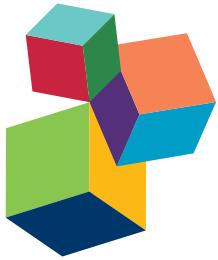


PLANT ORGAN ABSCISSION: FROM MODELS TO CROPS

EDITED BY: Timothy J. Tranbarger, Mark L. Tucker, Jeremy A. Roberts
and Shimon Meir

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PLANT ORGAN ABSCISSION: FROM MODELS TO CROPS

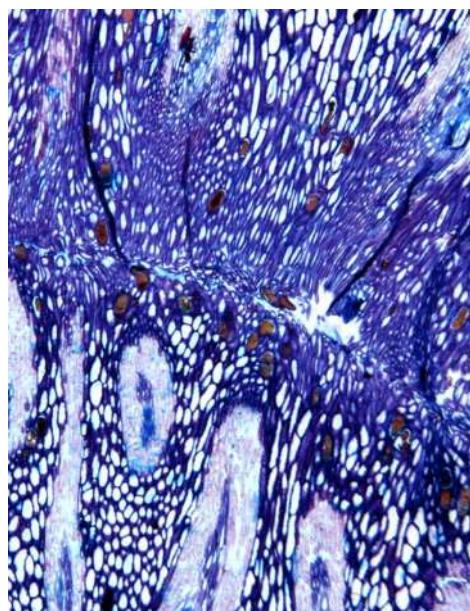
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Longitudinal section of the base of the oil palm fruit stained with Alcian Blue and periodic acid-Schiff showing cell separation that occurs in the abscission zone between the mesocarp (above separation) and the pedicel (below separation) during oil palm fruit abscission.

Image by Peerapat Roongsattham.

Plant organ abscission is a developmental process regulated by the environment, stress, pathogens and the physiological status of the plant. In particular, seed and fruit abscission play an important role in seed dispersion and plant reproductive success and are common domestication traits with important agronomic consequences for many crop species. Indeed, in natural populations, shedding of the seed or fruit at the correct time is essential for reproductive success, while for crop species the premature or lack of abscission may be either beneficial or detrimental to crop

productivity. The use of model plants, in particular *Arabidopsis* and tomato, have led to major advances in our understanding of the molecular and cellular mechanisms underlying organ abscission, and now many workers pursue the translation of these advances to crop species. Organ abscission involves specialized cell layers called the abscission zone (AZ), where abscission signals are perceived and cell separation takes place for the organ to be shed. A general model for plant organ abscission includes (1) the differentiation of the AZ, (2) the acquisition of AZ cells to become competent to respond to various abscission signals, (3) response to signals and the activation of the molecular and cellular processes that lead to cell separation in the AZ and (4) the post-abscission events related to protection of exposed cells after the organ has been shed. While this simple four-phase framework is helpful to describe the abscission process, the exact mechanisms of each stage, the differences between organ types and amongst diverse species, and in response to different abscission inducing signals are far from elucidated. For an organ to be shed, AZ cells must transduce a multitude of both endogenous and exogenous signals that lead to transcriptional and cellular and ultimately cell wall modifications necessary for adjacent cells to separate. How these key processes have been adapted during evolution to allow for organ abscission to take place in different locations and under different conditions is unknown. The aim of the current collection of articles is to present and be able to compare recent results on our understanding of organ abscission from model and crop species, and to provide a basis to understand both the evolution of abscission in plants and the translation of advances with model plants for applications in crop species.

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Table of Contents

Editorial

07 Editorial: Plant Organ Abscission: From Models to Crops

Timothy J. Tranbarger, Mark L. Tucker, Jeremy A. Roberts and Shimon Meir

Reviews, Perspectives and Hypothesis and Theory

11 Seed shattering: from models to crops

Yang Dong and Yin-Zheng Wang

24 Development and regulation of pedicel abscission in tomato

Yasuhiro Ito and Toshitsugu Nakano

30 The IDA Peptide Controls Abscission in Arabidopsis and Citrus

Leandro H. Estornell, Mari Wildhagen, Miguel A. Pérez-Amador, Manuel Talón, Francisco R. Tadeo and Melinka A. Butenko

37 Understanding the Physiology of Postharvest Needle Abscission in Balsam Fir

Rajasekaran R. Lada and Mason T. MacDonald

48 Are We on the Right Track: Can Our Understanding of Abscission in Model Systems Promote or Derail Making Improvements in Less Studied Crops?

Sara E. Patterson, Jenny L. Bolivar-Medina, Tanya G. Falbel, Janet L. Hedtcke, Danielle Nevarez-McBride, Andrew F. Maule and Juan E. Zalapa

Original Research

Arabidopsis Organ Abscission and Comparisons to Selected Crop Species

56 Allele-Specific Interactions between CAST AWAY and NEVERSHED Control Abscission in Arabidopsis Flowers

William D. Groner, Megan E. Christy, Catherine M. Kreiner and Sarah J. Liljegren

63 Examination of the Abscission-Associated Transcriptomes for Soybean, Tomato, and Arabidopsis Highlights the Conserved Biosynthesis of an Extensible Extracellular Matrix and Boundary Layer

Joonyup Kim, Srivignesh Sundaresan, Sonia Philosoph-Hadas, Ronghui Yang, Shimon Meir and Mark L. Tucker

78 Conservation of the abscission signaling peptide IDA during Angiosperm evolution: withstanding genome duplications and gain and loss of the receptors HAE/HSL2

Ida M. Stø, Russell J. S. Orr, Kim Fooyontphanich, Xu Jin, Jonfinn M. B. Knutsen, Urs Fischer, Timothy J. Tranbarger, Inger Nordal and Reidunn B. Aalen

Fruit Abscission

Tomato

- 90 De novo Transcriptome Sequencing and Development of Abscission Zone-Specific Microarray as a New Molecular Tool for Analysis of Tomato Organ Abscission**
Srivignesh Sundaresan, Sonia Philosoph-Hadas, Joseph Riov, Raja Mugasimangalam, Nagesh A. Kuravadi, Bettina Kochanek, Shoshana Salim, Mark L. Tucker and Shimon Meir
- 119 Distribution of XTH, expansin, and secondary-wall-related CesA in floral and fruit abscission zones during fruit development in tomato (*Solanum lycopersicum*)**
Mutsumi Tsuchiya, Shinobu Satoh and Hiroaki Iwai

Subtropical and Tropical Fruits

- 128 Ethephon induced abscission in mango: physiological fruitlet responses**
Michael H. Hagemann, Patrick Winterhagen, Martin Hegele and Jens N. Wünsche
- 139 Genome-wide digital transcript analysis of putative fruitlet abscission related genes regulated by ethephon in litchi**
Caiqin Li, Yan Wang, Peiyuan Ying, Wuqiang Ma and Jianguo Li
- 155 Cellular and Pectin Dynamics during Abscission Zone Development and Ripe Fruit Abscission of the Monocot Oil Palm**
Peerapat Roongsatham, Fabienne Morcillo, Kim Fooyontphanich, Chatchawan Jantasuriyarat, Somvong Tragoonrung, Philippe Amblard, Myriam Collin, Gregory Mouille, Jean-Luc Verdeil and Timothy J. Tranbarger
- 170 Two abscission zones proximal to *Lansium domesticum* fruit: one more sensitive to exogenous ethylene than the other**
Prapinporn Taesakul, Jingtair Siriphanich and Wouter G. van Doorn
- 180 Burst of reactive oxygen species in pedicel-mediated fruit abscission after carbohydrate supply was cut off in longan (*Dimocarpus longan*)**
Ziqin Yang, Xiumei Zhong, Yan Fan, Huicong Wang, Jianguo Li and Xuming Huang

Flower, Leaf and Root Abscission

- 190 Transcriptome Profiling of Petal Abscission Zone and Functional Analysis of an Aux/IAA Family Gene RhIAA16 Involved in Petal Shedding in Rose**
Yuerong Gao, Chun Liu, Xiaodong Li, Haiqian Xu, Yue Liang, Nan Ma, Zhangjun Fei, Junping Gao, Cai-Zhong Jiang and Chao Ma
- 203 Transcriptome Analysis of Soybean Leaf Abscission Identifies Transcriptional Regulators of Organ Polarity and Cell Fate**
Joonyup Kim, Jinyoung Yang, Ronghui Yang, Richard C. Sicher, Caren Chang and Mark L. Tucker
- 219 Flower abscission in *Vitis vinifera* L. triggered by gibberellic acid and shade discloses differences in the underlying metabolic pathways**
Sara Domingos, Pietro Scafidi, Vania Cardoso, Antonio E. Leitao, Rosario Di Lorenzo, Cristina M. Oliveira and Luis F. Goulao
- 237 Bimodal effect of hydrogen peroxide and oxidative events in nitrite-induced rapid root abscission by the water fern *Azolla pinnata***
Michael F. Cohen, Sushma Gurung, Giovanni Birarda, Hoi-Ying N. Holman and Hideo Yamasaki

245 Primary and Secondary Abscission in *Pisum sativum* and *Euphorbia pulcherrima*—How Do They Compare and How Do They Differ?

Anne K. Hvoslef-Eide, Cristel M. Munster, Cecilie A. Mathiesen, Kwadwo O. Ayeh, Tone I. Melby, Paoly Rasolomanana and YeonKyeong Lee

262 Auxin is a long-range signal that acts independently of ethylene signaling on leaf abscission in *Populus*

Xu Jin, Jorma Zimmermann, Andrea Polle and Urs Fischer



Editorial: Plant Organ Abscission: From Models to Crops

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Editorial on the Research Topic

Plant Organ Abscission: From Models to Crops

The shedding of plant organs is a highly coordinated process essential for both vegetative and reproductive development (Addicott, 1982; Sexton and Roberts, 1982; Roberts et al., 2002; Leslie et al., 2007; Roberts and Gonzalez-Carranza, 2007; Estornell et al., 2013). Research with model plants, namely floral organ abscission in *Arabidopsis* and leaf, flower and fruit abscission of tomato, and seed shattering in rice has provided new insights into the molecular mechanisms underlying abscission. However, little is known about how these mechanisms that take place in the abscission zone (AZ) have diversified during plant evolution and differs between species and plant families, and within diverse tissues and developmental contexts. A major aim of this topic was to examine diverse organ abscission examples from a wide range of species in order to provide a format for comparisons between model and important crop species.

To set the stage, the Topic was spearheaded by a timely mini review on seed shattering (Dong and Wang) and a review on tomato pedicel abscission (Ito and Nakano). Dong and Wang cover recent knowledge about dry fruit dehiscence in *Arabidopsis*, a model dicot, and some legumes compared to seed shattering in the model monocot rice and other cereal crops and also to pedicel abscission in tomato. The review provides an overview of the importance of AZ development as a common denominator for all organ abscission to occur, pointing out commonalities and gene neo-functionalization of MADS-box genes in particular that give rise to these different dispersal systems.

Tomato flower and leaf abscission are excellent model systems to study abscission in horticultural crops: (1) tomato abscission is well characterized at the anatomical, physiological and molecular levels, especially tomato flower abscission that has a distinct flower AZ (FAZ) at the midpoint of the pedicel; (2) tomato has a high-quality, well-annotated genome available; (3) tomato plants offer opportunities for functional analyses using Virus Induced Gene Silencing (VIGS), stable transformation and recently developed gene editing methodologies; and (4) ethylene and auxin interactions in tomato abscission are well documented, which makes tomato an excellent model system for comparison to abscission in other crops discussed in this topic series (Jiang et al., 2008; Meir et al., 2010; Ma et al., 2015). Ito and Nakano provide a mini-review that describes in detail the recent advances in research on the mechanisms regulating the formation of the tomato pedicel AZ, focusing on the role of the MADS-box family transcription factors that regulate pedicel-AZ development. The review also covers recent results from transcriptome analyses that identified pedicel AZ specific transcription factors (TFs) that connect abscission-inducing signals with abscission processes.

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In the article by Sundaresan et al., a *de novo* AZ transcriptome assembly provides the functional annotation of transcripts expressed in the tomato FAZ and leaf AZ (LAZ) during abscission. The results from this study revealed distinct patterns of transcriptional regulation that occur in the FAZ and LAZ, and the identification of common and distinct TFs not previously related to abscission. The AZ-specific transcript information was utilized to design a robust customized AZ microarray chip with a greater number of transcript probes than any commercial chip, including probes designed in both sense and antisense direction that can be used to explore the expression of antisense transcripts (NATs). Kim et al. used this chip to identify prominent changes in gene expression in the tomato FAZ and LAZ, in addition to parallel analysis of the soybean leaf and *Arabidopsis* floral AZs, linked to cell wall disassembly and synthesis, and deposition of a waxy-like boundary layer that defines the fracture plane for organ separation. They proposed that abscission is an adaptation of a more primal organogenesis process occurring in the meristem. In addition, they propose that IDA (Inflorescence Deficient in Abscission) gene expression, which appears to be a common occurrence in abscission processes, may play a role in the synthesis of the boundary layer. Tsuchiya et al. used tomato to compare the pedicel AZ cell walls during flower and fruit abscission. They provide evidence that expansin and xyloglucan endotransglucosylase/hydrolase (XTH) play important, but different roles in the FAZ during the flower abscission process, while no AZ-specific expansin and XTH expression was observed during fruit abscission, and that lignification occurred in the AZ of over-ripe tomato fruit pedicels.

The *Arabidopsis* floral organ abscission model was used to further explore the signaling mechanisms that control cell separation processes, with Groner et al. using suppression analysis to examine the allelic dependent restoration of organ abscission in the *nev* (NEVERSCHED) mutant by specific alleles of CAST AWAY (CST). This work provides insight into allelic-specific interactions of key regulators of abscission, and provides a basis for nuanced applications to control abscission in crop plants. In addition, two articles focused on the *Arabidopsis* INFLORESCENCE DEFICIENT IN ABSCISSION (AtIDA) peptide ligand, and the leucine-rich repeat (LRR) receptor-like kinases (RLKs) HESA (HAE) and HESA-LIKE 2 abscission-signaling pathway (HSL2) (Jinn et al., 2000; Cho et al., 2008; Stenvik et al., 2008). In a Perspective article Estornell et al. investigated the functional conservation of the citrus IDA ortholog *CitIDA3* by over expression in *Arabidopsis* wild-type and *ida* mutant plants. Their results demonstrated that overexpression of the *CitIDA3* in wild-type *Arabidopsis* produced similar phenotypes as overexpression of *AtIDA* in wild-type *Arabidopsis*, which included shorter plants and siliques and early floral abscission; moreover, *CitIDA3* rescued the abscission phenotype in the *ida-2* mutant background, which provides evidence that the IDA-HAE/HSL2 abscission-signaling pathway is conserved in citrus. Furthermore, Stø et al. examined the IDA-HAE/HSL2 abscission-signaling pathway in *Populus*, a dicot tree, and palm (*Elaeis guineensis*), a monocot tree, and provide further evidence for conservation of function for this pathway, and that

whole genome duplications gave rise to functionally divergent IDA-like (IDL) peptides during higher plant evolution. The results of these papers provide pertinent evidence that the IDA-HAE/HSL2 abscission-signaling pathway functions in higher plants in diverse phylogenetic groups. However, to what extent the IDA pathway regulates organ abscission in combination with other pathways in these species is still not clear.

Auxin and ethylene are well known to play inhibitory and positive roles, respectively, during organ abscission, and several articles further established their role during organ abscission of diverse species and organ types. Jin et al. examined the role of auxin transport during *Populus* leaf abscission and provided evidence that auxin transport is required to delay abscission and because the transport mechanism functions in the absence of ethylene signaling, auxin appears to act independent of ethylene on cell separation. Gao et al. performed a large-scale transcript profiling analysis during rose petal abscission, and identified the transcriptional control of important pathways in addition to 150 TFs that are differentially expressed during petal abscission in rose. In particular, down-regulation of the Aux/IAA gene *RhIAA16* in rose promoted petal abscission, suggesting that *RhIAA16* plays a negative regulatory role in rose petal abscission.

The role of ethylene was examined in four manuscripts, while one manuscript examined the involvement of reactive oxygen species (ROS) during the process of fruit abscission in tropical crop species. Taesakul et al. observed different ethylene sensitivities in two distinct AZs of the tropical Longkong (*Lansium domesticum*) fruit. One AZ is more sensitive to ethylene than another adjacent AZ, the latter of which is proposed to allow animals to remove ripe fruit from the tree with minimal force without a requirement for ethylene. Hagemann et al. examined the role of ethylene signaling during ethephon (an ethylene-releasing compound) induced mango fruitlet abscission. Their findings suggest a role for novel versions of the Mango ethylene receptor *MiERS1* expressed in the pedicel during premature fruit abscission. Ethylene perception in the fruitlet AZ is proposed to first reduce polar auxin transport capacity in the pedicel, followed by an up-regulation of ethylene receptors and finally a decrease in fruitlet sucrose content. Fruitlet abscission was also examined in litchi by Li et al., who performed a genome-wide digital transcript abundance analysis to identify genes regulated during ethephon-induced premature fruit abscission. The study gives insight into the major cellular processes transcriptionally regulated during ethephon-induced fruitlet abscission providing a host of candidate genes to target for control of abscission including those involved in ethylene biosynthesis and signaling, auxin transport and signaling, transcriptional regulation, protein ubiquitination, ROS response, calcium signal transduction, and cell wall modification. Yang et al. examined the involvement of ROS during the induction of longan (*Dimocarpus longan* Lour.) fruit abscission by carbon restriction caused through girdling, defoliation or detached fruit clusters. Their data suggest a role for H_2O_2 in the up-regulation of cellulase activity during fruit abscission, and that fruit abscission induced by carbohydrate stress is mediated by ROS. Cohen et al. examined a similar role for H_2O_2 in the rapid abscission of *Azolla pinnata* roots, and propose that metabolic products of nitrite

and NO react with H₂O₂ in the apoplast that leads to free-radical-mediated cleavage of structural polysaccharides and the consequent rapid root abscission. Roongsattham et al. examined and compared the cellular processes that occur in the multi-cell layered boundary region AZ between the pedicel and mesocarp during ethylene induced ripe fruit abscission of the oil palm (*Elaeis guineensis* Jacq.). Their results highlight the dynamic developmental processes that occur in the AZ during oil palm fruit abscission, in particular changes to the pectin portion of the cell wall including a decrease in methylesterified homogalacturonan.

Flower abscission in grapevine (*Vitis vinifera* L.) induced independently by two contrasting abscission-inducing treatments: shade and gibberellic acid (GAc) was monitored (Domingos et al.). The results highlight specificities and common links in the metabolic pathways that control abscission by both inducers. While oxidative stress remediation mechanisms and a change in indolacetic acid (IAA) concentration across the AZ are common to the two abscission stimuli, GAc application and C-starvation induced abscission do not occur through the same metabolic pathways.

In the second Kim et al., a genome analysis identified 1,088 differentially regulated TFs in the soybean leaf AZ during abscission. Network analysis found that most TFs expressed early in abscission were linked to key determinants involved in the maintenance of organ polarity, lateral growth and cell fate, similar to what occurs during organogenesis in the meristem.

Hvoslef-Eide et al. compares the primary AZ in the seed funiculus of pea with the inducible secondary AZ in the poinsettia (*Euphorbia pulcherrima*) flower pedicel. The comparative analysis yielded the identification of abscission specific genes expressed in both poinsettia flower pedicel and pea seed AZs, suggesting widespread conservation of function for these gene products during organ abscission.

Lada and MacDonald provide a Hypothesis and Theory article on postharvest needle abscission in the conifer Balsam Fir. They concluded that postharvest needle abscission cannot be prevented entirely; however, abscission related processes

including ethylene and other pathways identified to be involved in abscission represent targets that may delay abscission.

Finally, Patterson et al. provided a Perspective article that explores the question whether the use of model organ abscission systems such as tomato and Arabidopsis will necessarily provide applications to alter abscission in non-model understudied crop species. Their opinion highlights the challenges to translate model derived knowledge to crop species, and that it is essential for breeders and molecular biologists to work together to provide an understanding of the unique development of each species as well as the targeted genes or pathways of interest.

Overall, the current topic series of articles covered abscission of different organs in a variety of plant species including both model plants and crops. In particular, one emerging example of a common mechanism is the IDA-HAE/HSL2 signaling pathway, which is present in the AZ of a variety of species and may interact with ethylene and auxin to regulate organ abscission in these species. Articles in this series provide further evidence that this pathway functions in plants from divergent evolutionary histories, including both monocot and dicot species. Importantly, a recent article corroborates further the conservation of this pathway in the tropical fruit species litchi (Ying et al., 2016). If indeed this pathway is universally plant kingdom wide, inhibition at critical points of the pathway may allow either elimination or at least delayed organ abscission in divergent crop species. One significant development for agriculturally important but less studied crop species that is observed in this series is the next generation sequencing (NGS) based transcriptome analysis that allows rapid validation of model derived gene candidates in addition to new resources to identify targets to manage abscission in particular species and insight into the regulatory processes that may be unique to divergent species.

