folic Acid Enhances the Resistance of Barley to Powdery Mildew (Bgh)

In *Arabidopsis* Fol triggers SA-mediated immunity against hemi-biotrophic bacteria [35]. In barley, SA and in particular its functional analogue BTH enhances immunity against the biotrophic fungus *Bgh* while Fol enhances barley susceptibility to *Xtc* (Figures 1 and 2; [46]). Here, we compared the effects of SA, Fol, and AzA on propagation of *Bgh* in barley. To this end, the first true leaves of 3-week-old barley plants were syringe-infiltrated with 1 mM SA, 500 μM Fol, 1 mM AzA, or 0.025% MeOH as control treatment. Five days later, the plants were inoculated with *Bgh*. The resulting *Bgh* infection was quantified on a fluorescence microscope 6 days later. We used the fluorescent dye DAF-FM DA which usually is used to detect and quantify NO [70] and, as shown in Figure 3, also has a high affinity for *Bgh*. If leaf discs from *Bgh*-infected leaves were stained with DAF-FM DA, the resulting fluorescence intensities reflected visual differences in *Bgh* infection levels and were quantified as a measure for fungal growth (Supplemental Figure S1). To this end, the stained leaf discs were loaded onto 96-well plates and scanned by a spinning disc (confocal) microscope (Figure 3).
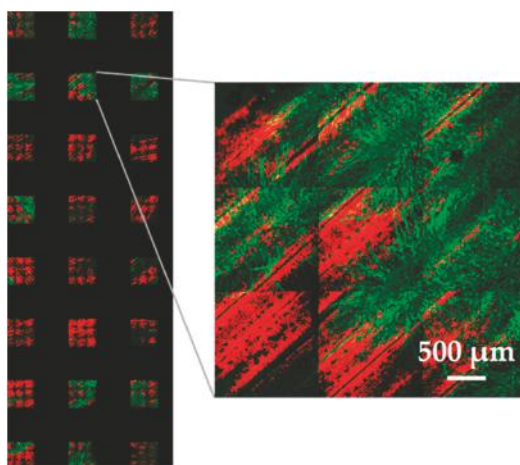
Figure 3. Merged *z*-stack 3 × 3 tiled images of 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM-DA) stained discs of *Blumeria graminis* f. sp. *hordei* (*Bgh*)-infected barley leaves in the first 3 columns of a 96-well plate. Enlarged: merged image of a single well. Chlorophyll fluorescence is shown in red, DAF-FM DA fluorescence in green.

A local infiltration of Fol in leaf 1 of barley reduced *Bgh* growth on the Fol-treated leaf (Figure 4a) as well as on systemic leaf 2 of the treated plants (Figure 4b) as evidenced by a ~50% decrease in DAF-FM-DA fluorescence on the leaves of *Bgh*-infected Fol-treated compared to mock-treated plants. In contrast, SA and AzA infiltration did not induce significant changes in the DAF-FM-DA fluorescence of the treated leaves and thus had no local effect on *Bgh* growth (Figure 4a). Nevertheless, SA and AzA appeared to reduce *Bgh* propagation, although the differences to the control were not significant (p = 0.0518 in the case of SA, Figure 4a). In the systemic leaves, *Bgh*-associated DAF-FM-DA fluorescence was decreased by SA and AzA to a similar extent as by Fol (Figure 4b). Thus, Fol might induce local and systemic resistance to *Bgh*, while SA and AzA appear to trigger systemic resistance.

(**a**)

Figure 4. *Cont.*

(**b**)
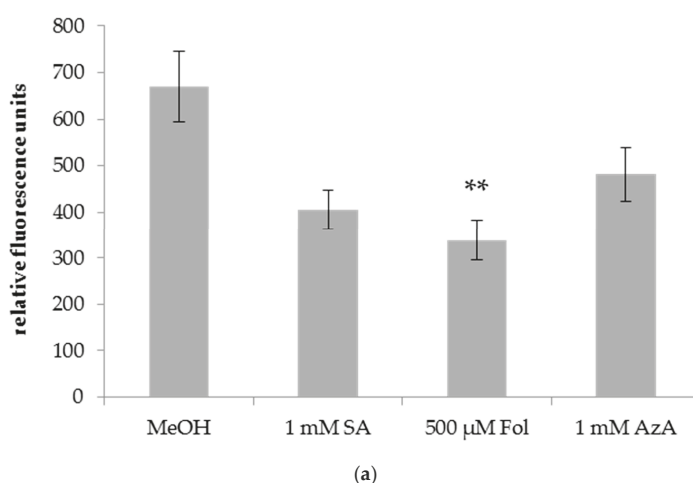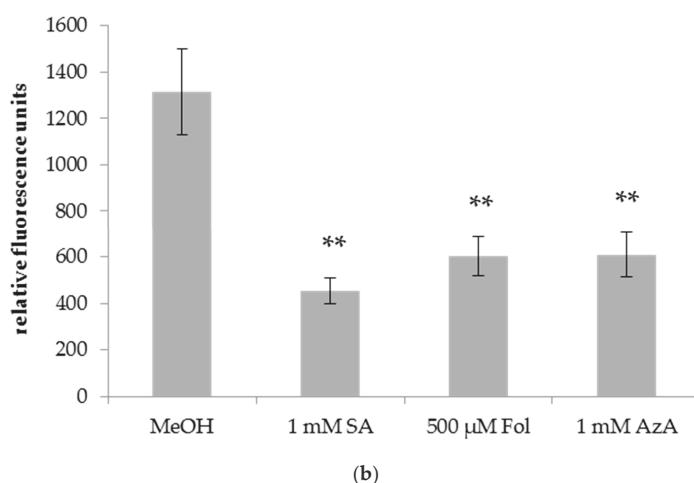
Figure 4. Relative fluorescence of DAF-FM DA staining of *Bgh* in leaves of barley plants after application of SA, Fol, or AzA to leaf 1. Barley cultivar GP plants were infiltrated in the first true leaf with 0.025% MeOH as control, 1 mM SA, 500 μM Fol, or 1 mM AzA in 10 mM MgCl$_2$ as indicated below the panel. Five days later, the plants were infected with *Bgh* spores. Leaf discs were cut out of the first (local) (**a**) and second (systemic) (**b**) true leaf and stained with DAF-FM DA. Fluorescence was recorded using a spinning disc (confocal) microscope. Bars represent the average of 37–64 replicates from 2 (SA treatment in **b**) to 3 (all other treatments) independent experiments ± standard error; replicates were as follows: (**a**) MeOH: 57 (22 + 14 + 21), SA: 62 (22 + 16 + 24), Fol: 55 (19 + 19 + 17), AzA: 58 (19 + 18 + 21); (**b**) MeOH: 55 (20 + 22 + 13), SA: 37 (17 + 20), Fol: 64 (21 + 23 + 20), AzA: 53 (6 + 24 + 23). Asterisks above the bars indicate statistically significant differences from the mock control treatment (one-way ANOVA and poct hoc Dunnett's test, ** $p < 0.005$).

3.3. Arabidopsis Immunity-Related Compounds Do Not Alter Barley Susceptibility to P. Teres

In *Arabidopsis*, SA induces local, but not systemic susceptibility to the necrotrophic fungal pathogen *Alternaria brassicicola* [35,63]. This most likely happens due to negative crosstalk between the SA and JA pathways, with SA inhibiting JA-mediated defence against *A. brassicicola*. A similar effect was observed for Fol, as its application increased the size of lesions caused by *A. brassicicola* on the treated, but not on systemic leaves [35]. Here, we examined the effects of SA, Fol, and AzA on barley local and systemic defence responses to the necrotrophic fungus *P. teres*. To this end, leaves of 3-week-old barley plants were syringe-infiltrated with 1 mM SA, 50 or 500 μM Fol, 1 mM AzA, or 0.025% MeOH as control treatment. The same or systemic leaves were inoculated with *P. teres* 1 or 5 days later, respectively, and necrotic lesions were measured at 4 dpi using the ImageJ macro PIDIQ [69], which was modified to recognize the brown lesion caused by *P. teres*. The outcome varied strongly between different replicate experiments. Strikingly, SA application caused increases in *P. teres* lesion sizes in 4 out of 8 experiments if leaves systemic to the site of SA treatment were inoculated. However, taking all data together SA, Fol, and AzA did not significantly influence *P. teres* lesion sizes either locally (Figure 5) or systemically (Supplemental Figure S2), suggesting that these compounds do not affect the susceptibility of barley to *P. teres*.