AUTHOR CONTRIBUTIONS

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Seed shattering: from models to crops

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Seed shattering (or pod dehiscence, or fruit shedding) is essential for the propagation of their offspring in wild plants but is a major cause of yield loss in crops. In the dicot model species, *Arabidopsis thaliana*, pod dehiscence necessitates a development of the abscission zones along the pod valve margins. In monocots, such as cereals, an abscission layer in the pedicel is required for the seed shattering process. In the past decade, great advances have been made in characterizing the genetic contributors that are involved in the complex regulatory network in the establishment of abscission cell identity. We summarize the recent burgeoning progress in the field of genetic regulation of pod dehiscence and fruit shedding, focusing mainly on the model species *A. thaliana* with its close relatives and the fleshy fruit species tomato, as well as the genetic basis responsible for the parallel loss of seed shattering in domesticated crops. This review shows how these individual genes are co-opted in the developmental process of the tissues that guarantee seed shattering. Research into the genetic mechanism underlying seed shattering provides a premier prerequisite for the future breeding program for harvest in crops.

Keywords: seed shattering, fruit shedding, pod dehiscence, domestication, domestication syndrome, indehiscent fruit, genetic regulation

Introduction

The emergence of fruit represents a major evolutionary innovation in angiosperms, and the evolutionary success of wild plant species depends essentially on their capacity to scatter their offspring (Nathan and Muller-Landau, 2000). The seed shattering or fruit shedding is usually used to describe the detachment of the fruit from the pedicel in cereals and fleshy fruit species, respectively. While in dry dehiscent fruit taxa, such as Legumes and crucifers, pod dehiscence refers to the shattering of the pod shell, which enable the successful shattering of seeds. Although these processes happen in non-homologous tissues, the abscission layer is an essential tissue both for the shattering or shedding process (Estornell et al., 2013).

The fruit morphology and associated dispersal strategies are of significant adaptive importance, which are under strong selective pressures. While in seed crops, premature seed shattering is an undesired character and has been selected against during the domestication process of distinct crops. Most of our knowledge on the genetic regulation of pod dehiscence has been obtained in the model organism *Arabidopsis thaliana*, a Brassicaceae species with a characteristic dry dehiscent fruit that shatters the seeds through the dehiscence zones (DZs) along the siliques after maturity (Ferrández et al., 1999). The differentiation of the DZ is under the control of intricate regulatory networks involving multiple transcription factors. Recent investigations in pod dehiscence regulation have uncovered another layer of the regulatory network that include phytohormones in specifying the DZs (Sorefan et al., 2009; Arnaud et al., 2010; Marsch-Martínez et al., 2012).

Evidence from comparative studies in the taxa related to *Arabidopsis* suggests modest genetic changes in the key regulatory component could be responsible for the phenotypic changes that are associated with fruit function and novel dispersal strategies (Avino et al., 2012; Fourquin et al., 2013; Mühlhausen et al., 2013). Studies on the fruit shedding process in tomato, a model for fleshy fruits, have provided new insights into the regulatory networks responsible for the control of cell separation (Mao et al., 2000; Nakano et al., 2012; Liu et al., 2014). These findings reveal that there are strong similarities between dry and fleshy fruits in the molecular networks governing fruit dehiscence and maturation. Meanwhile, our understanding about the genes involved in the loss of seed shattering in crops has increased dramatically, offering us a great opportunity to examine the details regarding the molecular basis of such convergent morphological adaptation in the face of artificial selection in a wide array of species.

In this review, we try to incorporate the recent insights into the molecular and hormonal regulation of tissues that are necessary for seed shattering and fruit shedding in model species and discuss how the genetic modification of the regulatory genes is co-opted in the evolutionary process to generate altered fruit morphologies with novel dispersal strategies. We also review the recent findings in the genetic control of non-shattering (indehiscent) fruit in crop species and highlight the prevalence of parallel molecular evolution in plant domestication. A comprehensive understanding of the factors influencing the seed shattering process is particularly important, as it might have great potential in the facilitation of future crop domestication and breeding procedures to prevent unwanted seed loss.

Genetics of Pod Dehiscence in *Arabidopsis thaliana* and its Relatives

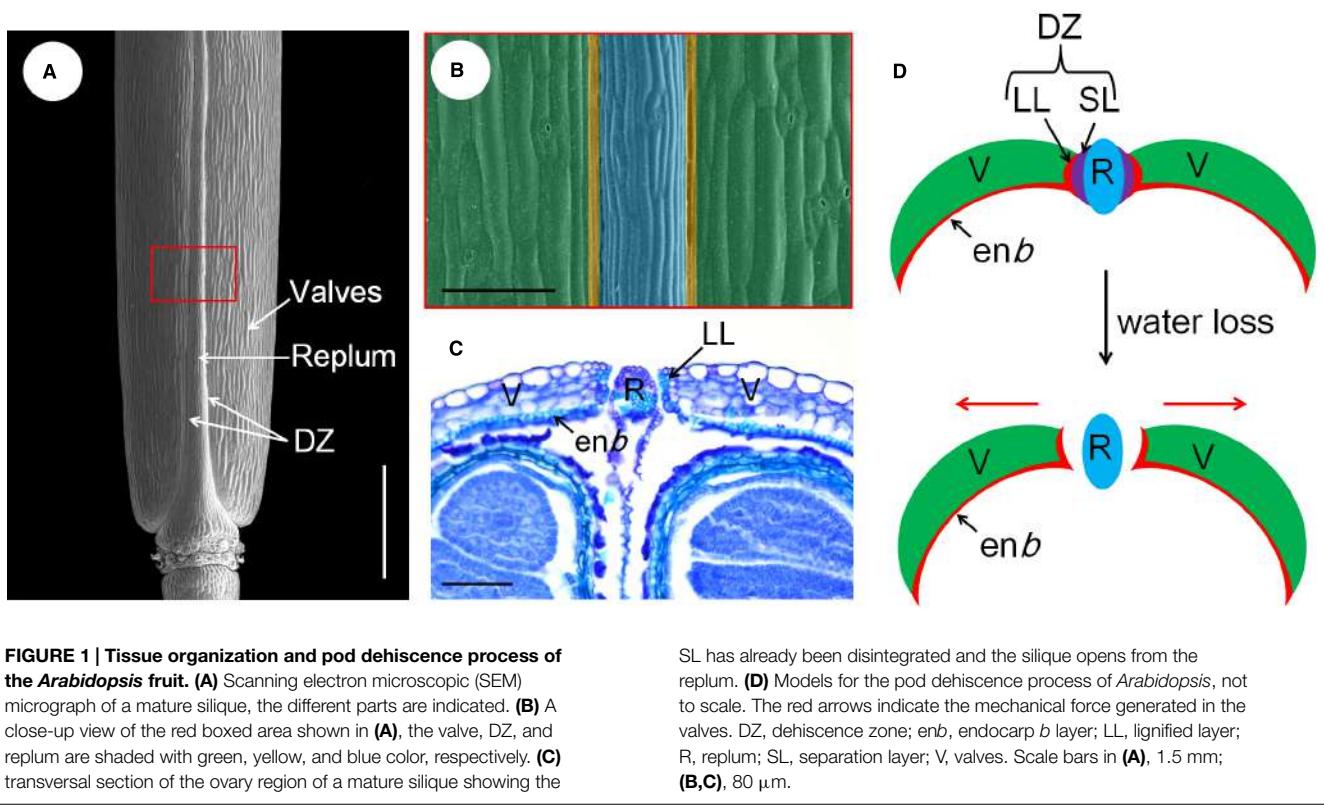
The model species *A. thaliana* belongs to the Brassicaceae family, which develops a typical dry dehiscent fruit called the siliques. Essentially, the siliques develop from the gynoecium composed of two congenitally fused carpels (Ferrández et al., 1999). The developmental program of the fruit initiates from fertilization of the ovules. In the transverse view of the mature fruit, the outer layer consists of three principal tissues, the valves, the replum, and the valve margins. The valve margins are sandwiched between the valve and replum and are further differentiated into lignified layer (LL) and separation layer (SL), which together form the DZ along the siliques (**Figures 1A–C**; Ferrández et al., 1999). The LL cells are connected with the endocarp *b* (enb) layer of the valves, which is also rigidly lignified. The SL is composed of several isodiametric cells, and will be degraded autonomously before pod dehiscence (Seymour et al., 2013). When the siliques become dry with loss of water, these highly organized structures produce a spring-like tension within the pod valves that force the siliques to shatter from the weakest position, the SL (**Figures 1C,D**). Therefore, the siliques dehiscence is a dynamic process that depends on the proper positioning and formation of the DZs along the siliques (Ferrández, 2002).

The Genetics of DZ Development and Pod Dehiscence in *Arabidopsis*

The spatial specification of DZ, valve cells and replum is under the control of a complex genetic regulatory network and dynamic hormonal interactions with several transcription factors involved (**Figure 2**; Lewis et al., 2006; Østergaard, 2009; Ferrández and Fourquin, 2014). This regulatory network has recently been extended to include genes that are involved in the leaf development and the establishment of dorsoventral axes of the lateral organs (e.g., *FILAMENTOUS FLOWER*, *YABBY3*, *ASYMMETRIC LEAVES1/2*) as well as the meristematic potential maintenance (*BREVIPEDICELLUS*) (Hay et al., 2006; Alonso-Cantabrina et al., 2007). This review mainly focuses on the core regulatory genes specific to siliques dehiscence, thus those remotely related genes are not included in this article. A thorough description of all these interactions can be found elsewhere in the literatures (Dinneny et al., 2005; Lewis et al., 2006; Østergaard, 2009).

Two MADS-box transcription factor encoding genes *SHATTERPROOF1* (*SHP1*) and *SHP2* act redundantly to control the pod dehiscence as neither single mutant displays a detectable phenotype from wild type (Liljegren et al., 2000). The *shp1/2* double mutant produces indehiscent fruit devoid of cell differentiation in the DZ (Liljegren et al., 2000). Expressions of *SHP1/2* are specifically localized in the DZs and developing seeds during late fruit development (Liljegren et al., 2000; Colombo et al., 2009). Further genetic analysis shows that *SHP1/2* act at the top of the genetic cascade that direct the development of DZ for pod dehiscence (**Figure 2**; see below; Ferrández, 2002; Lewis et al., 2006).

Acting down-stream of and in parallel with *SHP1/2* are two b-HLH transcription factors, *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*; **Figure 2**). *IND* directs the differentiation of DZ into LLs and SLs. Similar to *shp1/2* double mutant, *ind* mutation fully abolishes the specification of DZs and results in indehiscent fruits (Liljegren et al., 2000, 2004). By contrast, *ALC* specifically establishes the cell identity in the separation layer and mutation in *ALC* leads to partially indehiscent fruits (Rajani and Sundaresan, 2001). Both *IND* and *ALC* are specifically expressed in the DZ during late fruit development. Evidence indicates that *IND* acts downstream of *SHP1/2* to control pod dehiscence, as illustrated by the observation that *IND* expression is completely lost in the *shp1/2* mutant (Liljegren et al., 2000, 2004; Rajani and Sundaresan, 2001). The valve identity is regulated by the activity of the *FRUITFULL* (*FUL*) MADS-box gene (Gu et al., 1998; Ferrández et al., 2000). Expression of *FUL* initiates in the carpel primordia very early in flower development, and soon after becomes restricted in the gynoecium and further in the carpel valves (Gu et al., 1998). In the *ful* mutant, the valves fail to elongate and are cracked by the inner developing seeds (Gu et al., 1998). *FUL* negatively regulates *SHP1/2* expression thus delimitates the boundary of *SHP1/2* expression in the valves (Ferrández et al., 2000). When *FUL* is mutated, *SHP1/2* and *IND* are ectopically expressed in the valves promoting the mesocarp cells to adopt lignified valve margin cell identity instead of normal parenchymatous cell identity (Gu et al., 1998; Ferrández et al., 2000). The *ful* mutant phenotype can be partially rescued by combining the *ful* mutant with mutations in the *SHP1/2* genes,

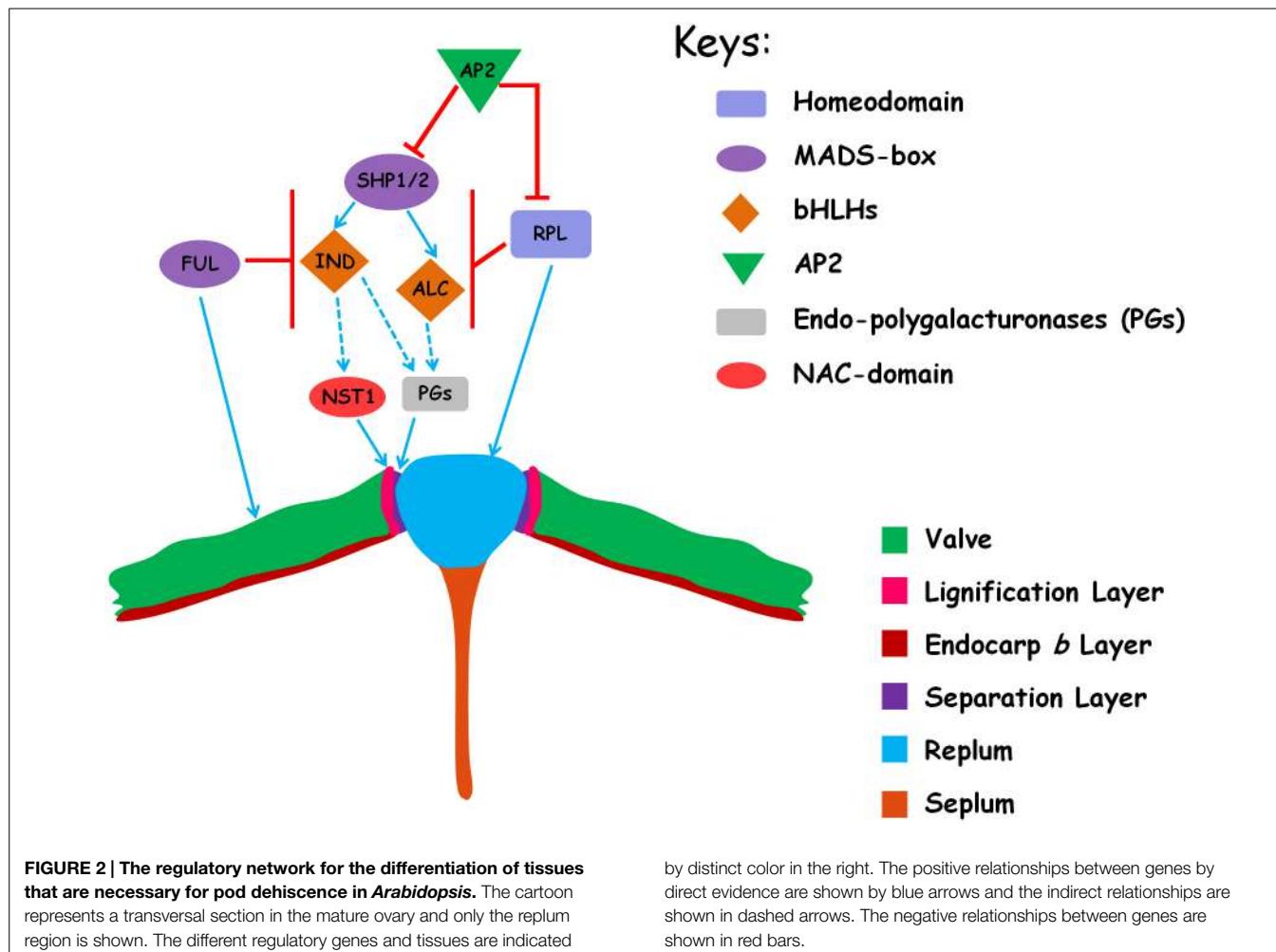


and largely rescued in the *ind* mutant background, suggesting that *IND* has a more specialized role in DZ cell specification than *SHP1/2*. On the other hand, fruits of 35S::*FUL* transgenic lines are indehiscent as the result of complete conversion of DZ cells into valve cells (Ferrández et al., 2000). Interestingly, the activity of *SHP1/2*, *IND*, *ALC*, and *FUL* is all necessary for the lignification of cells in the *enb* layer (Ferrández et al., 2000; Liljegren et al., 2004).

In addition to *FUL*, the DZ-specific expression of *SHP1/2* and *IND* is also restricted by the *REPLUMLESS* (*RPL*), which encodes a homeodomain transcription factor and contributes to the specification of replum identity (Roeder et al., 2003). Expression of *SHP1/2* and *IND* is expanded into the replums in the *rpl* mutant genetic background (Figure 2; Roeder et al., 2003). Fruits from the *rpl* mutant are partially indehiscent due to loss of replum identity with ectopic cell lignification, in which the replum-lignified cells are coalesced into a single stripe that is connected with the lignified valve margin cells (Roeder et al., 2003). The loss of replum identity in *rpl* mutant can be largely rescued by further removal of *SHP1/2* activity, suggesting that the ectopic expression of *SHP1/2* is responsible for the *rpl* mutant phenotype (Roeder et al., 2003). Thus, both *RPL* and *FUL* are necessary for the proper development of a functional DZ by restricting the expression of *SHP1/2* in the valve margins. Recently, it was demonstrated that the *rpl* mutant phenotype can be rescued largely by *ap2* mutation (Ripoll et al., 2011). *AP2*, well known for its role in floral organ identity determination, encodes a transcription factor belonging to AP2/ERF family. *AP2* acts to prevent replum and valve margin overgrowth by negatively regulating replum and valve margin identity gene expression, respectively (Ripoll et al., 2011).

After the differentiation instruction of specific cell identity is established, the next step should be the final differentiation of distinct cell types. *NAC SECONDARY WALL THICKENING PROMOTING FACTOR1* (*NST1*) and *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN1* (*SDN1*, also called *NST3*) are the master transcriptional switches controlling secondary cell wall thickening (Zhong et al., 2010). In the fruits, *NST1* and *SDN1* are expressed in the valve *enb* layer, while only *NST1* is specifically expressed in the developing LL cells of DZs (Mitsuda and Ohme-Takagi, 2008). In the *nst1* null mutant, the fruits are indehiscent due to the loss of lignification of valve margin cells, and all the lignified cells except the vessel cells in the replums are lost in the *nst1* *sdn1* double mutant (Mitsuda and Ohme-Takagi, 2008). Expression of *SHP1/2* and *IND* appears to be normal in the *nst1* *sdn1* double mutant, suggesting that *NST1* and *SDN1* act downstream of these transcription factors. Mitsuda and Ohme-Takagi (2008) further show that ectopic cell wall thickening in the valve cells in the *ful* mutant can be eliminated by mutation of *NST1*. Taken together, these data suggest *SHP1/2* regulate the lignification of valve margin cells by the path of *NST1* (Figure 2).

Intriguingly, *NST1* and *SDN1* are predominantly expressed in the interfascicular fibers and xylems in the stems where *SHP1/2* are not expressed and are responsible for the secondary cell wall thickening in these cells (Zhong et al., 2006; Mitsuda et al., 2007). Furthermore, *NST1* and *SDN1* are also identified as master regulators for xylem fiber differentiation (Zhong et al., 2006; Mitsuda et al., 2007; Oda and Fukuda, 2012). It is apparent that the developmental program of the stem interfascicular



fibers and lignified valve margin cells are distinct. It seems that the valve margin specific expression of *NST1* represents an evolutionary innovation in the *cis*-regulatory elements that correlate with the establishment of lignified valve margin cells. How the *NST1* gene is co-opted in the *SHP1/2*-regulated network that direct the lignified valve margin cell development is an interesting question and worthy to be clarified in the future.

Prior to pod dehiscence, the cells in the separation layer secret enzymes to degrade the cell wall matrix, which bring about a reduction in cell-to-cell adhesion, thus facilitate the fruit to commit to dehiscence (Roberts et al., 2002). *ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1* (*ADPG1*) and *ADPG2* encode plant specific endo-polygalacturonases (PGs) and are expressed in the separation layer of flower organs and fruit DZs (Ogawa et al., 2009). *ADPG1* and *ADPG2* are essential for enzymatic breakdown of cell middle lamella and are necessary for siliques dehiscence, as genetic lesion in either genes leads to indehiscent fruits (Ogawa et al., 2009). *IND* is required for normal expression of *ADPG1* in the siliques DZs (Ogawa et al., 2009). Thus, it seems that ADPGs are the final regulators of pod dehiscence in the separation layers (Figure 2).