(a)

(b)
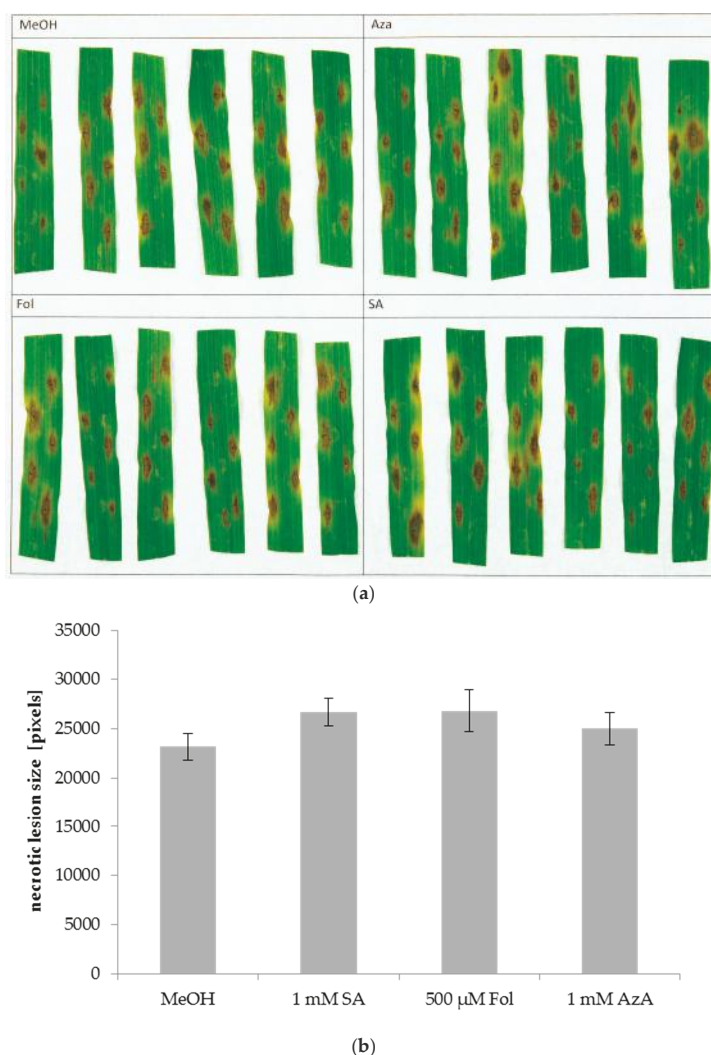
Figure 5. Lesions caused by *Pyrenophora teres* on the second leaves of barley after application of SA, Fol, or AzA on leaf 2. Barley cultivar GP plants were infiltrated in the second true leaf with 0.025% MeOH as control, 1 mM SA, 500 µM Fol, or 1 mM AzA in 10 mM MgCl$_2$ as indicated. One day later, the same leaves were inoculated with *P. teres* by pipetting droplets of a solution containing *P. teres* spores onto the leaf surface. The resulting necrotic lesions were photographed at 4 dpi (**a**) and measured using ImageJ (**b**). Bars in (**b**) represent the average of 35 replicates from 6 (Fol) or 42 replicates from 7 (all other treatments) independent experiments (each experiment with 6 replicates per treatment, except one experiment with Fol comprising 5 replicates) ± standard error.

4. Discussion

4.1. Salicylic Acid Has Differential Effects on Pathogens with Different Lifestyles

Here, we report on the role of SA, Fol, and AzA on barley defence against pathogens with different lifestyles. The three tested compounds are involved in *Arabidopsis* SAR and known for

inducing resistance to (hemi-)biotrophic pathogens in the model plant. In contrast, it has been reported that a local application of SA does not induce systemic resistance to the hemi-biotrophic bacterium *Xtc* in barley [47]. In order to find out if SA is involved in (systemic) defence responses in barley, we performed inoculations with two additional pathogens.