Hormonal Regulation of DZ Specification

Hormonal homeostasis and interactions are recently found as immediate downstream outputs from the core genetic network. Expression of *IND* is responsible for the formation of local auxin minimum in the valve margin by coordinating auxin efflux in the separation layer cells (Sorefan et al., 2009). Further analysis shows that another b-HLH transcription factor *SPATULA* (*SPT*), which is required for the carpel fusion early in female reproductive organ development, can interact with *IND* physically (Girin et al., 2011). The interaction of *IND* and *SPT* promotes the localization of PIN3 in the plasma membrane of valve margin cells to create the auxin depletion in the valve margin thus offering a proper hormonal environment for specific cell differentiation (Sorefan et al., 2009; Girin et al., 2011). Auxins and cytokinins often play an antagonistic role in plant development (Bishopp et al., 2011). Consistent with this scenario, the cytokinin signalling pathway is recently found to be active in the valve margin, and such a signaling pathway is disrupted in *shp1/2* and *ind* mutant. However, local application of cytokinin in developing fruits can restore valve margin formation and further increases dehiscence in *shp1/2* and *ind* mutants, suggesting that cytokinins play a crucial role in valve margin differentiation (Marsch-Martinez et al., 2012). In

addition to auxins and cytokinins, gibberellins (GAs) have also recently been implicated as having roles in the establishment of separation layer cell identity (Arnaud et al., 2010). According to the “relief of restraint” model, GA-mediated degradation of DELLA protein is central to GA signaling and also required to activate downstream genes (Harberd, 2003; Sun and Gubler, 2004). GA3ox1, which catalyzes the final step in the synthesis of bioactive GAs, is demonstrated as the direct target of *IND*. *ALC* physically interacts with DELLA repressors, and the local production of GAs destabilize the DELLA protein and relieve the *ALC* to exert its function in SL cell specification (Arnaud et al., 2010). Taken together, these findings indicate involvement of several phytohormones in the specification of DZs and suggest that a precise balance between their biosynthesis and response is of fundamental importance. Notwithstanding with the investigations where the role of hormones in DZ development has been extensively explored, very few reports on how these hormonal signals are coordinated in the DZ are available. Therefore, one of the main challenges for future work remains to decipher the complete picture of the molecular mechanisms and interactions of plant hormones underlying DZ differentiation in dry fruits.

Evolutionary Origin of Novel Fruit Characters by Modification of DZ Specification Genes

The family Brassicaceae contains over 300 genera, including a number of important vegetables and crops, such as broccoli and cauliflower (*Brassica oleracea*), oilseed rape (*Brassica napus*), and common radish (*Raphanus sativus*). As noted above, the basic fruit type in Brassicaceae is dry dehiscent silique, while there still exist bountiful morphological fruit variations within this family.

Heteroarthrocarpic fruit is a two-segmented fruit with an indehiscent distal part containing rudimentary ovules and a dehiscent proximal part consisting of normal ovules that develop into seeds. Phylogenetic reconstruction combined with morphological analysis shows that heteroarthrocarpic fruit has evolved multiple times within the tribe Brassiceae with nearly half of genera being heteroarthrocarpic (Hall et al., 2011). *Erucaria erucarioides* and *Cakile lanceolata* produce heteroarthrocarpic fruit with different dehiscent patterns. Avino et al. (2012) isolated the homologs of *SHP1/2*, *IND*, *ALC*, *FUL*, and *RPL* from both species and conducted comparative expression examinations. They found that the expression patterns of these genes in the fruit dehiscent segments are largely conserved between these species and in *Arabidopsis*, especially the genes that are involved in the establishment of valve margin identities (Avino et al., 2012). On the other hand, the fruit indehiscent segment is correlated with loss of gene expression of the entire valve margin genetic pathway. These expression data support the hypothesis that heteroarthrocarpy is evolved from dehiscent fruit via repositioning the valve margins (Avino et al., 2012).

Loss of fruit dehiscence has independently evolved in several genera across Brassicaceae (Appel and Al-Shehbaz, 2003). In the genus *Lepidium*, two phylogenetically related species, *L. campestris* and *L. appelianum*, bear dehiscent and indehiscent siliques, respectively (Mummenhoff et al., 2009). Mühlhausen et al. (2013)

conducted a comparative analysis of the expression patterns of *SHP1/2*, *IND*, *ALC*, *FUL*, and *RPL* orthologs in these two species. They found that the expression patterns of these orthologous genes are highly conserved between *L. campestris* (dehiscent fruit) and *A. thaliana* (Mühlhausen et al., 2013). Transgenic plants of *L. campestris* with down-regulation of *SHP1/2*, *IND*, *ALC*, *FUL*, and *RPL* are found to be defective in fruit dehiscence; further anatomical examinations reveal that the fruit structure of these transgenic plants are similar to that of respective *Arabidopsis* mutant (Lenser and Theissen, 2013a). By contrast, the expression of these respective orthologs is completely abolished in the corresponding tissues of indehiscent *L. appelianum* fruit (Mühlhausen et al., 2013). These studies support the notion that the dehiscence network is basically conserved in Brassicaceae and further suggest that genetic changes in the upstream components of *SHP*-regulated pathway are responsible for the evolutionary origin of novel fruit characters (Mühlhausen et al., 2013). This idea is further supported from studies of Brassica species. *B. rapa* and *B. oleracea* produce dehiscent fruits and share similar anatomical structure with *A. thaliana* fruits. Functional analysis shows that *BraA.IND.a* and *BolC.IND.a* are orthologous to *IND* since mutation or down-regulation of either genes results in valve margin defect (Girin et al., 2010). Sequence alignment of the promoters of *IND*-like genes of *A. thaliana* and *B. rapa* reveals a 400-bp conserved sequence, which direct valve margin-specific expression of *IND* in *A. thaliana*. Further analysis shows that the specific activity of the 400-bp promoter sequence depends on the *SHP1/2* and *FUL* (Girin et al., 2010). An independent study in Brassica species reveals that loss of *RPL* gene expression is responsible for the evolutionary origin of the typical narrow replum in this genus. It is found that a point mutation in the promoter region significantly reduces *RPL* expression in the fruits and is associated with the narrow replum character (Arnaud et al., 2011). More recently, an independent research found that the genomic regions that encompass the key regulators of DZ specifying genes are associated with the natural variations in the pod dehiscence character in *Brassica napus* (Raman et al., 2014).

In *Medicago*, a genus of the legume family with a close phylogenetic relationship with Brassicaceae, some species develop coiled pods representing a novel strategy of collective seed dispersal. It is observed that the coiled pod morphology is tightly correlative with increased valve margin lignification, which is associated with a change in the protein sequence of *SHP* orthologs (Fourquin et al., 2013). Further analysis shows that the protein sequence modification alters the properties of the protein by affecting the affinity for other protein partners involved in a high-order complex (Fourquin et al., 2013). It is possible that *SHP*-directed secondary cell wall thickening is an evolutionary conserved module in Rosids (Ferrández and Fourquin, 2014). Nonetheless, it remains to determine the exact cellular and genetic basis that contributes to this indehiscent fruit morphology.

On the whole, the evidence outlined above points to a conserved genetic network controlling the pod dehiscence process and modifications of gene expression and protein properties in the core genetic components are associated with the origin of novel fruit characters.

Regulation of Fruit Ripening and Shedding of Fleshy Fruits in Tomato

Genetics of Fruit Ripening in Tomato

Like pod dehiscence in dry fruit species, the emergence of fleshy fruit represents another evolutionary innovation in which they attract animals for seed dispersal (Dilcher, 2000). Fleshy fruit can be divided into two classes, non-climacteric (e.g., strawberry and grape) and climacteric fruits (e.g., tomato and apple). In the fleshy model plant tomato (*Solanum lycopersicum*), the initiation of fruit ripening process is signified by a concomitant increase in respiration and biosynthesis of ethylene (Giovannoni, 2004; Seymour et al., 2008). In recent years, great advances have been made in dissecting the transcriptional regulation of ripening by the identification of genes with mutations that abolish the normal ripening process. Evidence shows that fruit ripening is a well-orchestrated process with the initiation of multiple genetic and biochemical pathways, which finally brings about the remarkable changes to the metabolic and physiological traits in a ripening fruit. The genetic regulation of the fruit ripening process has recently been thoroughly reviewed by several authors (Seymour et al., 2013; Ferrández and Fourquin, 2014). Here we only briefly introduce the genetic mechanisms underlying the fruit ripening process.

The *SEPALLATA4* clade of MADS-box gene *RIPENING INHIBITOR* (*RIN*) gene is demonstrated to act as the master switch of the fruit ripening process by directly activating the expression of *ACC Synthase 2* (*ACS2*), which is involved in the switch to system-2 ethylene production (Vrebalov et al., 2002; Martel et al., 2011). The spontaneous epigenetic modification of the promoter sequence of the *SQUAMOSA* Promoter Binding (SPB) protein encoded by the *COLORLESS NON-RIPENING* (*CNR*) gene decreases the expression level of *CNR* in the developing fruits, which effectively blocks the ripening process and results in fruits that fail to produce elevated ethylene at the onset of fruit ripening and an insensitivity to ethylene applications (Manning et al., 2006). Similar to the *rin* mutant, genetic lesions in the NAC transcription factor *NON-RIPENING* (*NOR*) gene lead to a non-ripening phenotype with a green fruit (Tigchelaar et al., 1973).

TOMATO AGAMOUS LIKE1 (*TAGL1*), which encodes the orthologous gene of *AtSHP1/2*, is a positive regulator of fruit ripening (Itkin et al., 2009; Vrebalov et al., 2009). *TAGL1* interacts with *RIN* to regulate the ethylene production by directly activating *ACS2* expression (Leseberg et al., 2008; Vrebalov et al., 2009). Overexpression of *TAGL1* in *Arabidopsis* results in an array of phenotypes that are similar to *SHP1/2* over-expressors, which points to a basically conserved role of *SHP*-like genes in organ identity determination (Pinyopich et al., 2003). However, the expression of *TAGL1* in *shp1/2* mutant genetic background only partially rescues the indehiscent fruit phenotype, indicating that *TAGL1* has evolved a novel function in fruit development compared with the *Arabidopsis* counterparts. Other positive regulators of fruit ripening include two closely related *FUL*-like homologs *FUL1* (also known as *TDR4*) and *FUL2* (also known as *MBP7*), which interact with *RIN* protein to regulate fruit ripening by coordinating the expression of genes involved in

cell wall modification, cuticle production, volatile production, and glutamate accumulation (Bemer et al., 2012; Shima et al., 2013). Interestingly, the expression of *TAGL1* is found to be up-regulated in the pericarp of *FUL1/2* RNAi fruits, indicating a negative feedback loop from *FUL1/2* to *TAGL1* (Bemer et al., 2012). The negative regulation of *FUL* to *SHP* is also evident in the valve of *Arabidopsis* (Ferrández et al., 2000). These data point to a conservation of the regulatory network in the *FUL* and *SHP* between *Arabidopsis* and tomato. On the other hand, the homologs of the AP2-ERF protein *SIAP2a* are demonstrated to act as negative regulators of ripening by inhibiting ethylene biosynthesis and signaling pathways (Chung et al., 2010; Karlova et al., 2011). *SIAP2a* seems likely to act downstream of *CNR* as *CNR* protein can bind to the promoter of *SIAP2a* *in vitro* (Karlova et al., 2011).

As outlined above, it appears that genes (including *AP2*, *SHP*, and *FUL*) in fruit development are functionally conserved between *Arabidopsis* and tomato. In the case of the *SHP-FUL* module, it is plausible to assume that the genetic interaction between *SHP* and *FUL* in fruit development might have been established before the split of rosids and asterids. In the fleshy tomato, *SHP* and *FUL* are further co-opted in the *RIN*-regulated ethylene pathway to regulate fruit ripening subsequent upon sub-functionalization and neo-functionalization after lineage-specific gene duplication. The broad conservation of the *SHP-FUL* functional module in dry and fleshy fruits further suggest that fruit dehiscence and ripening may share a common origin and are parallel evolutionary innovations by recruiting a deeply conserved regulatory network (Ferrández and Fourquin, 2014).

Genetic Control of Fruit Shedding in Tomato

In tomato (*Lycopersicon esculentum*), fruit shedding requires the proper development of the abscission zone (AZ) in the knuckle region of the pedicle (see reviews in Roberts et al., 2002; Estornell et al., 2013). The AZ is composed of several layers of smaller and densely cytoplasmic cells (Figure 3, lower panel). Cells in the AZ appear to be predetermined very early in development and are arrested in the following differentiation process (Roberts et al., 2002; Nocker, 2009). Several genes are found to be associated with the initial establishment and further differentiation of the AZ (Figure 3; also see reviews in Roberts et al., 2002). *JOINTLESS* (*J*), which encodes a *SVP/AGL24* clade MADS-box gene, is required for the proper AZ development, as *j* mutant fails to develop the AZ in the pedicle and fruit shedding does not occur normally (Mao et al., 2000). *MACROCALYX* (*MC*) encodes another MADS-box protein that falls into the *AP1/FUL* clade. Similar to the *j* mutant, the AZ is completely lost in the pedicle of *MC* RNAi plants (Nakano et al., 2012). Further analysis shows that the *MC* protein interacts physically with *J* to form a heterodimer with DNA-binding activity. It seems that *J* and *MC* regulate a common set of target genes, including transcription factors regulating meristem maintenance. These data further suggest the AZ cells possess meristematic potential (Roberts et al., 2002; Nakano et al., 2012). The *J-MC* protein complex has recently been extended to incorporate the *SEP*-like MADS-box protein *SLMBP21*. The *SLMBP21* protein interacts with *J* and *MC* to form a higher-order protein complex to confer

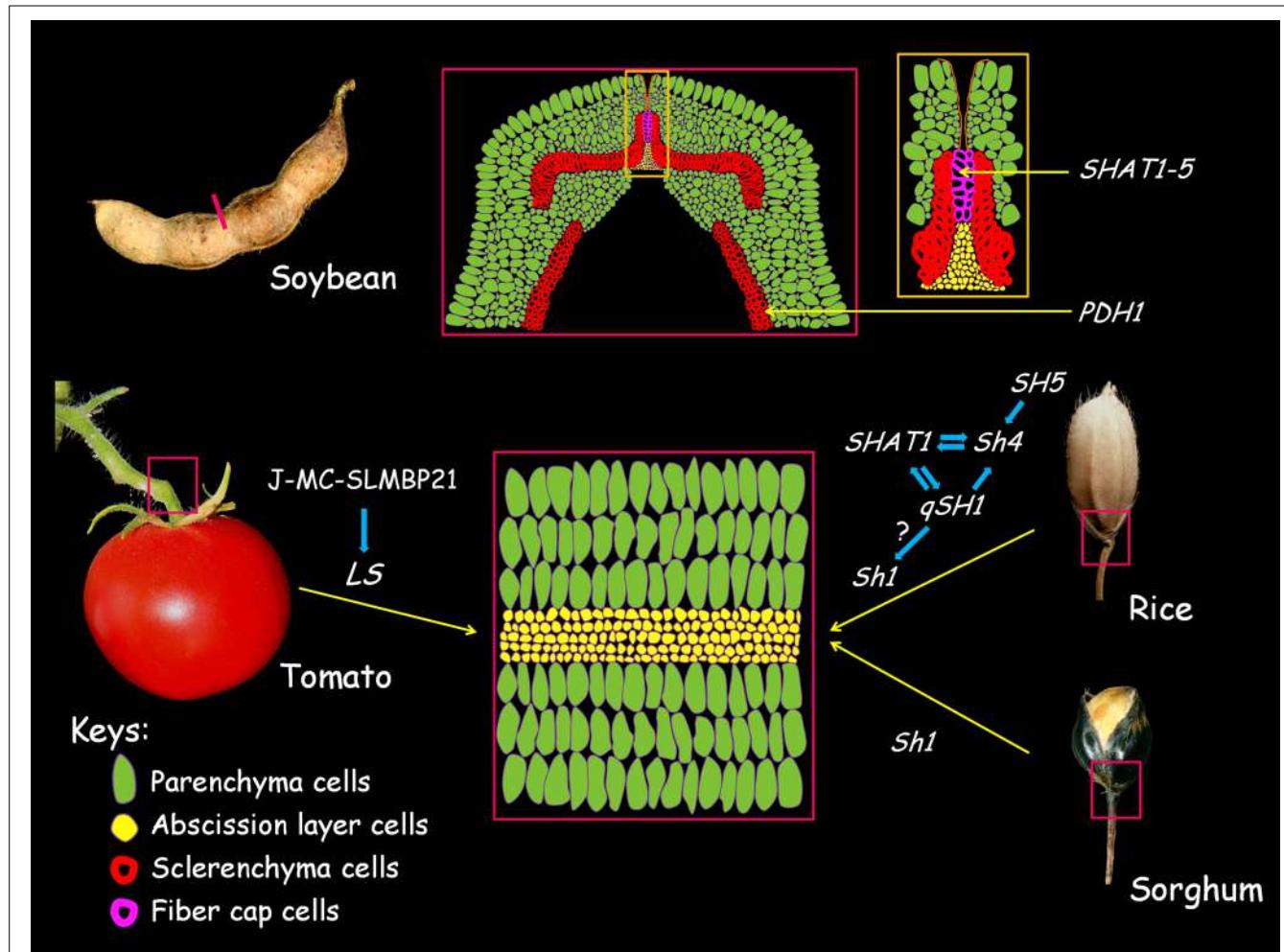


FIGURE 3 | Cellular basis of seed shattering in crops. In soybeans, the loss of pod dehiscence is caused by the excessive lignification of the fiber cap cells (FCCs) and cell wall modification of the inner sclerenchyma cells in the pod valves (upper panel). The middle cartoon of the upper panel shows the transverse section of the pod ventral sutures with the yellow box shows the enlarged photo of the FCC and abscission layer. The loss of fruit shedding and seed shattering

are due to the malfunction of the abscission zone (AZ) development of the pedicels (lower panel). The cartoon in the middle represents a transversal section of the AZ region as boxed in tomato, sorghum, and rice; genes with possible functional relationships that are involved in the AZ development in respective species are shown. The positive relationships between genes were shown by blue arrows. The figures and cartoons are not to scale.

transactivation activity (Liu et al., 2014). Knockdown of *SLMBP21* completely abolishes AZ development, while overexpression of *SLMBP21* results in ectopic AZ-like cells at the proximal region of the pedicel (Liu et al., 2014). Because *J*, *MC*, and *SLMBP21* regulate a common set of target genes, it is possible that the obligate *J-MC-SLMBP21* complex works synergistically to direct the expression of AZ development genes. In line with this notion, the expression of *LATERAL SUPPRESSOR (LS)*, which encodes a VHID protein of the GARS transcription factor family, is found to be down-regulated in *j*, *mc*, and *SLMBP21* RNAi pedicels (Nakano et al., 2012; Liu et al., 2014). The *LS* was initially identified as a positive regulator of axillary meristem maintenance and the *ls* mutant also brings about impaired AZ development (Schumacher et al., 1999). It will be interesting to address how *LS* is co-opted in AZ cell meristematic potential maintenance under the control of *J-MC-SLMBP21* complex-directed pathway.

Convergent Evolution of the Non-Shattering Character in Domesticated Crops

From the evolutionary perspective, natural selection enables the wild plant species to possess elaborate mechanisms to disperse their seeds and fruits. While from the agronomic perspective, the natural seed dispersal is an undesired trait in crops as it leads to severe seed loss in harvest. As a result, natural seed dispersal is severely selected against by ancient humans to assure efficient cultivation during the domestication process (Harlan, 1992; Purugganan and Fuller, 2009; Lenser and Theissen, 2013b). The non-shattering or indehiscent character has been regarded as the milestone of domestication in the seed crops (such as cereals and legumes) as it renders the domesticated species more dependent on human activity for propagation and further facilitates the fixation of other domestication characters

(Doebley et al., 2006; Purugganan and Fuller, 2009). In the seed crops, the reduction of seed shattering capability is evolved independently and is a convergent morphological adaptation to artificial selection (Doebley et al., 2006; Purugganan and Fuller, 2009; Lenser and Theissen, 2013b; Olsen and Wendel, 2013). In Section “Parallel Evolution of the Non-Shattering Trait in Cereal Crops,” we will review the cellular and genetic mechanisms underlying the morphological transition from shattering to non-shattering in domesticated crops (**Figure 3**, lower panel).

Parallel Evolution of the Non-Shattering Trait in Cereal Crops

In cereal crops (such as rice and sorghum), the fruit dehiscence or seed shattering is implemented by an abscission layer in the joint between lemma and pedicel (**Figure 3**, lower panel). In rice (*Oryza sativa*), several transcription factor coding genes have been found to be associated with the reduction of seed shattering (**Figure 3**). *Shattering4* (*Sh4*) encodes a transcription factor with homology to Myb3 and is necessary for the development of a functional abscission layer in the pedicel (Li et al., 2006). A single amino acid change in the putative DNA binding domain is closely associated with the reduction in seed shattering in domesticated rice. In addition, the expression of the domesticated allele is also remarkably decreased compared with the wild allele (Li et al., 2006). Thus, it appears that the combination of coding and regulatory change of *Sh4* impairs the developmental program of the abscission layer, thus weakens the shattering phenotype (Li et al., 2006). *qSH1* is a major QTL on chromosome 1 controlling seed shattering in rice. The underlying gene, *qSH1*, encodes a BEL1-type homeobox transcription factor that is highly homologous to *AtRPL* (Konishi et al., 2006). *qSH1* is required for formation of the abscission layer in the pedicel. A single nucleotide polymorphism (SNP) in the 5'-regulatory region completely eliminates *qSH1* expression in the provisional abscission layer early in the development process and results in non-shattering trait in domesticated rice (Konishi et al., 2006). Notably, the regulatory SNP in the promoter sequence of *RPL* homologs is also responsible for the difference in seed dispersal structures produced by natural selection in *Brassica* species with reduced replum development (Arnaud et al., 2011). These examples demonstrate a remarkable convergent mechanism in which the same regulatory SNP can explain the developmental variations in seed dispersal structures relevant to both domestication and natural selection in a distantly related species (Arnaud et al., 2011; Gasser and Simon, 2011).

SH5 is another BEL1-type homeobox gene with high homology to *qSH1*. *SH5* is highly expressed in the abscission layer (Yoon et al., 2014). Silencing of *SH5* suppresses the development of the abscission layer and inhibits seed shattering. Overexpression of *SH5* gives rise to an increase in seed shattering, a consequence of decreased lignin levels in the pedicel (Yoon et al., 2014). The expression of *Sh4* is found to be significantly up-regulated in the *SH5*-overexpressor, suggesting *SH5* positively regulates *Sh4* to direct abscission layer development (Yoon et al., 2014). Recently, the regulatory pathway of the abscission layer development was extended to include an AP2-transcription factor coding gene,

SHATTERING ABORTION1 (*SHAT1*, Zhou et al., 2012). *SHAT1* is required for seed shattering through specifying the abscission layer. The expression of *SHAT1* in the abscission layer is positively regulated by *Sh4*. *qSH1* expression is completely lost in the abscission layer in either *shat1* and *sh4* mutant background, suggesting *qSH1* functions downstream of *SHAT1* and *Sh4* in the establishment of the abscission layer (Zhou et al., 2012). Interestingly, *qSH1* is also required for the expression of *SHAT1* and *Sh4* in the abscission layer. Therefore, *qSH1* is probably involved in a positive feedback loop of *SHAT1* and *Sh4* by maintaining the expression of *SHAT1* and *Sh4* in the abscission layer (Zhou et al., 2012). Although *SH5* and *SHAT1* play roles in the differentiation of abscission layer, it remains to be determined whether these two genes are domestication genes targeted by artificial selection.

Similar to rice, the reducing of seed shattering in domesticated sorghum (*Sorghum bicolor*) results from the loss of abscission layer in the joint connecting the seed hull and pedicel. Seed shattering in sorghum is controlled by a single gene, *Shattering1* (*Sh1*), which encodes a YABBY transcription factor. The non-shattering character can be accounted for by one of three distinct loss-of-function mutations that are independently selected upon during the sorghum domestication process (Lin et al., 2012). Notably, the *Sh1* orthologs in rice and maize (*Zea mays*) harbor mutations that are possibly associated with the shattering reduction in respective crops (Paterson et al., 1995; Lin et al., 2012). Whether *Sh1* is rewired into the *SH5*-directed seed shattering network in rice remain to be explored in the future (**Figure 3**, lower panel). In *Sorghum propinquum*, a wild sorghum relative, seed shattering is conferred by the *SpWRKY* gene. It is postulated that *SpWRKY* negatively regulates cell wall biosynthesis genes in the abscission layer. Nonetheless, the *SpWRKY* has not been crafted by artificial selection to make a contribution to the non-shattering trait in domesticated sorghum (Tang et al., 2013). Taken together, these above findings have raised an intriguing possibility that the convergent domestication of non-shattering crops might have achieved through parallel selection on the same underlying genetic targets (**Figure 3**, lower panel; Lin et al., 2012; Lenser and Theissen, 2013b).

The *Q* gene in domesticated wheat (*Triticum aestivum*) is an important domestication gene as it confers the free-threshing character (the loss of tendency of the spike shattering; Simons et al., 2006). *Q* gene encodes a member of AP2-family transcription factor. The cultivated *Q* allele is transcribed more abundantly than the wild *q* allele. Furthermore, the two alleles also differ in a single amino acid that significantly enhances the homodimerization capacity of the domestication allele (Simons et al., 2006). Thus, similar to the case of *Sh4*, the evolution of the free-threshing trait in domesticated wheat may have attributed to the combination of both coding and regulatory changes in the domestication gene. The expression difference between *Q* and *q* seems more important as it can largely explain the free-threshing trait in the domesticated wheat (Simons et al., 2006; Zhang et al., 2011). Although the mutation that gives rise to *Q* had a profound effect in the domestication process of wheat as it enables the farmers to harvest the grain more efficiently, the exact cellular basis leading to the free-threshing trait is still unknown.

The Domestication of Indehiscent Fruit in Legume Crops

In addition to cereals, loss of pod dehiscence also occurs in dicot crops, such as legumes. Species in the Legume family develop a characteristic dry dehiscent fruit (a legume or more generally a pod), which is derived from a monocarpellate pistil. The legume species disperse seeds by shattering the pod along the ventral suture after maturation (Tiwari and Bhatia, 1995). In cultivated soybean (*Glycine max*), the indehiscent pod is a major domestication trait that is targeted by artificial selection (Hymowitz, 1970; Harlan, 1992). The cellular basis and molecular mechanisms leading to the indehiscent pod have very recently been characterized. It is shown that the excessive lignification of the fiber cap cells (FCCs) in the ventral suture is responsible for the indehiscent fruit character (Figure 3, upper panel; Dong et al., 2014). Unexpectedly, the abscission layer is found to be functionally unchanged in the cultivated soybeans (Dong et al., 2014). *SHATTERING1-5* (*SHAT1-5*), which is homologous to *AtNST1/2* that acts as master transcriptional activator of secondary cell wall biosynthesis, resides in a QTL controlling pod dehiscence. Expression of *SHAT1-5* is specifically localized in the developing FCCs. The lack of any fixed amino acid difference between the cultivated allele and wild allele, and that both alleles are capable of fully restoring the secondary cell wall thickening in the interfascicular fibers of *nst1-1;nst3-1* double mutant suggest that the differential expression of *SHAT1-5* in the FCC upon regulatory changes might be important for the indehiscent fruit. Using Laser Capture Microdissection system, Dong et al. (2014) reveal that a significant up-regulation of *SHAT1-5* in FCC of cultivated soybean is responsible for the excessive cell wall deposition in the FCC, which in turn prevents the pod from committing dehiscence after maturation (Figure 3, upper panel). Further analysis show that the over transcription of *SHAT1-5* in cultivated soybean FCC is attributable to the disruption of a repressive *cis*-regulatory element in the 5'-promoter region (Dong et al., 2014). Expression of *SHAT1-5* is related to the organs with severe secondary cell wall thickening, which is a common process during plant development (Dong et al., 2013). It seems that artificial selection would have discarded the null mutant in this gene due to pleiotropic effect, leaving a change in the specific regulatory element as a preferred mechanism for producing the desired phenotype.

PDH1 (QTL for Pod Dehiscence 1) is another major QTL controlling pod dehiscence in soybean that have very recently been cloned and shown to encode a dirigent-like protein with a possible function in lignin biosynthesis (Suzuki et al., 2010; Funatsuki et al., 2014). Expression of *PDH1* is correlated with the lignin deposition in the inner sclerenchyma of the pod walls (Figure 3, upper panel). *PDH1* promotes pod dehiscence by increasing the twisting force in the pod wall, which serves as a driving force for pod dehiscence (Funatsuki et al., 2014). In cultivated soybean, the indehiscent fruit is attributable to a premature stop codon in *PDH1*, which generates a non-functional protein (Funatsuki et al., 2014). Although the exact cellular and biochemical mechanisms leading to indehiscent pod by *PDH1* remain to be elucidated, it is apparent that artificial selection might have targeted multiple cellular mechanisms

and the controlling genes, including *SHAT1-5* and *PDH1*, to minimize seed loss during soybean domestication. Meanwhile, these findings also raise an intriguing question as to how *SHAT1-5* and *PDH1* interact genetically to fine-tune the indehiscence degree of cultivated soybean that are adapted in different environments. Future analysis of allele frequency combined with careful phenotypic evaluation in a large collection of cultivated soybean germplasms would help to address this question.

The domesticated common bean (*Phaseolus vulgaris*) originated in the Mesoamerican and Andean regions independently (Schmutz et al., 2014). Similar to other legume crops, the reduction of pod dehiscence represents a key domestication syndrome in the domesticated common bean. The indehiscent fruit results from the loss of fibers in the sutures (“stringless”), which is under the control of a major QTL, *St* locus (Koinange et al., 1996). *PvIND1*, a homolog of *AtIND* in common bean, was recently mapped in a region near the *St* locus. It appears that *PvIND* may not be directly involved in the control of pod dehiscence and may not be the causal gene underlying *St*, as polymorphism in the *PvIND* gene fails to link with the genotype on *St* locus and co-segregate with the dehiscent/indehiscent phenotype (Gioia et al., 2013). While *PvIND* is postulated as the *AtIND* homolog based on sequence homology in the conserved b-HLH domain, the *IND*-related transcription factors are specific to Brassicaceae and its role in valve margin cell lignification may have been acquired since the duplication event happened recently in the *HECATE3* (*HEC3*) gene clade in Brassicaceae (Liljegren et al., 2004; Girin et al., 2010). Therefore, it is possible that polymorphisms in other *AtIND* homologs in the common bean genome may have been associated with pod indehiscence. Alternatively, considering that the fibers are mainly composed of sclerenchyma cells with well-developed secondary cell walls, it is also likely that genes involved in the regulation of secondary cell wall deposition or fiber cell differentiation may have contributed to the *St* locus in controlling pod dehiscence. Future work is necessary to discriminate these possibilities.

Conclusions and Future Perspectives

In the past 15 years, our understanding of the genetic control and evolution of the seed shattering/pod dehiscence processes has been advanced significantly by the implement of a combination of multiple experimental approaches. In *Arabidopsis*, the homostasis and interaction of hormones is revealed to work in another regulation layer in establishing the DZs. The core regulatory module (*SHP-FUL*) controlling DZ development is found to be largely conserved in dry fruit species that are closely relative to *Arabidopsis* while modification of the key regulatory genes frequently contributes to the evolution of specialized fruit morphology with novel dispersal strategies. Studies in the genetic control of the fleshy fruit maturation process further extend the conservation of *SHP-FUL* module into angiosperms and suggest that fruit dehiscence and ripening are in parallel evolved characters by co-opting the same underlying regulatory networks. Although we can now begin to understand the molecular and biochemical basis of fruit dehiscence and ripening in model species, a challenge remains to obtain greater molecular data from

TABLE 1 | Summary of genes involved in the seed shattering process.

Species	Gene(s)	Gene category	Molecular function	Phenotypic effect	References
<i>Arabidopsis thaliana</i>	<i>SHATTERPROOF1/2</i>	Transcription factor	Transcriptional regulator (MADS)	Indehiscent pod	Liljeblad et al. (2000)
	<i>INDEHISCENT</i>	Transcription factor	Transcriptional regulator (bHLH)	Indehiscent pod	Liljeblad et al. (2004)
	<i>ALCATRAZ</i>	Transcription factor	Transcriptional regulator (bHLH)	Partially indehiscent pod	Rajani and Sundaresan (2001)
	<i>FRUITFULL</i>	Transcription factor	Transcriptional regulator (MADS)	Premature bursting pod	Gu et al. (1998)
	<i>REPLUMLESS</i>	Transcription factor	Transcriptional regulator (homeodomain)	Partially indehiscent pod	Poeder et al. (2003)
	<i>NST1/3</i>	Transcription factor	Transcriptional regulator (NAC)	Indehiscent pod	Mitsuda and Ohme-Takagi (2008)
	<i>ADPG1/2</i>	Endo-polygalacturonase	Degradate cell wall matrix	Indehiscent pod	Ogawa et al. (2009)
	<i>GA3ox1</i>	Catalytic enzyme	GA biosynthesis	Partially indehiscent pod	Arnaud et al. (2010)
	<i>Glycine max</i>	Transcription factor	Transcriptional regulator (NAC)	Indehiscent pod	Dong et al. (2014)
	<i>PDH1</i>	Dirigent-like protein	Lignin biosynthesis	Indehiscent pod	Funatsu et al. (2014)
	<i>JOINTLESS</i>	Transcription factor	Transcriptional regulator (MADS)	Non-shedding fruit	Mao et al. (2000)
	<i>MACROCALyx</i>	Transcription factor	Transcriptional regulator (MADS)	Non-shedding fruit	Nakano et al. (2012)
	<i>SLMBP21</i>	Transcription factor	Transcriptional regulator (MADS)	Non-shedding fruit	Liu et al. (2014)
	<i>LATERAL SUPPRESSOR</i>	Transcription factor	Transcriptional regulator (GARS)	Non-shedding fruit	Schumacher et al. (1999)
<i>Solanum lycopersicum</i>	<i>Shattering4</i>	Transcription factor	Transcriptional regulator (Myb)	Non-shattering seed	Li et al. (2006)
	<i>qSH1</i>	Transcription factor	Transcriptional regulator (homeodomain)	Non-shattering seed	Konishi et al. (2006)
	<i>SH5</i>	Transcription factor	Transcriptional regulator (homeodomain)	Non-shattering seed	Yoon et al. (2014)
	<i>SHATTERING ABORTION1</i>	Transcription factor	Transcriptional regulator (AP2)	Non-shattering seed	Zhou et al. (2012)
	<i>Shattering1</i>	Transcription factor	Transcriptional regulator (YABBY)	Non-shattering seed?	Lin et al. (2012)
<i>Sorghum bicolor</i>	<i>Shattering1</i>	Transcription factor	Transcriptional regulator (YABBY)	Non-shattering seed	Lin et al. (2012)
	<i>SpWRKY</i>	Transcription factor	Transcriptional regulator (WRKY)	Non-shattering seed	Tang et al. (2013)
<i>Zea mays</i>	<i>Shattering1</i>	Transcription factor	Transcriptional regulator (YABBY)	Non-shattering seed?	Lin et al. (2012)
	<i>Q</i>	Transcription factor	Transcriptional regulator (AP2/ERF)	Free-threshing character	Simons et al. (2006), Zhang et al. (2011)

other non-model species, to unveil the evolutionary mechanisms of fruit diversification widespread in nature.

In the domesticated crops, it is apparent that the convergent evolution of non-shattering (indehiscent) fruit is often employed by the same gene or strikingly, the same mutation, while non-homologous genes are also frequently evident in different crops. In the future, with the growing interest in the molecular mechanisms of domesticated syndromes that arise as the result of evolutionary implications and their agriculture importance, an equally important and complementary issue will be the advances in the application of high throughput sequencing technology (next-generation sequencing, NGS) combined with genotype-phenotype associations (genome-wide association analysis, GWAS) to zoom in on the exact mutations leading to the non-shattering character in additional crops. Overall, the list of genes that participate in the seed shattering process has experienced an unprecedented explosion in the past few years (**Table 1**), we can

now begin to think about how to translate this basic knowledge into practice in crop breeding programs to feed the world in the face of growing population pressures. Exemplary work has been done in *Brassica juncea* by over-expression *AtFUL* to make pods resistant to shattering (Østergaard et al., 2006).

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Development and regulation of pedicel abscission in tomato

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To shed unfertilized flowers or ripe fruits, many plant species develop a pedicel abscission zone (AZ), a specialized tissue that develops between the organ and the main body of the plant. Regulation of pedicel abscission is an important agricultural concern because pre-harvest abscission can reduce yields of fruit or grain crops, such as apples, rice, wheat, etc. Tomato has been studied as a model system for abscission, as tomato plants develop a distinct AZ at the midpoint of the pedicel and several tomato mutants, such as *jointless*, have pedicels that lack an AZ. This mini-review focuses on recent advances in research on the mechanisms regulating tomato pedicel abscission. Molecular genetic studies revealed that three MADS-box transcription factors interactively play a central role in pedicel AZ development. Transcriptome analyses identified activities involved in abscission and also found novel transcription factors that may regulate AZ activities. Another study identified transcription factors mediating abscission pathways from induction signals to activation of cell wall hydrolysis. These recent findings in tomato will enable significant advances in understanding the regulation of abscission in other key agronomic species.

Keywords: abscission, pedicel, MADS-box, ERF, tomato

Introduction

Similar to leaves, flowers and young fruits shed when the organs become unneeded or as a result of environmental stresses; for example, failure of pollination results in abscission of the unfertilized flowers. Also, in "June drop" in apple (*Malus × domestica*), some young fruitlets abscise at an early developmental stage (Bangerth, 2000). Just after flowering, apple trees often bear more fruits than they can support to maturity; thus the plants shed some fruits to limit fruit set. In addition, when fruits on a plant ripen, abscission of the fruits helps to disperse the seeds. The pedicel, a stem, or a stalk structure, connects at the base of the flower or fruit, attaching the organ to the plant body. In many species, an abscission zone (AZ) forms in the pedicel to enable regulated separation of the fruit or flower from the main plant body (Sexton and Roberts, 1982; Tabuchi et al., 2001; Roberts et al., 2002).

In agricultural applications, pedicel abscission is a critical trait directly affecting crop yields; thus the regulation of abscission has been important since ancient times. During the domestication of cereal crops such as rice (*Oryza sativa*), maize (*Zea mays*), or wheat (*Triticum aestivum*), early farmers selected for plants with reduced abscission (Doebley et al., 2006; Li et al., 2006; Lin et al., 2012). Cultivars carrying the trait conferring resistance to grain abscission retain the grain on the stalk, rather than dropping it on the ground.

Regulation of abscission also remains an important trait in modern breeding programs. Research in tomato has identified several mutations that block formation of the pedicel AZ, producing a "jointless" phenotype (Butler, 1936; Rick, 1967; Roberts et al., 2002), which has proven useful in

tomato cultivars grown for industrial processing of tomato puree or juice. In these cultivars, fruits can be mechanically harvested without the pedicel and sepals because, in the absence of a breaking point in the pedicel AZ, the fruit detaches at the next breaking point, the calyx AZ at the proximal end of the fruit, and the green organs remains on the plant. This reduces the labor and time required to remove the pedicel and sepals during harvesting (Zahara and Scheuerman, 1988).

Fruit abscission is also an important trait for tree fruit production. In apple, abscission affects the fruit yield at several stages (Celton et al., 2014). The trees shed young fruitlets as “June drop,” as described above. Thinning of young fruits is an important practice to control fruit load and chemicals that induce partial abscission of fruit have been developed to reduce the labor required for thinning (Bangerth, 2000). After the early developmental stages, fruits at the expanding stage remain stably attached to the plant via the pedicels but the attachment gradually loosens during the initiation of ripening. However, severe weather can cause fruit to drop prematurely. For example, in Japan, the autumn fruit harvest coincides with the typhoon season and large numbers of fruits just before harvest time drop by the strong winds, which break a boundary between the plant body and pedicel, where the AZ is localized, resulting in severe damage to production (Yamamoto et al., 2012; Fujisawa et al., 2015).

Pedicel AZ Structure and Development in Tomato

The AZ, a specialized tissue for organ abscission, forms at a predetermined site on the organ that will abscise. Anatomical studies revealed that an AZ includes several layers of small, densely cytoplasmic cells that forms at an early stage of pedicel development and proliferation of the cells is observed during fruit development (Addicott, 1982; Sexton and Roberts, 1982; Tabuchi and Arai, 2000; Patterson, 2001). These properties suggest that these cells may be arrested in an undifferentiated state (van Nocker, 2009). In tomato, initial differentiation of the pedicel AZ occurs when the flower sepal differentiates from the primordium. AZ cells first form in the inner region of the young pedicel and then the AZ structure gradually extends to the outer tissues (Tabuchi, 1999; Liu et al., 2014). The innermost cell layer has a critical role in AZ development, as examination of chimeric plants consisting of layers of jointless mutant cells and wild-type cells showed that the genotype of the inner layer (L3) determines cell fates of overlaying layers L1 (outer layer) and L2 (middle layer) and whether they differentiate into AZ tissue (Szymkowiak and Irish, 1999). At the flower anthesis stage, pedicel AZ tissues have developed into six to eight cell layers that extend across the pedicel. The AZ cells around the vascular tissue and cortex can still divide (Tabuchi and Arai, 2000), suggesting that the AZs in flower pedicels maintain meristem-like activity.

Normally, pedicel abscission is induced if flower fertilization fails or the fruit ripens fully. Pedicel abscission can also be induced artificially by flower removal (Roberts et al., 1984; Meir et al., 2010; Nakano et al., 2013) or ethylene treatment (Roberts

et al., 1984; Wang et al., 2013); several studies have used these treatments to analyze abscission. Roberts et al. (1984) observed that cell separation for abscission first took place at the cortex within the distal side of the AZs if the pedicel was treated with ethylene. Also, Tabuchi et al. (2001) reported that pedicel abscission occurred first at the epidermis of the AZ if abscission was induced by emasculation. Dissolution of the middle lamella commonly occurred in response to either treatment, and cell wall hydrolysis enzymes and remodeling proteins, such as polygalacturonase (tomato abscission-related polygalacturonase; TAPG), endo- β -1,4-glucanase (also referred as cellulase; Cel), xyloglucan endotransglucosylase/hydrolase (XTH), and expansin, play a critical role in abscission (Roberts et al., 2002; Tucker et al., 2007; Cai and Lashbrook, 2008). The abscission-inducing treatment also caused enlargement of the epidermal cells in tomato (Tabuchi et al., 2001). Cell enlargement during abscission also occurs in other plant systems such as bean leaves (McManus et al., 1998) and *Arabidopsis* flower organs (Shi et al., 2011), and this enlargement may confer mechanical force to facilitate abscission (Shi et al., 2011). The abscised surface of the proximal side formed thickened and lignified cell walls, implying that a protective layer forms to prevent pathogen invasion (Tabuchi et al., 2001).