Although a local application of SA did not affect growth of the hemi-biotrophic bacterium *Xtc* in the systemic tissue, the same treatment reduced the disease burden of the biotrophic fungal pathogen *Bgh* in the systemic leaves by more than 50% (Figure 4). There might also be a local effect of SA on *Bgh* propagation, for which we observed a clear, but statistically insignificant trend. Thus, SA might enhance the resistance of barley to the powdery mildew pathogen *Bgh*. Similar to SA, its functional analogue BTH does not affect barley resistance to *Xtc* [47], but it induces resistance to *Bgh* [46]. Additionally, NPR1, the master regulator of SA responses in *Arabidopsis* [7,9,10], is important for barley defence responses against *Bgh*, but not against *Xtc* [47], further supporting a possible role of SA in barley defence against *Bgh* but not *Xtc*. Previous studies had reported only a minor effect on *Bgh*, if any, after SA treatment of barley plants [46,55]. The relatively robust effect of SA on systemic *Bgh* propagation in barley that we observe in this study can have one or more of three reasons. First, the apparent difference in *Bgh* burden between SA- and mock-treated plants might be exaggerated by the method used for evaluation of the *Bgh* infections. While other studies rely on pustule counts, we used the fluorescent dye DAF-FM-DA to quantify fungal material. It is known that barley produces NO as part of the plants early defence responses against *Bgh* [71]. Additionally, at certain stages of its life cycle the fungus itself produces NO [72]. However, it seems unlikely that this NO interferes with our quantification. The production of NO is very short-lived and happens mostly in early defence responses and early life stages of *Bgh*, whereas we stain with DAF-FM-DA at 6 dpi, a relatively late stage of the infection, at which time *Bgh* displays significant hyphal growth (Figure 3). Second, previous studies used soil-drench treatment for SA application [46,55] while in this study, syringe-infiltration of leaves was used. Finally, and perhaps most importantly, plant age differed between both prior and the current studies. In both of the cited publications, seedlings of 5 or 7 days of age were used while we worked with 3-week-old plants. It is known that plant age can positively affect plant resistance against *Bgh* [73]. In support of this hypothesis, we observed robust effects of SA and the other tested compounds on *Bgh* propagation and *Xtc* growth in 3-week-old plants, but did not reproducibly observe the same effects in 2-week-old plants. Comparing our data to those presented in [46,55], it is possible that SA more effectively enhances barley resistance to *Bgh* if applied directly to the leaves rather than the soil and/or if applied to 3-week-old rather than younger plants.

Although SA locally enhances the susceptibility of *Arabidopsis* plants to a necrotrophic fungal pathogen [35,63], it had no effect on *P. teres* lesion sizes either locally or systemically in barley (Figure 5 and Figure S2). Thus, the trade-off between plant defence responses to biotrophic and necrotrophic pathogens that is observed in *Arabidopsis* does not appear to influence growth of the necrotrophic fungus in SA-treated barley plants.

In summary, SA appears to induce systemic resistance against *Bgh* in barley but likely does not contribute to resistance against *Xtc* or *P. teres*.

4.2. Folic Acid Has Differential Effects against Bacteria and Fungi with Similar Lifestyles

Fol application is known to induce local and systemic resistance to hemi-biotrophic bacteria in *Arabidopsis* [35]. This effect is dependent on SA biosynthesis and signalling and on the SAR-associated compound glycerol-3-phosphate. Similar to SA, Fol application triggers local susceptibility to necrotrophic *A. brassicicola*. Here, we infiltrated or sprayed barley with Fol and monitored the effects on local and systemic propagation of bacterial and fungal barley pathogens.

In contrast to SA, Fol application enhanced barley susceptibility to *Xtc* both systemically and to a lesser extent also in the local treated tissue (Figures 1 and 2). In *Arabidopsis*, Fol enhances resistance, probably through the SA pathway [35]. Here, SA and Fol differentially affected *Xtc* growth in barley, which was not affected by SA [47] and was enhanced (rather than reduced) by Fol. It is conceivable

that *Xtc* can take up Fol, which is needed for nucleotide biosynthesis. Such supplementation could directly enhance bacterial growth, mimicking the induction of plant susceptibility. However, because the effect of Fol application on *Xtc* growth was stronger in the systemic compared to the local, treated tissue (Figures 1 and 2), the data argue in favour of a Fol-induced effect on plant immunity.