MADS-Box Family Transcription Factors Regulate Pedicel AZ Development in Tomato

The most important breakthrough in abscission research was the identification of the *jointless* (*j*) mutant locus (Mao et al., 2000), which causes the plant to fail to develop pedicel AZs. The *j* locus was isolated by map-based cloning and the wild-type gene encodes a MADS-box transcription factor. In the same year, independent work on an early-flowering mutant identified *Arabidopsis* SHORT VEGETATIVE PHASE (*SVP*), which encodes a MADS-box protein with high similarity to *J* (Hartmann et al., 2000). Although *J* and *SVP* have high amino acid sequence similarity, they have distinct functions, with *SVP* acting as a repressor of the floral transition. Moreover, *Arabidopsis* plants do not shed fruits from the pedicels. Also, in several tree fruit species, *SVP* homologs may play roles in bud dormancy (Li et al., 2009; Yamane et al., 2011; Wu et al., 2012).

Further studies identified two additional tomato MADS-box genes regulating pedicel AZ development, *Macrocalyx* (*MC*) and *SlMBP21*. *MC* was originally identified in a study of *rin* (ripening inhibitor), which regulates fruit ripening. The *rin* mutation produces non-ripening fruits with large sepals (Vrebalov et al., 2002). The cloning study identified two nearby genes, *RIN* and *MC*, both of which encode MADS-box genes. *RIN* regulates ripening and *MC* regulates sepal size (Vrebalov et al., 2002). The *rin* mutation also shows a weak effect on pedicel AZ development and antisense-mediated knockdown revealed that *MC* also plays a role in pedicel AZ development (Nakano et al., 2012). A comprehensive interaction study of tomato MADS-box proteins using yeast two-hybrid system initially identified *SlMBP21* as a MADS-box protein interacting with *J* (Leseberg et al., 2008). A gene knockdown study revealed that *SlMBP21*

also participates in pedicel AZ development (Liu et al., 2014). These studies showed physical interactions among J, MC, and SLMBP21, suggesting that these three MADS-box proteins form a complex. At an early stage of AZ initiation, these MADS-box genes are co-expressed in vascular tissue derived from the L3 layer required for AZ development (Szymkowiak and Irish, 1999; Liu et al., 2014). In *Arabidopsis*, the J homolog SVP and the MC homolog AP1 likely form a dimer as an active form to regulate floral identity (Gregis et al., 2009). SEP family proteins, including SLMBP21, play an important role in forming multimers of MADS-box proteins by acting as a glue (Immink et al., 2009). Thus, multimer formation of J, MC, and SLMBP21 may be a conserved activity among plant species, although the targets of biological regulation by homologous MADS-box proteins may differ in each plant.

Is the regulation of pedicel AZ development by the MADS-box transcription factors conserved in other plant species, or is it specific to tomato? Ectopic expression of the apple SVP family MADS-box gene *MdJb* in a tomato *j* mutant restored the formation of pedicel AZ structure in the *j* mutant (Nakano et al., 2015). The restored AZs showed abscission-associated expression of cell wall hydrolysis enzyme genes and complete pedicel abscission, as in wild-type tomato plants. The results suggest that the regulation of pedicel AZ development in plants by the MADS-box transcription factors may be conserved, but other plant systems remain to be examined. Further investigation will be required to understand the mechanism of AZ development in other plant species.

Genes Expressed in Tomato Pedicel AZs

Before abscission, pedicel AZs attach the flowers firmly to the plant body, but when the AZ cells perceive an abscission-stimulating signal, the adhesion immediately starts to loosen. During abscission, the gene expression pattern in the AZ changes drastically; genes for cell wall hydrolysis enzymes, such as TAPG and Cel, and for factors regulating programmed cell-death increase intensely and specifically at the AZ (Roberts et al., 2002; Cai and Lashbrook, 2008; Meir et al., 2010; Bar-Dror et al., 2011). In addition to these genes, a transcriptome study during initiation of abscission found many genes possibly responsible for regulatory roles in abscission, such as genes for transcription factor families of ARF, Aux/IAA, KNOX, HAT, bHLH, AP2, NAC, AGL, and WRKY, genes for components of signal transduction pathways such as a LRR-RLK and a Ser/Thr protein kinase, and a gene for a component of a RNA-induced silencing complex, AGO1 (Meir et al., 2010). The analyses also provided specific expression patterns of phytohormone-related genes, which confirmed and improved a conventional abscission-inducing model with the substantial evidence (Patterson, 2001; Roberts et al., 2002; Meir et al., 2010); a decrease in auxin provides the first signal for abscission, and reactions to the decrease in auxin, including down-regulation of genes induced by auxin (such as *Aux/IAA* genes and other transcription factor genes) and up-regulation of genes repressed by auxin, confers ethylene-sensitivity and abscission competence to the AZ. Then increased ethylene production, due to the up-regulation of genes for ethylene biosynthesis (such

as ACS, encoding 1-aminocyclopropane-1-carboxylate (ACC) synthase), leads to AZ-specific up-regulation of the genes for abscission, such as genes encoding cell wall-modifying proteins and pathogenesis-related proteins, development of a protective layer on the surface of the abscised tissue, and so on.

Before the onset of pedicel abscission, the plant maintains firm cell-to-cell adhesion at the AZs to allow continuous growth from the flower to the mature fruit. To maintain the adhesion and the competence to react to an abscission-inducing signal, the AZ cells might undergo specific regulation. A transcriptome analysis comparing gene expression between pedicel AZs and the flanking pedicel regions at anthesis (Nakano et al., 2013) identified about 90 genes specifically expressed in AZ cells, including genes for transcription factors, phytohormone-related proteins, cell wall modification enzymes, lipid metabolism, and others. Most interestingly, the AZ-specific gene set included transcription factor genes that encode key regulators of meristem-associated functions, including a tomato homolog of *WUSCHEL* (*LeWUS*), *GOBLET* (*GOB*), *LATERAL SUPPRESSOR* (*Ls*), and *Blind* (*Bl*). *WUS* expressed in *Arabidopsis* shoot apex is required for maintenance of stem cells in an undifferentiated state (Mayer et al., 1998). *GOB* is a member of the NAC family transcription factor genes and its *Arabidopsis* homolog genes, *CUP-SHAPED COTYLEDONS* (*CUCs*), are involved in shoot meristem formation and specification of organ boundaries (Aida et al., 1997; Blein et al., 2008; Berger et al., 2009). *Ls* and its *Arabidopsis* homolog are known to regulate axillary meristem initiation (Schumacher et al., 1999; Greb et al., 2003). *Bl* and its *Arabidopsis* homolog of *REGULATOR OF AXILLARY MERISTEM* (*RAX*) also involved in axillary meristem formation (Schmitz et al., 2002; Keller et al., 2006). These transcription factors were suppressed in the pedicels of AZ-deficient plants, the *j* mutant, and *MC*- and *SLMBP21*-suppressed plants (Nakano et al., 2012; Liu et al., 2014). Also, *LeWUS*, *GOB*, and *Ls* were down-regulated immediately after an abscission-inducing treatment while *Bl* was up-regulated (Nakano et al., 2013). These characteristic expression patterns suggest that these transcription factor genes play important roles in the AZs. Similar to meristems, AZs include small cells that likely exist in an undifferentiated state (van Nocker, 2009); thus, these transcription factors may regulate the maintenance of these undifferentiated cells in both tissues. In rice flower pedicels, homologs of *Bl*, *GOB*, and *Ls* are expressed specifically in the AZs, indicating that the mechanism of regulation by these transcription factors may be conserved in monocots and dicots (Nakano and Ito, 2013).

A recent study showed an intriguing result on the undifferentiated properties of the AZ cells. Constitutive expression of a miRNA-resistant form of a tomato homolog of the *REVOLUTA* gene, encoding a Class III homeodomain-leucine zipper (HD-ZIP III) transcription factor, caused the transgenic plants to produce ectopic flowers from the pedicel AZs (Hu et al., 2014). In the AZs at anthesis, the transgenic plants expressed *Bl* and *GOB* at significantly higher levels than the wild-type plants. The results imply that pedicel AZs include undifferentiated cells that have the potential to develop into flower primordia, and the transcription factors expressed in the AZs may coordinately

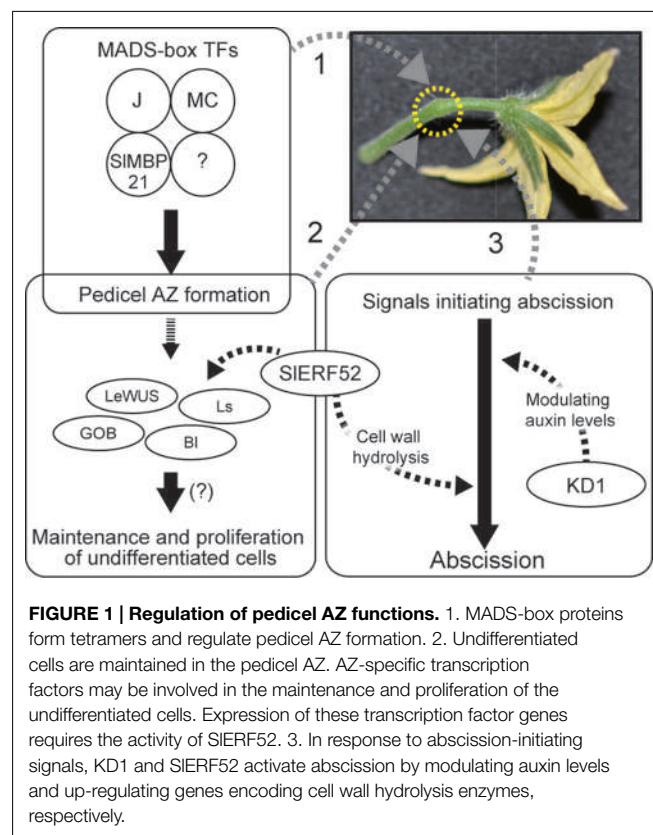
regulate the maintenance and proliferation of the undifferentiated AZ cells.

These transcriptome analyses identified genes specifically expressed in tomato pedicel AZs, and of them, two transcription factor genes, *SIERF52* and *KD1*, were further analyzed for their effect on AZ functions, as described in the next section.

Transcription Factors Connecting Abscission-Inducing Signals and Abscission Processes

Of the transcription factor genes expressed in tomato pedicel AZs, the ERF family transcription factor gene *SIERF52* was further investigated by RNAi-mediated knockdown assays (Nakano et al., 2014). The *SIERF52*-knockdown plants developed pedicel AZ structures similarly to wild-type plants; however, the responses to an abscission-inducing treatment differed in the knockdown and wild-type plants. In wild-type plants, removing the anthesis-stage flower from the pedicel usually induces pedicel abscission within 2 days; in the *SIERF52*-knockdown plants, pedicel abscission took significantly longer. The knockdown disturbed the abscission-specific up-regulation of the genes for hydrolytic enzymes, such as TAPG and Cel, indicating that the suppression of the hydrolytic enzymes caused the delay in abscission. The result suggests that the *SIERF52* ERF transcription factor functions as a component of a signaling pathway for pedicel abscission and plays a key role in the induction of expression of genes involved in cell wall hydrolysis. The induction of the hydrolytic enzyme genes during abscission, however, may require an additional factor to activate *SIERF52*. The expression levels of *SIERF52* did not differ before and after the abscission-inducing treatment; thus, the expression level of *SIERF52* cannot explain the activation of abscission. On the other hand, before the onset of abscission, the AZ-specific expression of *LeWUS*, *Ls*, and *GOB* requires *SIERF52*, implying that *SIERF52* acts before and during abscission, but the transcriptional targets of *SIERF52* apparently differ in the two stages. Explaining the functional switching of *SIERF52* may require additional factors, such as stage-specific co-factors of *SIERF52* or repressor proteins at *SIERF52*-binding sites. The identification of the switching mechanism will provide further insights into the regulation of pedicel abscission.

Another transcription factor gene expressed specifically in the tomato pedicel AZ, *KD1*, a *KNOTTED1-LIKE HOMEOBOX* (*KNOX*) family gene, was investigated for function in pedicel and petiole AZs (Ma et al., 2015). Down-regulation of *KD1* significantly delayed abscission of the pedicel and even petiole and up-regulation of *KD1* promoted abscission. The investigation suggested that *KD1* controls abscission by regulating genes that modulate auxin levels (Ma et al., 2015). Identification of the regulator of abscission in both pedicels and petioles provides substantial evidence that abscission in these tissues involves identical regulatory mechanisms, in contrast to their distinct mechanisms regulating AZ development (Szymkowiak and Irish, 1999). Further investigation of the relationship between *SIERF52* and *KD1* may reveal their activities in abscission processes more clearly.



Conclusion

These recent advances in our understanding of the regulation of pedicel abscission revealed key factors involved in AZ development and signal transduction in the initiation of abscission. **Figure 1** shows a current model of development of AZs and induction of abscission in tomato pedicels. The MADS-box transcription factor complex regulates pedicel AZ development. The developed AZ contains undifferentiated cells, probably maintained by a mechanism similar to that found in meristems. The signals of decreased auxin and increased ethylene induce abscission and *SIERF52* and *KD1* possibly connect the phytohormone signaling pathway and abscission processes. A remaining mystery is another tomato jointless mutation, *jointless-2* (*j-2*), which is the best used mutation in practical breeding programs of processing tomatoes. A candidate gene for the mutation was reported but it has not been fully identified yet (Yang et al., 2005).

The outline of the current model constructed in tomato will facilitate further detailed studies on pedicel functions in tomato and other plants and these studies will provide new applications for fruit crops to improve their productivities.

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The IDA Peptide Controls Abscission in *Arabidopsis* and *Citrus*

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Organ abscission is an important process in plant development and reproduction. During abscission, changes in cellular adhesion of specialized abscission zone cells ensure the detachment of infected organs or those no longer serving a function to the plant. In addition, abscission also plays an important role in the release of ripe fruits. Different plant species display distinct patterns and timing of organ shedding, most likely adapted during evolution to their diverse life styles. However, it appears that key regulators of cell separation may have conserved function in different plant species. Here, we investigate the functional conservation of the citrus ortholog of the *Arabidopsis* peptide ligand INFLORESCENCE DEFICIENT IN ABSCISSION (AtIDA), controlling floral organ abscission. We discuss the possible implications of modifying the citrus IDA ortholog for citrus fruit production.

Keywords: IDA, HAESA, receptor-like kinases, *Citrus*, floral abscission, fruit production, crop yield

INTRODUCTION

Small peptides are used as important signaling ligands to control plant growth and development and more than 1000 genes encoding putative peptides have been discovered in *Arabidopsis thaliana* (*Arabidopsis*; Lease and Walker, 2006; Murphy et al., 2012; Grienberger and Fletcher, 2015). One such peptide ligand, INFLORESCENCE DEFICIENT IN ABSCISSION (AtIDA), was found to be important for the regulation of floral organ abscission in *Arabidopsis* as the *ida* mutant failed to abscise its floral organs (Butenko et al., 2003). In wild type (wt) plants the abscission process takes place at the boundary between the organ to be shed and the main plant body, in cell files constituting the abscission zone (AZ). After the formation of an AZ, which occurs early and simultaneously with the development of lateral organs from the apical meristem, the AZ cells can be distinguished from their neighbors by being small, densely cytoplasmic and lacking vacuoles (Addicott, 1982; Sexton and Roberts, 1982; Osborne, 1989; Roberts et al., 2000; Liljegren, 2012). Once the abscission process is initiated these cells go through three sequential developmental stages; (i) cell fate determination and acquisition of competence to respond to abscission signals; (ii) cell wall loosening and expansion by cell wall remodeling (CWR) enzymes followed by organ separation; and (iii) differentiation of a protective lignified layer (Patterson, 2001; Aalen et al., 2013; Gubert et al., 2014).

In the *ida* mutant, the cell separation event fails to take place (Butenko et al., 2003). In accordance with this, an IDA overexpression line (35S:AtIDA) exhibited ectopic abscission and displayed an enlarged floral AZ in *Arabidopsis* (Stenvik et al., 2006). Additionally, organ loss was observed at the bases of the pedicel, branches of the inflorescence, and cauline leaves; places where

abscission normally does not occur in *Arabidopsis*. AtIDA signaling and the 35S:AtIDA phenotypes are dependent on the two leucine-rich repeat (LRR) receptor-like kinases (RLKs) HAESA (HAE) and HAESA-LIKE 2 (HSL2; Jinn et al., 2000; Cho et al., 2008; Stenvik et al., 2008). Upon receptor activation a MAP kinase signaling event is turned on ultimately leading to the induction of genes encoding CWR enzymes (Cai and Lashbrook, 2008; Cho et al., 2008; Kumpf et al., 2013). Recently, it was shown that a hydroxypyrolylated AtIDA peptide of 12 amino acids was sufficient to mediate AtIDA signaling. Furthermore, this same peptide was shown to bind the HSL2 receptor with high affinity (Butenko et al., 2014).

The gene encoding the AtIDA peptide belongs to a family of five *AtIDA-LIKE* (*AtIDL*) genes in *Arabidopsis* that are expressed in a variety of tissues in the *Arabidopsis* plant body, including the base of the pedicel, in the floral tissue AZ, in the funicle AZ and in the main root tip (Stenvik et al., 2008). Interestingly, some of the regions of expression represent cell files where abscission takes place in other plants such as in *Citrus* (citrus) species (Lewis et al., 2006; Estornell et al., 2013), where, unlike for *Arabidopsis*, leaf abscission does take place. Flower and fruit abscission in citrus occurs at the pedicel AZ (called AZ-A), located close to the boundary between the pedicel and the twig, and at the ovary/fruit AZ (called AZ-C), located in the calyx between the pericarp and the nectary or floral disk, respectively (Tadeo et al., 2008). Cultivated citrus trees usually display heavy flowering. However, a high number of flowers and young fruits are shed during the fruit set period (physiological drop) thus maintaining only those fruits that can be nurtured until maturity. The attachment force by which a fruit is held to the calyx shows a large reduction in value at the end of the maturation phase of fruit development in almost all citrus species. However, early and mid-season varieties of sweet orange are especially prone to a premature decline in the attachment force of the fruit leading to pre-harvest fruit abscission. This problem has a serious economic impact especially in those citrus producing areas dedicated to the fresh fruit market as the Mediterranean Basin. The premature decline in the attachment force of the fruit prevents on-tree storage of fruit which shortens the harvesting season and hinders the orderly fruit marketing. Therefore, understanding the mechanisms controlling fruit abscission in citrus, with the prospect of genetic engineering (transgenic or CRISPR gene editing technologies), is of importance for two reasons; one for controlling fruit loss during the physiological drop and pre-harvest abscission, and two to facilitate shedding of unmarketable fruits remaining on the tree once the harvesting season is over.

INFLORESCENCE DEFICIENT IN ABSCISSION and the IDL peptides are evolutionary conserved across the plant kingdom and they exhibit sequence similarity in their conserved C-terminal domain (PIP domain) containing the highly active AtIDA peptide (Butenko et al., 2003; Stenvik et al., 2008; Butenko et al., 2014). Furthermore, the HSL receptors are found across the plant kingdom and in dicots the HSL receptor

sequences are conserved (Stø et al., 2015). It has previously been proposed that the presence of *IDL* and *HSL* transcripts at sites where cell separation occur in *Arabidopsis* may indicate that these genes play a more general role in cell separation (Butenko et al., 2009). Here, we explore on the possibility that this may not only be the case in *Arabidopsis* but also in citrus.

We show that the C-terminal domain of AtIDA is highly conserved in the IDA and IDL citrus orthologs. We also provide evidence that the citrus IDA (CitIDA) most similar to AtIDA, CitIDA3, has a function in abscission. We discuss the potential agriculture implications of modifying CitIDA3 and additional downstream signaling components of the IDA pathway identified in *Arabidopsis*.

MATERIALS AND METHODS

Plant Material and Generation of Transgenic Lines

The *CitIDA3* CDS was amplified from clementine cDNA, obtained from total RNA isolated from post-anthesis floral buds, by amplification of a 270 bp fragment with primers: 5' CACCATGGCTTCTTCTTCTTC 3', 5' TCAATTGAGT AGAATCCACAACAGA 3'. In parallel, screening of a clementine BAC collection (Terol et al., 2008) was carried out, and as a result BAC clones CCL005I18 and CCH3006E10 were identified to contain the genomic region of *CitIDA3*. A 3 kb *CitIDA3* promoter fragment was amplified from BAC clone CCL005I18 using 5' CACCGAATTGTAAATTAACTTGTCTTCTT 3' and 5' ATAAA TTGTTGTTGGGTTGGC 3' and cloned into pKGWFS7.0 (Plant Systems Biology, Ghent, Belgium). 35S:*CitIDA3* lines were made by cloning the CDS fragments into pK2GW7.0 (Plant Systems Biology, Ghent, Belgium). Sixteen independent lines were obtained and two independent lines were investigated in detail. The *Agrobacterium tumefaciens* C58 strain was used for *Arabidopsis* transformation by flower dipping (Clough and Bent, 1998).

Plant Material for Complementation of the *ida* Mutation

Two independent 35S:*CitIDA3* lines were crossed into the *ida-2* mutant background (Cho et al., 2008). The F₂ progeny of both lines were phenotypically scored based on their ability to abscise and genotyped by the *ida-2* genotyping primers 5' CGGTGTTGGTGGATCCAAGTC 3' and 5' CCCTCATTTCCG CCACACTTA 3' and the T-DNA LBb1 primer 5' ATTTTGCG CGATTTCGGAAC 3'. The presence of the 35S:*CitIDA3* transgene was verified with primers: 5' CGCACAATCCCCTATCC TT 3', 5' TCAATTGAGTAGAACACAGA 3'.

Petal Breakstrength Measurements

The measurements were performed using a petal breakstrength meter, as previously described (Stenvik et al., 2008). The petal breakstrength was quantified as the force, in gram equivalents, required to remove a petal from a flower (Butenko et al., 2003).

Identification of IDL and HSL Citrus Orthologs

We employed two different strategies to identify IDL citrus orthologs. We first performed TBLASTN searches against the citrus (sweet orange and clementine) genome at the Join Genome Institute (JGI¹) using the amino acid sequences corresponding to the variable region and the EPIP motif of the *Arabidopsis* IDL proteins. Second, we used only the amino acid sequences of the EPIP motifs of the *Arabidopsis* IDL proteins as query to perform TBLASTN searches in the NCBI GenBank dbEST database for the expressed sequence tag (EST) set of citrus and *Poncirus trifoliata*. Selected EST sequences (query coverage > 85%; max identity > 55%) were downloaded from the GenBank database and assembled using the CAP3 program (Huang and Madan, 1999). We used the sequences of the unigenes to query against the citrus (sweet orange and clementine) genome at the JGI.¹ Signal peptide predictions of all putative citrus IDL proteins were carried out using the SignalP 4.0 server².

To identify the citrus HSL orthologs TBLASTN (six-frame translation) searches against the citrus (sweet orange and clementine) genome were performed at the JGI¹ using the amino acid sequences of the *Arabidopsis* HAE (AT4G28490), HSL1 (AT1G28440), and HSL2 (AT5G65710) proteins.

Protein Alignments and Phylogenetic Analysis

Multiple sequence alignments of the IDL and HSL proteins were performed using ClustalW tools offered by GenomeNet³ with default parameters and displayed with GENEDOC. Based on the aligned sequences of the citrus IDL and HSL proteins, Neighbor Joining trees were constructed using MEGA version 6.0 (Tamura et al., 2013) with a bootstrap of 1000 replicates.

RESULTS

Identification of the Citrus Members of the IDA-HAE/HSL2 Signaling Module

In the citrus (sweet orange and clementine) genome five *IDL* genes were identified. They were named *CitIDA1* to *CitIDA5* depending on the location of their sequences in the citrus chromosomes or scaffolds (Supplementary Table S1). The phylogenetic relationship between the six *Arabidopsis* and five citrus IDL proteins were investigated, showing *CitIDA3* to be the closest related to *AtIDA* (Figure 1A). This gave rise to the possibility that *CitIDA3* could have a function in regulating cell separation during organ abscission. Supporting this idea the complete nucleotide sequence of *CitIDA3* can be reconstructed from two ESTs derived from AZ libraries (Supplementary Table S3).

All of the citrus *IDL* genes showed uninterrupted open reading frames encoding translation products with a predicted

N-terminal signal peptide sequence followed by a variable region and a highly conserved C-terminal PIP domain (Stenvik et al., 2008; Butenko et al., 2014; Figure 1B). Within the twelve amino acid residues that make up the PIP domain, there were invariant amino acid residues (positions 3, 5, 7, 8, and 12) and others in which the substitutions were conservative (positions 1, 2, and 6). *CitIDA3* was the IDA-like protein with highest similarity to the PIP motif of *AtIDA* differing only in one amino acid (Figure 1B). Given these similarities we hypothesized that *CitIDA3* has a conserved function similar to that of *AtIDA*.

Our genome-wide analysis identified three HSL proteins in the citrus (sweet orange and clementine) genome highly similar to each of the *Arabidopsis* proteins (Figure 1A and Supplementary Table S2). Therefore, the IDA-HAE/HSL2 signaling module could also work in citrus.

Function and Expression of *CitIDA3* Phenocopies *AtIDA*

To investigate whether *CitIDA3* has a conserved function to *AtIDA* in regulating cell separation during organ abscission, *Arabidopsis* plants were transformed with a construct driving *CitIDA3* expression with the strong constitutive cauliflower mosaic virus 35S promoter. Fully developed 35S:*CitIDA3* plants showed reduced stature and shorter siliques in comparison to wild-type plants (Figures 2A,B). The 35S:*CitIDA3* transgenic plants abscised their floral organs at an earlier stage than wild-type plants. Petal breakstrength (pBS), the force needed to remove petals from a flower, decreases along the length of the inflorescence. Changes in progression of abscission are determined by pBS (Bleecker and Patterson, 1997). In wt pBS reaches zero at floral position 8, while for 35S:*CitIDA3* this occurs at position 4, comparable to that observed for 35S:*AtIDA* (Figure 2C; Stenvik et al., 2006). Furthermore, the premature floral organ abscission observed for 35S:*CitIDA* was associated with an increase in the size of the AZ similar to that observed for 35S:*AtIDA* (Figure 2D; Stenvik et al., 2006; Shi et al., 2011).

In an additional experiment performed to investigate the spatial and temporal activity of the *CitIDA3* promoter, we monitored the expression of a *ProCitIDA3:GUS* construct in *Arabidopsis* plants. In the flowers, *CitIDA3* was expressed in the style and in floral organ AZs (Supplementary Figure S1A). GUS expression was absent in flowers from positions 1–4 but detected in the AZ of flowers from positions 5–8 (Figure 2G). *CitIDA3* was also expressed in cotyledons, developing leaves and roots of 1-week-old transgenic *Arabidopsis* seedlings (Supplementary Figure S1B). The expression of *CitIDA3* in *Arabidopsis* flowers is similar to that of *AtIDA* (Butenko et al., 2003).

35SCitIDA3 Complements the Abscission Deficiency of the *ida* Mutant

In order to investigate to what extent *CitIDA3* could replace the function of *AtIDA*, 35S:*CitIDA3* plants were crossed into the *ida-2* mutant background. The F₂ progeny of two independent lines was genotyped in order to identify *ida-2* plants. 6 out of 29 and 5 out of 27 plants were homozygous for the *ida-2*

¹<http://phytozome.jgi.doe.gov/>

²<http://www.cbs.dtu.dk/services/SignalP/>

³<http://www.genome.jp/tools/clustalw/>

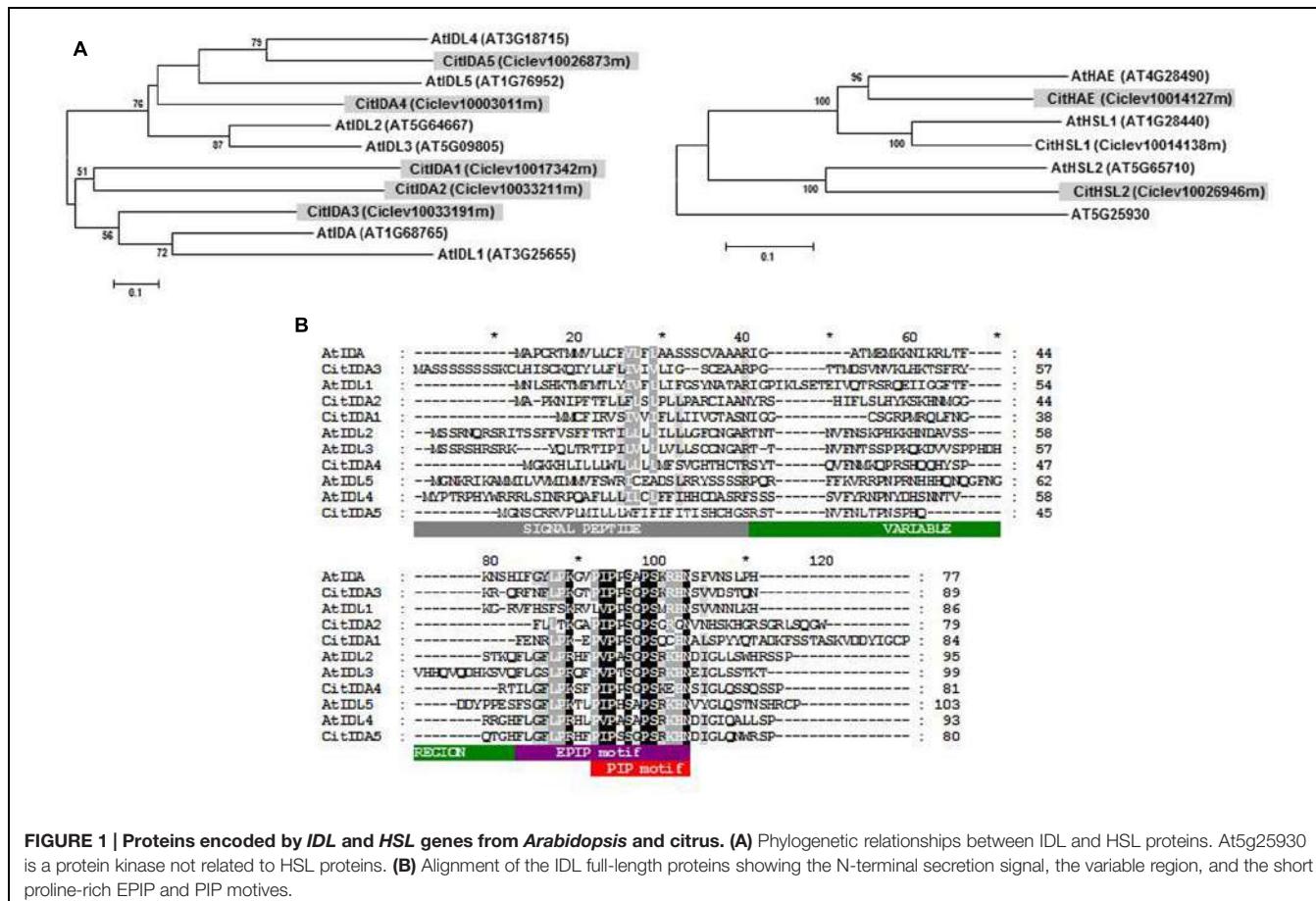


FIGURE 1 | Proteins encoded by IDL and HSL genes from *Arabidopsis* and *citrus*. **(A)** Phylogenetic relationships between IDL and HSL proteins. At5g25930 is a protein kinase not related to HSL proteins. **(B)** Alignment of the IDL full-length proteins showing the N-terminal secretion signal, the variable region, and the short proline-rich EPIP and PIP motives.

allele. Furthermore, the plants were scored for their abscission phenotype. Two and three of the *ida-2* plants from each cross, respectively, showed the *ida-2* phenotype, with attached petals after anthesis (**Figures 2E,F**). The remaining *ida-2* homozygotes showed wt abscission (**Figures 2E,F**). All of these plants contained the 35S:*CitIDA3* transgene indicating that the presence of 35S:*CitIDA3* is sufficient to induce abscission in *ida-2* mutant plants.

DISCUSSION

Pre-harvest abscission is a problem in citrus producing areas dedicated to the fresh fruit market, preventing on-tree storage of fruit and thereby shortening the harvesting and marketing season. On the other hand, the force required to remove mature fruits from the trees in citrus varieties dedicated to the processing market (juices and concentrates) is too large for effective mechanical harvesting. Therefore, new citrus genotypes with different abscission behavior or plants derived from genetic engineering (transgenic or CRISPR gene editing technologies) aiding in timing or execution of citrus fruit specific abscission would be favorable.

In *Arabidopsis*, the small peptide IDA forms a ligand-receptor module with the two RLKs HAE and HSL2 to initiate a

signaling pathway in floral organ abscission (Stenvik et al., 2008). Even though IDA was first found to regulate floral organ abscission, it has later been shown to play a role in cell separation during lateral root emergence, providing this peptide with a more general function in the control of cell separation (Kumpf et al., 2013). The discovery that IDA, HSL and additional downstream components of the IDA signaling system are found across the plant kingdom (Butenko and Simon, 2015; Stø et al., 2015) makes it plausible that orthologs in citrus have a conserved function. Indeed, several auxin response factor (ARF) genes conserved between citrus and *Arabidopsis* have been shown to have a role in fruit abscission in citrus and floral abscission in *Arabidopsis* (Ellis et al., 2005; Tadeo et al., 2015). Thus, different cell separation processes appear to share common signaling elements that are conserved across plant species.

The results shown here indicate that *CitIDA3* plays a role in floral abscission when overexpressed in *Arabidopsis*. The *Arabidopsis* 35S:*CitIDA3* transgenic plants were characterized by reduced plant and siliques growth and precocious floral organ abscission together with an increased size of the AZ (**Figures 2B–D**). Overexpression of *CitIDA3* resulted in a highly similar phenotype to that observed in *Arabidopsis* 35S:*AtIDA* plants (Stenvik et al., 2006). Furthermore expression of the *CitIDA3* transgene in an *ida-2* mutant background was

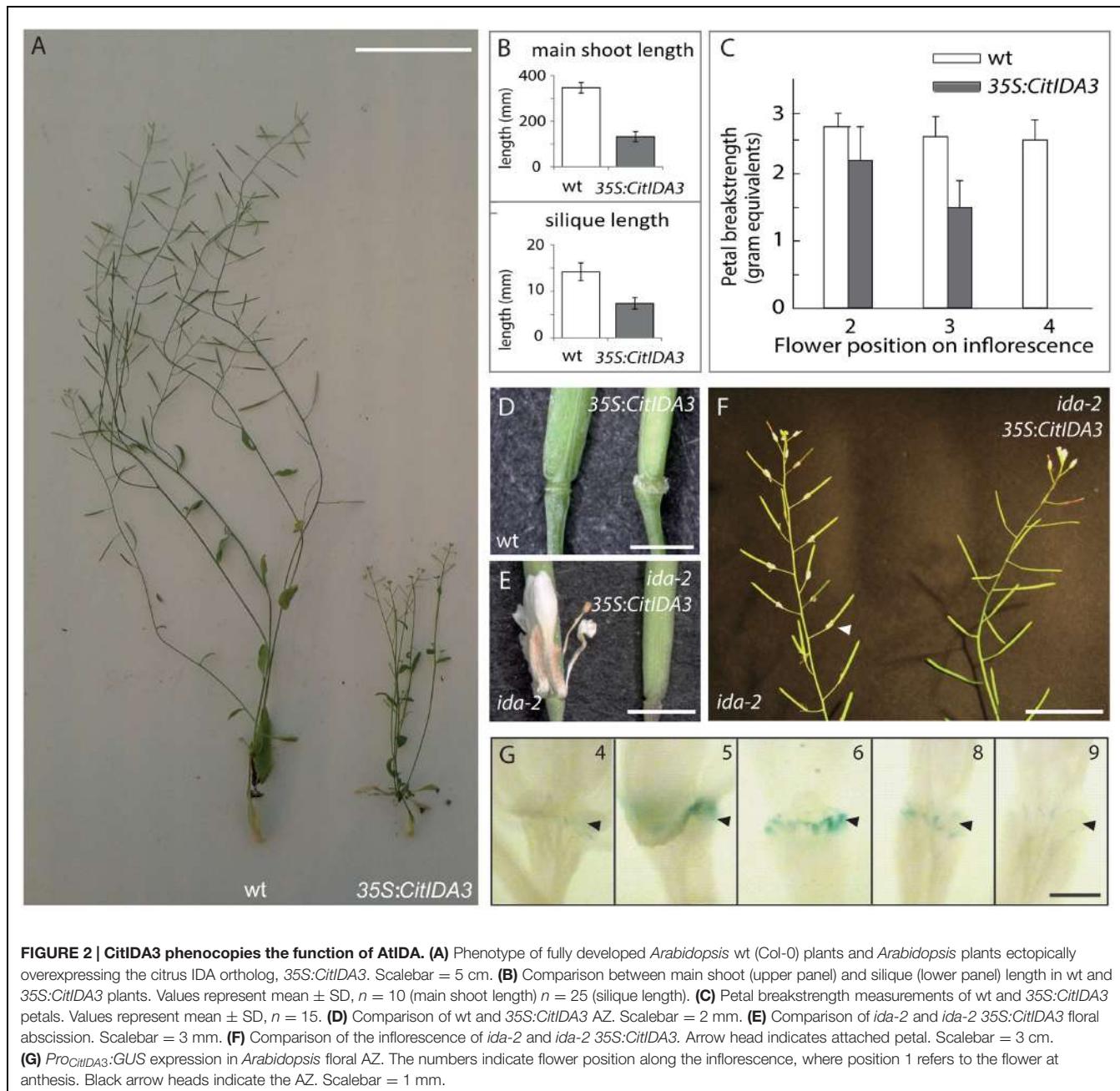


FIGURE 2 | **CitIDA3 phenocopies the function of AtIDA.** **(A)** Phenotype of fully developed *Arabidopsis* wt (Col-0) plants and *Arabidopsis* plants ectopically overexpressing the citrus IDA ortholog, 35S:CitIDA3. Scalebar = 5 cm. **(B)** Comparison between main shoot (upper panel) and siliques (lower panel) length in wt and 35S:CitIDA3 plants. Values represent mean \pm SD, $n = 10$ (main shoot length) $n = 25$ (silique length). **(C)** Petal breakstrength measurements of wt and 35S:CitIDA3 plants. Values represent mean \pm SD, $n = 15$. **(D)** Comparison of wt and 35S:CitIDA3 AZ. Scalebar = 2 mm. **(E)** Comparison of ida-2 and ida-2 35S:CitIDA3 floral abscission. Scalebar = 3 mm. **(F)** Comparison of the inflorescence of ida-2 and ida-2 35S:CitIDA3. Arrow head indicates attached petal. Scalebar = 3 cm. **(G)** *ProCitIDA3:GUS* expression in *Arabidopsis* floral AZ. The numbers indicate flower position along the inflorescence, where position 1 refers to the flower at anthesis. Black arrow heads indicate the AZ. Scalebar = 1 mm.

sufficient to rescue the abscission defect of the mutant and the *ProCitIDA3:GUS* expression pattern in *Arabidopsis* plants is also consistent with a role of this gene in organ abscission (Figures 2D–G). Additionally, we identified two LRR-RLKs highly homologous in sequence to the *Arabidopsis* HAE and HSL2 in the citrus (sweet orange and clementine) genome (Figure 1A and Supplementary Table S2). All these results strongly suggest that the IDA-HAE/HSL2 abscission-signaling pathway characterized in *Arabidopsis* is conserved in citrus and therefore it could be feasible to manipulate *CitIDA3* in order to prevent or stimulate organ abscission in this fruit crop.

Downstream components of the IDA-HAE/HSL2 abscission-signaling pathway could also be potential candidates for manipulation in order to control abscission in citrus. There is genetic evidence for several KNOTTED LIKE HOMEOBOX (KNOX) transcription factors working downstream of the activated IDA-HAE/HSL2 pathway. These include *BREVIPEDICELLUS* (*BP*)/*KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1* (*KNAT1*), *KNAT2*, and *KNAT6* (Shi et al., 2011). There are two genes in the citrus (sweet orange and clementine) genome (LOC102615394/Ciclev10001508m and LOC102628261/Ciclev10001779m, respectively) highly homologous to *KNAT1* and *KNAT2/KNAT6* suggesting that the

known downstream components of the IDA-HAE/HSL2 abscission-signaling pathway also exist in citrus.

The MADS-domain transcription factor AGAMOUS-like 15 (AGL15) that previously was reported to play a role in regulating floral abscission in *Arabidopsis* has recently been shown to act downstream of the IDA-HAE/HSL2 and MAP kinase signaling module to regulate *HAE* expression (Fernandez et al., 2000; Patharkar and Walker, 2015). Phosphorylation of AGL15 relieves repression of *HAE* expression leading to production of *HAE* transcript in a positive feedback loop, thereby increasing the expression of the receptor (Patharkar and Walker, 2015). Manipulation of a citrus version of AGL15 (LOC102612882/Ciclev10032519m) could be a target to control abscission as could the CWR enzymes induced by IDA. Several members of different classes of CWR enzymes have been found in *Arabidopsis*, tomato and citrus (Estornell et al., 2013). By investigating the enzymes that are expressed in the citrus AZs, fruit abscission prevention or stimulation could be further modified.