Similar to SA, the application of Fol reduced *Bgh* propagation on barley both locally and systemically but did not affect *P. teres* lesion formation (Figures 4 and 5). Again, the effects of SA and Fol on *Bgh* propagation were more pronounced in tissues that were systemic to the site of SA/Fol treatment than in the treated leaves themselves. Although we cannot exclude direct effects of the compounds on fungal growth, the data argue in favour of SA- and Fol-induced plant defence mechanisms affecting *Bgh* propagation in barley. Importantly, Fol-related compounds can promote plant yield [36], whereas SA causes cell death when applied at high concentrations. Thus, although adverse effects on barley susceptibility to hemi-biotrophic bacteria such as *Xtc* should be considered, Fol could be used as an alternative to SA or BTH to enhance the resistance of barley to the economically relevant powdery mildew pathogen *Bgh*.

4.3. Azelaic Acid Moderately Affects Barley Defence Responses

Application of AzA to *Arabidopsis* confers local and systemic resistance to hemi-biotrophic bacteria [19,30,31]. AzA primes *Arabidopsis* to accumulate higher SA levels more quickly after a subsequent infection [19]. Similarly to Fol-induced responses in *Arabidopsis* [35], AzA-mediated SAR depends on SA [19]. Here, we found that AzA influences pathogen propagation in barley very similarly to Fol. Whereas Fol locally and systemically enhanced *Xtc* growth, AzA did the same only in the local treated tissue and not systemically (Figures 1b and 2). Nevertheless, this similarity in the effects of Fol and AzA on barley susceptibility to *Xtc* argues for a possible interference of these compounds with the barley defence response to *Xtc* rather than for direct effects of either compound on bacterial growth. Furthermore, in contrast to its effect on *Xtc* growth, AzA reduced *Bgh* propagation systemically but not locally (Figure 4). In this case, the systemic response induced by AzA is similar to the responses induced by SA and Fol, which also appear to induce systemic resistance to *Bgh*. Similar to SA and Fol, AzA application did not have an influence on fungal growth of *P. teres* (Figure 5 and Figure S2), neither in local treated nor in systemic tissues.

In *Arabidopsis*, SAR appears to be regulated by two parallel signalling pathways that are inter-dependent. One of these pathways depends on SA, the other one on AzA, G3P, reactive oxygen species, and nitric oxide [26,74]. While the SA pathway seems to be effective in barley at least against *Bgh* ([46] and Figure 4), the function of the other pathway, if existent in barley, is still unclear. Since SA did not affect barley resistance to *Xtc* while both Fol and to a minor extent AzA enhanced susceptibility rather than immunity to this hemi-biotrophic pathogen, it is possible that Fol and AzA influenced a SA-independent immune pathway. Such a pathway might rely on JA and/or ABA which are positively associated with barley defence against *Xtc* [47].

5. Conclusions

In this study, we investigated the role of resistance-inducing compounds from *Arabidopsis*, salicylic acid (SA), folic acid, and azelaic acid, on barley defence against the pathogens *Xanthomonas translucens*, *Bgh* (powdery mildew), and *Pyrenophora teres*. Azelaic acid appeared to induce local susceptibility to *X. translucens* and at the same time systemic resistance to powdery mildew. Also, we observed a possible activation of local and/or systemic resistance to powdery mildew after application of SA and folic acid. Because folic acid and azelaic acid, which both enhance SA-mediated immune responses in *Arabidopsis*, both enhance barley resistance to powdery mildew similarly to SA, the associated barley immune response might be related to SA. Importantly, the data show that folic acid- and azelaic acid-induced resistance is a double-edged sword that can at the same time induce resistance and susceptibility against different pathogens (powdery mildew and *X. translucens*). Also, folic acid and azelaic acid differentially influence the responses of *Arabidopsis* and barley to host-adapted

hemi-biotrophic bacterial pathogens. Thus, investigating induced defence responses in barley and studying the signalling pathways used to achieve resistance in this cereal crop will be challenging topics for future research.

Conflicts of Interest: The authors declare no conflict of interest.

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