Considering all the above, we can glimpse the possibilities available to manipulate the expression of *CitIDA3* and/or the citrus orthologs of the downstream components of the IDA-HAE/HSL2 abscission-signaling pathway as an agronomic tool that would affect positively on the economic benefit of growers and the citrus industry in general.

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AUTHOR CONTRIBUTIONS

LE, MW, MP-A, MT, FT, and MB designed the research. LE, MW, MP-A, MT, FT, and MB performed the research. MW, FT, and MB analyzed the data. MW, FT, and MB wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01003>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Understanding the Physiology of Postharvest Needle Abscission in Balsam Fir

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Balsam fir (*Abies balsamea*) trees are commonly used as a specialty horticultural species for Christmas trees and associated greenery in eastern Canada and United States. Postharvest needle abscission has always been a problem, but is becoming an even bigger challenge in recent years presumably due to increased autumn temperatures and earlier harvesting practices. An increased understanding of postharvest abscission physiology in balsam fir may benefit the Christmas tree industry while simultaneously advancing our knowledge in senescence and abscission of conifers in general. Our paper describes the dynamics of needle abscission in balsam fir while identifying key factors that modify abscission patterns. Concepts such as genotypic abscission resistance, nutrition, environmental factors, and postharvest changes in water conductance and hormone evolution are discussed as they relate to our understanding of the balsam fir abscission physiology. Our paper ultimately proposes a pathway for needle abscission via ethylene and also suggests other potential alternative pathways based on our current understanding.

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INTRODUCTION

Balsam fir (*Abies balsamea*) trees are conifers native to northeastern North America. Stands are centralized in southeastern Canada and northeastern United States, but there are isolated populations existing as far west as Wisconsin in the United States and Alberta in Canada (Bakuzis and Hansen, 1965). Balsam fir are harvested for multiple uses, such as pulp or medicine, but it is their use as a specialty horticultural species for Christmas trees that is of most interest from a postharvest quality perspective. Balsam fir is the preferred Christmas tree species in Canada, grown on approximately 130,000 acres and worth \$56 million per year (Statistics Canada, 2014). Almost 50% of all balsam fir grown in Canada are exported to other countries, such as the United States, Bermuda, Thailand, and Japan (Statistics Canada, 2014). Balsam fir is also one of the most popular species grown in the United States. A high percentage of American grown trees are also exported to a similar collection of countries as Canadian grown trees (Chastagner and Benson, 2000). Balsam fir are enjoyed due to their unique fragrance, color, and architecture.

Postharvest needle abscission is one of several challenges facing balsam fir and other conifers used as Christmas trees (Chastagner, 1986; MacDonald et al., 2010), although balsam fir is typically considered to have appreciable needle retention compared to other fir species. Chastagner and Riley (2007) had balsam fir ranked as one of the top needle retaining species, with only Noble fir and Korean fir having significantly better needle retention than balsam fir in a 3-year study.

Regardless, early harvest practices combined with warmer autumn temperatures contribute to accelerated postharvest abscission. It's been noted repeatedly that trees harvested in September or October tend to have lower needle retention than trees harvested in November or December (Mitcham-Butler et al., 1987; Chastagner and Riley, 2007; MacDonald et al., 2014b). MacDonald and Lada (2008) estimated that 1 in 3 trees shed all needles in less than 3 weeks in normal shipping/storage conditions. Such high postharvest losses emphasize the importance of understanding the abscission process.

The actual harvest itself, or root detachment, must be considered the initial stimulus for postharvest abscission. If a balsam fir tree was not cut down, then it would be unlikely to shed significant amounts of needles unless exposed to other stresses (e.g., pathogens, pests, drought). Beyond root detachment, the complete mechanisms and pathways that eventually culminate in abscission remain unknown. It has often been suggested that dehydration represents a major step toward abscission, as several studies have examined the link between critical moisture thresholds and needle loss (Hinesley and Snelling, 1997; Chastagner and Riley, 2003). But significant needle loss has also occurred even when antitranspirants were used (Chastagner and Riley, 1991; Duck et al., 2003) or when there was no discernable decrease in moisture status (MacDonald et al., 2012a). This has contributed to alternative or complimentary theories relating to the role of needle nutrition, volatile terpene compounds (VTCs), ethylene, or the lack of unknown root-derived factors no longer available after root-detachment that triggers or modulates postharvest needle abscission.

Postharvest needle abscission is a complex process, influenced by a number of external, internal, and management factors. The objective of this review is to provide insight into our current state of knowledge in postharvest abscission of balsam fir. We will discuss the dynamics of needle abscission in balsam fir while identifying key factors that modify abscission patterns, such as genotypic abscission resistance, nutrition, environmental factors, and postharvest changes in water conductance and hormone evolution. Ultimately we propose a model for postharvest needle abscission based on the information available and discuss key areas where research is still needed.

DYNAMICS OF NEEDLE ABSCISSION IN BALSAM FIR

The progress of postharvest needle abscission in balsam fir usually follows a logistic curve, as shown in **Figure 1** (MacDonald et al., 2010, 2011b; MacDonald and Lada, 2014). After harvest and display in water, typically no abscission occurs for between one and 2 weeks. Needle abscission commencement is usually noted by a loss of 1% dry needle weight and gradually accelerates until 100% needle loss, which has been referred to as needle retention duration or, more recently, needle abscission resistance (NAR; MacDonald et al., 2014a,b). The length of time between needle abscission commencement and completion has varied between 1 week (MacDonald et al., 2011b) and 4 weeks (MacDonald et al., 2014b), though 2 weeks is a reasonable estimate in most cases.

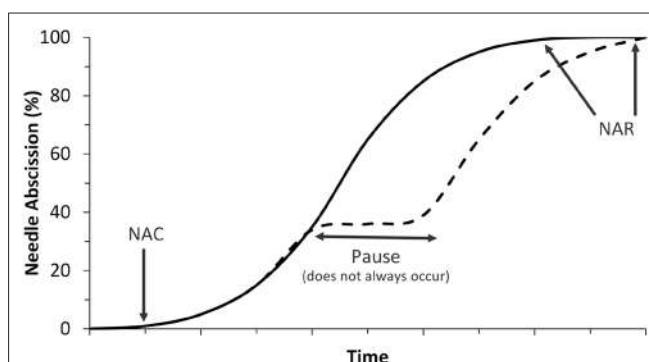


FIGURE 1 | Two most common postharvest needle abscission curves occurring in balsam fir. The solid line denotes the typical logistic pattern of abscission while the dashed line denotes the less common pause in abscission process. Time is given no units in this diagram because although the relative curves are consistent, the timing of events is highly variable. Also marked are indication of needle abscission commencement (1% needle loss) and needle abscission resistance (100% needle loss).

There are instances where abscission seems to pause for a length of time before resuming. A generic version of an alternative abscission curve including a pause in abscission is shown in **Figure 1** near 40% needle loss, but pauses in abscission have been observed at almost any portion of an abscission curve. For example, MacDonald et al. (2014b) observed a 2-week pause in abscission after 80% of needles had been shed before abscission resumed and eventually reached 100% needle abscission. In rare instances abscission will simply not occur or abscission will stop before reaching complete abscission and never resume (Heikkenen et al., 1986). Abscission is an energy-dependent process and if trees dehydrate too quickly then abscission cannot occur. Needles may still crumble or break off due to brittleness, but they do not truly abscise (Hinesley and Snelling, 1995).

The length of time to complete abscission has been shown to vary considerably in balsam fir, which has complicated the study of this phenomenon. In a study designed to evaluate 195 different balsam fir genotypes for NAR, it was found that some genotypes would complete abscission in as little as 6 days while others could take as long as 60 days (MacDonald and Lada, 2008). Similar results were found in a 3-year study of balsam fir, which found average NAR values between 12 and 60 days (MacDonald et al., 2014b). The variation in NAR in balsam fir led to the development of a genotype classification system where genotypes were labeled as having low NAR if they completed abscission in under 20 days, moderate NAR if they completed abscission between 20 and 40 days, and high NAR if they completed abscission in over 60 days (MacDonald, 2010). Though categorized based on needle retention, there are also certain physical differences between NAR genotypes. For instance, low NAR genotypes tend to have thicker branches, more densely distributed needles, and require more force to remove needles from fresh trees than their high NAR counterparts (MacDonald et al., 2014a). There are some physiological differences between NAR genotypes as well, which are discussed later in this article. The identification of NAR genotypes has been important as it is a major factor influencing postharvest abscission curves in

balsam fir. However, the identification of a number of external and internal factors influencing postharvest abscission has been equally important.

ENVIRONMENTAL FACTORS LINKED WITH POSTHARVEST ABSCISSION

The premier environmental factor to have an influence on postharvest needle abscission is cold acclimation. Cold acclimation is an adaptive process where plants acquire the ability to withstand cold temperatures through exposure to mild temperatures occurring during autumn (Gusta et al., 2005). A host of physiological changes are invoked in conifers by cold acclimation, including changes to lipid structure and composition (Senser, 1982), antioxidant molecules and enzymes (Tao et al., 1998), carbohydrates (Mitcham-Butler et al., 1987; MacDonald et al., 2014b), and abscisic acid (ABA; Thiagarajan et al., 2012, 2013). Balsam fir have been evaluated for several of these changes typically indicative of cold acclimation and were found to accumulate ABA, raffinose, and galactose in autumn months suggesting that balsam fir do undergo cold acclimation (Thiagarajan et al., 2013; MacDonald et al., 2014b). Further, needle retention also improved when subjected to a period of low temperature artificially prior to harvest (Thiagarajan et al., 2012).

Though the exact mechanism is unknown, cold acclimation generally increases postharvest needle retention in fir trees. Ten different fir species, including balsam fir, were evaluated for needle retention over 3 years and all 10 species had significantly improved postharvest needle retention in November, December, or January compared to October (Chastagner and Riley, 2007). Balsam fir had superior needle retention when harvested in November or December compared to any other month (MacDonald et al., 2014b). However, the effect of cold acclimation was not uniform on all genotypes. It was first noted by MacDonald and Lada (2008) that genotypes with poor needle retention in October tended to improve much more due to cold acclimation than genotypes with good needle retention. A comparison of needle retention in October and January harvested genotypes classified as low, moderate, and high NAR revealed that needle retention in low NAR genotypes improved by 10–15 days while there was never an improvement in high NAR genotypes (MacDonald et al., 2014b). It was speculated that high NAR genotypes may respond to environmental cues earlier than low NAR genotypes, thus high NAR genotypes may have already been acclimated in October or cold acclimation is not required for high NAR genotypes.

Photoperiod triggers a degree of cold acclimation often through a mechanism independent from temperature (Smit-Spinks et al., 1985; Welling et al., 2002). Thiagarajan et al. (2013) found that there was a significant negative relationship between photoperiod and postharvest needle retention in balsam fir in two different genotypes. MacDonald et al. (2014b) did not include a discussion of photoperiod in their study, but an examination of needle retention each month suggests that there would at least be a relationship with the reported average monthly needle retentions and photoperiod. Such a relationship was described

in a subsequent study where photoperiod was shown to have more influence than temperature in modifying needle retention (MacDonald and Lada, 2015). The above studies all found that a low photoperiod improves postharvest needle retention, which also corresponds with the superior needle retention observed in November or December harvested trees (MacDonald et al., 2014b).

Adjustments to postharvest environmental conditions, specifically lighting and vapor pressure deficit, also affected needle retention. Balsam fir branches stored in darkness or provided fluorescent lighting ($253.7 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$) had a NAR of 42.3 days and 38.0 days, respectively. However, exposure to white, red, or blue light emitting diodes significantly improved needle retention by over 50% (Veitch et al., 2012). Modifying vapor pressure deficit had an even greater impact on needle retention. When balsam fir branches were stored at vapor pressure deficits greater than 0.9 kPa, then NAR was 32 days or less. When branches were stored at vapor pressure deficits less than 0.9 kPa, then NAR was significantly increased. For example, at 0.23 kPa NAR had increased to 150 days (MacDonald et al., 2012a).

PREHARVEST NUTRITION LINKED WITH POSTHARVEST ABSCISSION

Preharvest nutrition has been a relatively minor area of study, though has shown a definitive impact on postharvest needle abscission in balsam fir. There is little standardization on fertilizer application rates, with only some emphasis placed on nitrogen (Georges, 2013). Most nutrients had no link to postharvest needle abscission. However, branches with higher foliar nitrogen, potassium, copper, and iron all had accelerated postharvest needle abscission (Georges, 2013). Nutrients were all present at concentrations lower than necessary to induce toxicity, thus the mode of action to explain a negative relationship with needle abscission remains unknown. Elevated ethylene evolution rate is one potential explanation, as iron and copper have each been linked to ethylene in other species (Mapson and Wardale, 1968; Ben-Yehosua and Biggs, 1970). External application of several nutrients also had minimal effect on postharvest needle abscission, though zinc significantly accelerated abscission (Georges, 2013).

One potential limitation in the work conducted by Georges (2013) was that experiments were often done with only one or two specific genotypes, in an effort to control experimental error. However, this also provided a more narrow range of foliar nutrient concentrations. The work from Georges (2013) was repeated in a natural tree stand, as opposed to clonal orchard. Several similar relationships were found, but higher concentrations of soil and foliar calcium were associated with delayed postharvest abscission (MacDonald and Lada, 2015). It is speculated that higher endogenous concentrations of foliar calcium delayed abscission by improving stability of cell walls and impeding cell wall degradation in abscission zones (Xu et al., 2009). The role of preharvest nutrient in needle abscission could be further explored by focusing on cell wall changes in the abscission zone.

POSTHARVEST CHANGES TO WATER CONSUMPTION AND HYDRAULIC CONDUCTIVITY

Water deficit and dehydration is an immediate postharvest concern in balsam fir. A freshly cut balsam fir tree consumes approximately $0.15\text{--}0.20 \text{ mL}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$ and has a stomatal conductance of $20\text{--}25 \text{ mmol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, but both rapidly decrease after harvest (MacDonald and Lada, 2014; Lada et al., 2015a; MacInnes, 2015). The first response is a decrease in stomatal conductance, which decreases by 50% within 4 days of harvest and decreases by 80% within the first week (MacInnes, 2015). The change in water consumption is slightly slower than the change in stomatal conductance, but within 2 weeks it is not uncommon for water consumption to decrease to $0.05 \text{ mL}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$ (MacDonald and Lada, 2014; Lada et al., 2015a). Postharvest needle abscission in balsam fir typically begins when water consumption falls below $0.05 \text{ mL}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$, which suggests any improvement to water status or water uptake may delay abscission (Lada et al., 2015a; MacDonald and Lada, 2015). The precise cause of decreased water consumption is not yet known, though cavitation, embolism, stomatal dysfunction, bacteria contamination, or blockage of xylem vessels have all been speculated.

Postharvest water status has been studied extensively in many root-detached conifers (Bates et al., 2004). Water status is conventionally assessed by percent moisture content, relative water content, or xylem pressure potential (XPP) of the branches. XPP is likely the most often used and is an indicator of tension at which water is held in the xylem conduits. Threshold values have been established for several conifers, which represent the XPP from which a tree is unable to recover and results in accelerated needle loss, discoloration, and a tendency to defoliate even under rehydration (Hinesley and Snelling, 1991). For example, the range in which trees could successfully rehydrate was -4.0 to -5.0 MPa in *Leyland cypress* (Hinesley and Snelling, 1995), -4.0 to -4.5 MPa in Fraser fir (Hinesley, 1984), and approximately -3.0 MPa in nordmann fir (Chastagner and Riley, 2003). Rehydration in balsam fir has not been studied as extensively as some other species, but the threshold in which rehydration may successfully occur is approximately 45% moisture content (Adams et al., 2013). As noted in earlier sections of the review, balsam fir inherently has a high degree of variation in its postharvest characteristics. The moisture content from which balsam fir could successfully rehydrate was also highly variable, though it was linked with NAR. Balsam fir genotypes characterized as high NAR could successfully rehydrate from a water content as low as 38% while balsam fir genotypes categorized as low could not rehydrate from moisture contents below 47% (Adams et al., 2013).

Postharvest needle abscission has occurred in several studies when XPP has been maintained above -1.0 MPa , which is not indicative of water stress (MacDonald et al., 2012a,b; MacDonald and Lada, 2014). However, though the final XPP values during abscission were not exceptionally low in those studies, they were significantly lower than fresh XPP values. Further, there were other studies where XPP fell as low as -6.0 MPa , which would indicate water stress (Lada et al., 2015a). XPP has not

had a strong relationship with needle abscission in some fir species (Bates et al., 2004), but there have been significant relationships with needle abscission in balsam fir (MacDonald et al., 2012a,b). Other evaluators, such as relative water content or percent moisture, all consistently decrease after harvest leading to abscission (MacDonald et al., 2012a; MacDonald and Lada, 2014) and there was a strong relationship between moisture content and postharvest needle abscission (MacDonald and Lada, 2015). Overall, there was consistently a decrease in water status in postharvest balsam fir that was highly linked to abscission.

Efforts to mitigate decrease in water status have a significant positive effect in limiting balsam fir needle abscission. Lada et al. (2015a) identified decreasing water quality in Christmas tree stands as having an adverse effect on needle retention, possibly due to an exponential increase in bacterial counts. When water was routinely drained and replaced with fresh water, then NAR was increased by 38%. Conversely, when water that was previously drained from a Christmas tree stand was provided to a freshly cut tree, then there was a 36% decrease in NAR (Lada et al., 2015a). An alternative method to maintain water status was to store branches in a low vapor pressure deficit environment, which effectively maintained XPP and relative water content at fresh harvest values. Storage at low vapor pressure deficit increased NAR fivefold (MacDonald et al., 2012a). Finally, a study was conducted that mounted balsam fir branches on a simulated root pressure system that could maintain water flow by generating positive pressure. Low levels of positive pressure were sufficient to delay abscission (MacInnes, 2015).

It is important to note that although a decrease in water status is a major factor that accelerates needle loss, hydration alone cannot retain needles indefinitely. Postharvest needle abscission still ultimately occurred in situations where water status was maintained through changes to water delivery, modifying vapor pressure deficit, or applying antitranspirants (Duck et al., 2003; MacDonald et al., 2012a; MacInnes, 2015). There must be a physiological signal that triggers abscission due to water stress, but also a signal that triggers abscission even if there is no water stress. The signal could be the same in both instances or could be triggered through different pathways. Ethylene triggers abscission in many species (Brown, 1997) and is a candidate for inducing postharvest abscission in balsam fir through one of the aforementioned pathways.

ETHYLENE AS A KEY SIGNAL FOR POSTHARVEST NEEDLE ABSCISSION

Ethylene, the simplest unsaturated hydrocarbon, is a plant hormone often produced in response to stress in many species, including conifers. For example, ethylene evolution was significantly increased in jack and white pines due to drought (Rajasekaran and Blake, 1999; Islam et al., 2003), in silver fir due to biotic stresses (Fuhrer, 1985), and Norway spruce due to ozone and drought stress (Van den Driessche and Langebartels, 1994). Though ethylene is involved in a host of physiological processes, ethylene evolution due to stress is often associated with senescence and abscission as a defense response (Brown, 1997).



FIGURE 2 | Comparison of a balsam fir branch after prolonged exposure to 1000 ppm ethylene (left) to a branch stored in the absence of ethylene (right) after 14 days (MacDonald et al., 2011a).

Ethylene evolution began slowly after harvest, but reached a peak several weeks after harvest in several conifers (Alvarez-Moctezuma et al., 2007). The pattern of ethylene evolution was very similar in balsam fir, with almost no detectable ethylene in the few days and then reaching a peak after several weeks (MacDonald et al., 2010). Due to evidence suggesting that ethylene in conifers is triggered via water stress (Rajasekaran and Blake, 1999), it is quite possible that deteriorating water status of postharvest balsam fir causes ethylene evolution in balsam fir. Interestingly, the progression of ethylene evolution increased in parallel with needle abscission, ultimately reaching its peak 1–3 days before complete abscission (MacDonald et al., 2010). Korankye (2013) also noted increased ethylene evolution along with other volatiles that were associated with abscission. Ethylene evolution has also been associated with genotypic differences in balsam fir needle retention; low NAR genotypes released significantly higher concentrations of ethylene and had accelerated abscission due to lower concentrations of exogenous ethylene (MacDonald et al., 2012b). Thus, endogenous ethylene increased in balsam fir and was, at the very least, associated with postharvest abscission.

There is strong evidence that ethylene is not only associated with abscission, but actually induces abscission in balsam fir (**Figure 2**). Prolonged exposure to exogenous ethylene has consistently decreased needle retention by 60–70% (MacDonald et al., 2010, 2011a,b). Exogenous ethylene also accelerated abscission uniformly regardless of NAR genotype (MacDonald et al., 2012b) and regardless of storage temperature or humidity (MacDonald et al., 2012a). Further, ethylene inhibition with aminoethoxyvinylglycine and 1-methylcyclopropene delayed abscission. Aminoethoxyvinylglycine inhibits ethylene synthesis by blocking the conversion of S-adenosyl-L-methionine to 1-aminocyclopropane-1-carboxylic acid (Boller et al., 1979). Xylem feeding aminoethoxyvinylglycine to balsam fir delayed abscission by up to 113%, though abscission was still accelerated 60–70% in the presence of exogenous ethylene (MacDonald et al.,

2010). Conversely, 1-methylcyclopropene blocks the effect of ethylene by competitively binding to ethylene receptors (Sisler and Serek, 1997). Foliar application of 1-methylcyclopropene to balsam fir delayed abscission by 73% in the absence of exogenous ethylene and delayed abscission by 118% in the presence of exogenous ethylene compared to respective controls (MacDonald et al., 2010). The only exception to exogenous ethylene inducing abscission in balsam fir was found in a study exploring the effect of acute ethylene exposure. Acute ethylene exposure actually helped to delay abscission, though the mechanism for this has not been explored (MacDonald et al., 2011b).

Ethylene is believed to facilitate abscission by promoting the production of cell wall hydrolytic enzymes, such as cellulase, in the abscission zone (Tucker et al., 1988). This mode of action may also occur in balsam fir, as there was an eightfold increase in cellulase activity due to endogenous ethylene exposure and a 12-fold increase in cellulase activity due to exogenous ethylene exposure (MacDonald et al., 2011a). It remains unknown whether ethylene may also stimulate production of other hydrolytic enzymes in balsam fir which may contribute to abscission.

The role of ethylene in postharvest abscission has practical implications as preharvest handling by industry includes shaking and baling of Christmas trees, which may act as a form of mechanical stress. One concern is that mechanical stress is associated with ethylene synthesis and abscission in many species (Mitchell and Myers, 1995), thus it is conceivable that normal Christmas tree handling processes contribute to postharvest abscission in balsam fir. There is a lack of information available in this area, but it has been shown that baling is linked to accelerated abscission patterns (Korankye et al., 2015). Non-baled balsam fir trees lost the least percentage of needles postharvest compared to any baled treatment, with some links to volatile terpene and ethylene evolution (Korankye et al., 2015). There is ongoing research in the area of preharvest handling and its link to abscission.

INVOLVEMENT OF OTHER PLANT HORMONES IN BALSAM FIR ABSCISSION

In addition to ethylene, ABA is likely the most studied hormone with respect to postharvest abscission in balsam fir. The concentration of ABA in fresh balsam fir need tissue was 350 $\text{ng}\cdot\text{g}^{-1}$ to 500 $\text{ng}\cdot\text{g}^{-1}$ (Thiagarajan et al., 2012; MacDonald and Lada, 2014). However, during abscission there is a 39-fold increase in ABA concentrations and even higher increase in ABA metabolites (**Table 1**; MacDonald and Lada, 2014). The increase in ABA during abscission has raised the possibility that ABA may be a trigger for abscission. Zhang et al. (2009) found that application of exogenous ABA application induced ethylene evolution and abscission. Likewise, use of the ethylene inhibitor silver thiosulfate in conjunction with ABA negated any ABA-induced abscission (Suttle and Abrams, 1993). These results suggest ABA could be an intermediary messenger for ethylene in the balsam fir abscission pathway. However, ABA is also well known to facilitate stomatal closure in response to drought (McAdam et al., 2011), which means that the increase in balsam fir ABA during abscission could simply be an artifact of postharvest water deficit.

TABLE 1 | Summary of known changes in phytohormones in fresh balsam fir compared to actively abscising balsam fir. Ethylene data is from MacDonald et al. (2010) and remaining hormone data is from MacDonald and Lada (2014).

Hormone	Fresh concentration	Abscising concentration	Change
Ethylene ($\mu\text{L}\cdot\text{g}^{-1}\cdot\text{d}^{-1}$)	0.5	15.1	30-fold increase
ABA ($\text{ng}\cdot\text{g}^{-1}$)	342	13,263	39-fold increase
Cytokinins ($\text{ng}\cdot\text{g}^{-1}$)*	51	143	3-fold increase
Auxins ($\text{ng}\cdot\text{g}^{-1}$)	99	5	20-fold decrease

*cytokinins represent the total of trans-zeatin-O-glucoside, trans-zeatin riboside (dominant species), cis-zeatin riboside, dihydrozeatin riboside, isopentenyl adenosine, and isopentenyl adenine.

Abscisic acid may also be associated with needle retention due to ABA's involvement with cold acclimation. MacDonald et al. (2014b) observed a twofold increase in foliar ABA and Thiagarajan et al. (2012) observed a fourfold increase in foliar ABA due to cold acclimation. The relative increase in ABA due to cold acclimation is much lower than the postharvest increase in ABA, but it was speculated that the increased ABA may play a role in the improved needle retention after cold acclimation. There was some support to this theory when exogenously supplied ABA at very low concentrations resulted in improved needle retention in balsam fir, while higher concentrations of ABA promoted needle abscission (Thiagarajan, 2012).

Auxins also appear to play a role in postharvest abscission of balsam fir. The predominant auxin in balsam fir is indole-3-acetic acid (IAA), which was detected $99 \text{ ng}\cdot\text{g}^{-1}$ in freshly harvested balsam fir (Table 1; MacDonald and Lada, 2014). However, there was a 95% decrease in IAA after harvest (MacDonald and Lada, 2014). Of interest is the antagonistic relationship between ethylene and auxin; IAA is considered to inhibit abscission while ethylene is thought to promote abscission. The general rule portrays that provided the flux of IAA to the abscission zone is maintained, then cell separation is inhibited and abscission does not occur (Taylor and Whitelaw, 2001). The postharvest decrease in balsam fir IAA is likely linked to ethylene-induced abscission.

Cytokinins are conventionally believed to delay abscission. As plants begin to senesce, cytokinin concentrations in the leaves and sap show a marked decrease (Noodén et al., 1990; Singh et al., 1992) and exogenous application of cytokinins on senescing tissue has demonstrated the ability to delay or reverse the process in some species (Adedipe and Fletcher, 1971). Further, endogenous cytokinins in root and needle tissue have a strong, positive correlation with needle retention (MacDonald and Lada, 2015). Roots are believed to be the major source of cytokinin synthesis (Chen et al., 1985), thus it reasons that root-detachment via harvest would impair cytokinin synthesis and contribute to abscission. However, postharvest changes in cytokinins exhibited the opposite response as expected (Table 1). Six species of cytokinins were identified in balsam fir needles, with trans-zeatin riboside contributing to 50% of the overall cytokinin concentration, and all species of cytokinins increased during abscission (MacDonald and Lada, 2014). As noted above with respect to auxins, the changes in cytokinins were noted during abscission compared to initial fresh concentrations, which

means it would be beneficial to understand exactly when the concentration of cytokinins increased with respect to abscission.

VOLATILE TERPENE COMPOUNDS AND THEIR ROLE IN POSTHARVEST NEEDLE ABSCISSION

Ethylene, through a variety of approaches, is shown to be a key signal involved in postharvest abscission of balsam fir (MacDonald et al., 2010). However, abscission still ultimately occurs in situations where ethylene is inhibited (MacDonald et al., 2010, 2012a), which has suggested that an ethylene-independent pathway must exist for postharvest needle abscission.

Volatile terpene compounds constitute a large class of secondary compounds produced by conifers which contribute to their characteristic smell (Schmelz et al., 2003). VTCs are most commonly associated with plant defense (Croteau et al., 2000), but may be linked to ethylene evolution and abscission. A total of 12 VTCs have been detected in balsam fir, which all have a tendency to increase postharvest corresponding with needle abscission (Korankye, 2013). When ethylene was inhibited, needle retention was delayed but eventually occurred following an increase in volatile terpene concentrations (Korankye, 2013). The concentration of volatile terpenes released was also correlated with percentage needle loss in baled versus non-baled trees, despite no significant difference in ethylene evolution rates (Korankye et al., 2015). It is speculated that volatile terpenes could be part of an ethylene-independent abscission pathway, though more work is needed to test that theory.

POSTHARVEST CHANGES IN MEMBRANE STABILITY, CHLOROPHYLL, AND LIPID PROFILES

Membranes are often one of the first sites of drought damage in conifers (Rajasekaran and Blake, 1999) and, in fact, are often deteriorated in response to any form of abiotic stress (Yang and Hoffman, 1984). The exact mechanisms are not fully understood, but it is generally accepted that stresses trigger the generation of free radicals and reactive oxygen species which in turn trigger lipid peroxidation, increased permeability of cellular membranes, and senescence (Droillard et al., 1987; Hodges et al., 2004). Harvest procedures, postharvest storage, water loss, and ethylene all contribute to oxidative stress and membrane damage in many species (Hodges et al., 2004). Thus, it is logical to suggest the aforementioned postharvest factors could induce oxidative stress in balsam fir. Further, lipid peroxidation is thought to contribute to ethylene synthesis (Paulin et al., 1986; Pell et al., 1997), which suggests reactive oxygen species and changes to membrane integrity could be a key step in the needle abscission pathway.

Measurements of the changes in reactive oxygen species or endogenous antioxidants in postharvest balsam fir have not been performed. However, there have been some measurements to assess changes in membrane stability. The main method of evaluating membrane stability in balsam fir has been to calculate the membrane injury index, which essentially reports

a ratio of the amount of electrolytes leaking from collected needles to the total electrolytes present (Rajasekaran and Blake, 1999). Membrane injury index of balsam fir does not change in autumn when trees experience colder temperatures, which suggests a level of membrane protection due to cold acclimation that may or may not be linked to superior needle retention (Thiagarajan et al., 2013; MacDonald et al., 2014b). Immediately after harvest, membrane injury was relatively low and remained low for almost 3 weeks. However, during abscission there was a 50% increase in membrane injury in balsam fir (MacDonald and Lada, 2014). Similar results were found in a different study, where membrane injury was relatively low until a 134% increase leading to abscission (MacDonald et al., 2015). The delay in membrane injury between harvest and abscission may be due to little discernable water stress since relative water content and XPP also remained relatively high for the first 3 weeks postharvest (MacDonald and Lada, 2014). As of yet, it remains undetermined whether membrane injury is a cause or symptom of abscission.

Membrane injury is apparent in chloroplasts of balsam fir. The chlorophyll index of postharvest balsam fir begins relatively high, but gradually decreases until abscission (MacDonald and Lada, 2014). Chlorophyll fluorescence is one tool to assess photosynthetic activity in a plant, chloroplast stability, and general physiological status of a plant (Ball et al., 1995). Postharvest fluorescence decreases more slowly in conifers than many other species (Richardson and Berlyn, 2002; Fangyuan and Guy, 2004), but there is a distinct relationship between fluorescence and needle abscission in balsam fir. Similar to changes in chlorophyll index, fluorescence gradually decreases postharvest until abscission. However, there is a very strong negative relationship ($R^2 > 90\%$) between fluorescence and needle abscission, which suggests at the very least that fluorescence and chloroplast integrity are associated with postharvest needle abscission (MacDonald and Lada, 2015). Further, galactolipids found primarily in the chloroplast (i.e., monogalactosyldiacylglycerol and digalactosyldiacylglycerol) significantly decreased during abscission, which also suggests chloroplast membrane breakdown associated with abscission (MacDonald et al., 2015). The relationship between membrane damage and onset of ethylene evolution has yet to be explored in balsam fir, but membrane protection via antioxidant application has suppressed ethylene evolution and decreased membrane leakage in other conifers (Borsos-Matovina and Blake, 2001; Islam et al., 2003).

SUMMARY OF POSTHARVEST ABSCISSION IN BALSAM FIR AND FUTURE RESEARCH

Postharvest needle abscission in balsam fir is a complex physiological event involving many different factors. Cold acclimation (Thiagarajan et al., 2013; MacDonald et al., 2014b), genotypic variability (MacDonald, 2010), soil and needle nutrition (Georges, 2013), volatile terpenes (Korankye, 2013), water status (Lada et al., 2015a; MacInnes, 2015), light (Veitch et al., 2012), vapor pressure deficit (MacDonald et al., 2012a), ethylene (MacDonald et al., 2010), and other hormones (MacDonald and

Lada, 2014; Lada et al., 2015b) all influence postharvest abscission in some manner. The proposed pathway for postharvest needle abscission in balsam fir is shown in **Figure 3**. There are six points of discussion labeled and research needed to further develop the theory is discussed below:

1. Root detachment during harvest is the initial trigger that leads to a decrease in root pressure, stomatal conductance, water content, and XPP (MacInnes, 2015; Lada et al., 2015a). There is also a postharvest increase in ABA, which may induce stomatal closure in an effort to conserve water (Thiagarajan et al., 2012). Transpiration is the driving force for water uptake in a branch, thus it is logical to suggest that decreased stomatal conductance leads to decreased water uptake (MacInnes, 2015). It has been shown that deteriorating water quality or bacteria accumulation in water supply can exacerbate the situation (Lada et al., 2015a). Ultimately, it is generally agreed that an immediate consequence of root detachment is dehydration.
2. The major pathway to postharvest balsam fir abscission is via ethylene (MacDonald et al., 2010, 2011a,b, 2012a,b; Korankye, 2013). Ethylene then triggers cellulase, which most likely weakens cell walls in the abscission zone to facilitate abscission (MacDonald et al., 2011a). It is hypothesized that other hydrolytic enzymes (i.e., polygalacturonase) are involved in degrading cell walls to perpetuate abscission. Further, understanding the order of postharvest events, such as oxidation, membrane injury, and ethylene synthesis is a key area of research to further develop the proposed pathway. Do reactive oxygen species cause membrane damage and then induce ethylene? Or is membrane damage another symptom of increased ethylene evolution?
3. Auxins decrease by 95% in balsam fir leading to abscission (MacDonald and Lada, 2014) and application of exogenous auxins, such as naphthalene acetic acid, delayed abscission in balsam fir (Lada et al., 2015b). The main area of research needed in this area is a temporal investigation into changes in endogenous auxins after harvest, which may then be compared to changes in ethylene synthesis and needle abscission.
4. It is not yet known whether volatile terpenes are a cause or a result of postharvest abscission. Are they part of a general defense response induced by harvest, or do they function as a complimentary or alternative pathway to ethylene? VTCs increased after harvest (alongside ethylene) and are highest in high NAR genotypes (Korankye, 2013). Both endogenous and exogenous volatile terpenes were associated with abscission when ethylene was inhibited (Korankye, 2013). Whether volatile terpenes induce abscission requires further study, though it is hypothesized that jasmonic acid could be involved. The role of jasmonic acid in regulating volatile terpenes and/or ethylene pathways should be explored.
5. Cytokinins are typically believed to delay senescence and abscission, but cytokinins increased two- to fourfold during abscission of balsam fir (MacDonald and Lada, 2014). It was once speculated that cytokinins, or rather lack of root-derived

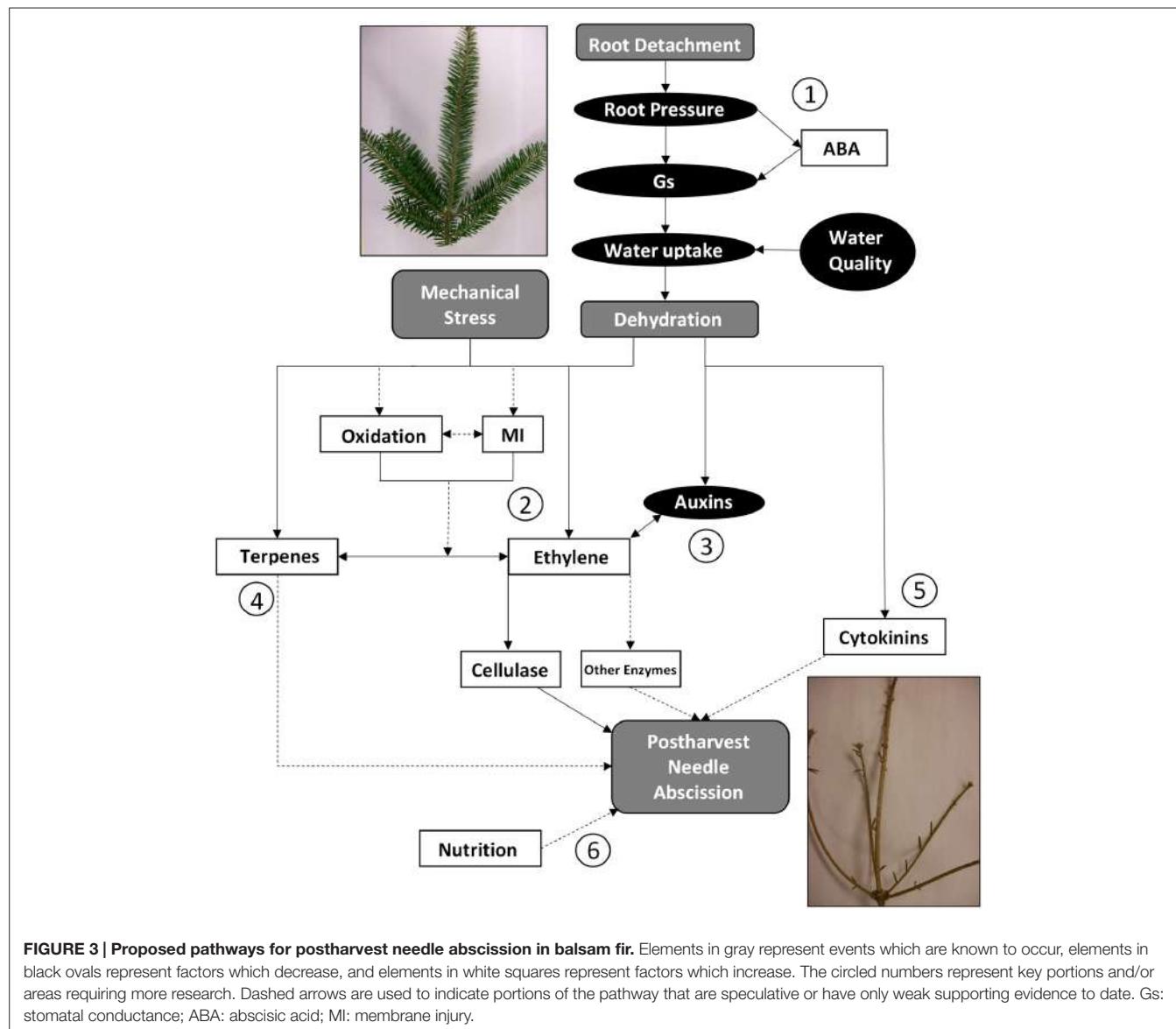


FIGURE 3 | Proposed pathways for postharvest needle abscission in balsam fir. Elements in gray represent events which are known to occur, elements in black ovals represent factors which decrease, and elements in white squares represent factors which increase. The circled numbers represent key portions and/or areas requiring more research. Dashed arrows are used to indicate portions of the pathway that are speculative or have only weak supporting evidence to date. G_s: stomatal conductance; ABA: abscisic acid; MI: membrane injury.

cytokinins, could contribute to postharvest abscission and could ultimately be an abscission pathway independent of dehydration (MacDonald et al., 2012a). That may still be a possible pathway, but there is a clear gap in our understanding of cytokinins. A temporal understanding of postharvest cytokinin changes in balsam fir would represent a major contribution to our understanding. It may be that cytokinins only increase at the very end of senescence to allow for some cell division or separation in the abscission zone. Or perhaps the increase in cytokinins represents a defensive reallocation of resources. Very little information is available regarding development of abscission zone in balsam fir, which may be linked to increases in postharvest cytokinins. Another useful contribution would be to investigation the effect of exogenous cytokinin application or manipulation of endogenous cytokinins through inhibition or genotypic investigations.

6. There is some evidence that foliar nitrogen, potassium, copper, and iron increased, then postharvest needle retention decreased in one study (Georgesom, 2013). The impact, mode of action, or interaction with other abscission-modifying factors has not yet been explored.

A complete understanding of the processes governing postharvest needle abscission is necessary to develop methods to halt or delay abscission. The abscission curve is described as a logistic curve and it is probably not realistic to believe postharvest needle abscission can be prevented. However, treatments that shift the abscission curve to the right would represent a method of delaying or inhibiting abscission. Referring again to Figure 3, anything that targets a major step in the postharvest balsam fir abscission pathway should, in turn, mitigate abscission. This has been observed by applying positive pressure to negate the effects of decreasing root pressure postharvest (MacInnes, 2015),

providing clean water to maintain water uptake and prevent xylem blockage (Lada et al., 2015a), storage in high vapor pressure deficit to delay dehydration (MacDonald et al., 2012a), or inhibit ethylene to maintain cell wall stability in abscission zones (MacDonald et al., 2010). All aforementioned methods significantly delayed abscission, which not only contribute to our scientific understanding and validation of the abscission pathway, but also represent potential abscission mitigating technologies.

AUTHOR CONTRIBUTIONS

RL and MM each contributed to gathering the literature for review, writing the initial draft of the article, and conceptual design of intellectual content. RL and MM reviewed the

manuscript independent from each other before meeting to decide which revisions to adopt. Both RL and MM approved the final article for submission and agree to be held accountable for all aspects of the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Are We on the Right Track: Can Our Understanding of Abscission in Model Systems Promote or Derail Making Improvements in Less Studied Crops?

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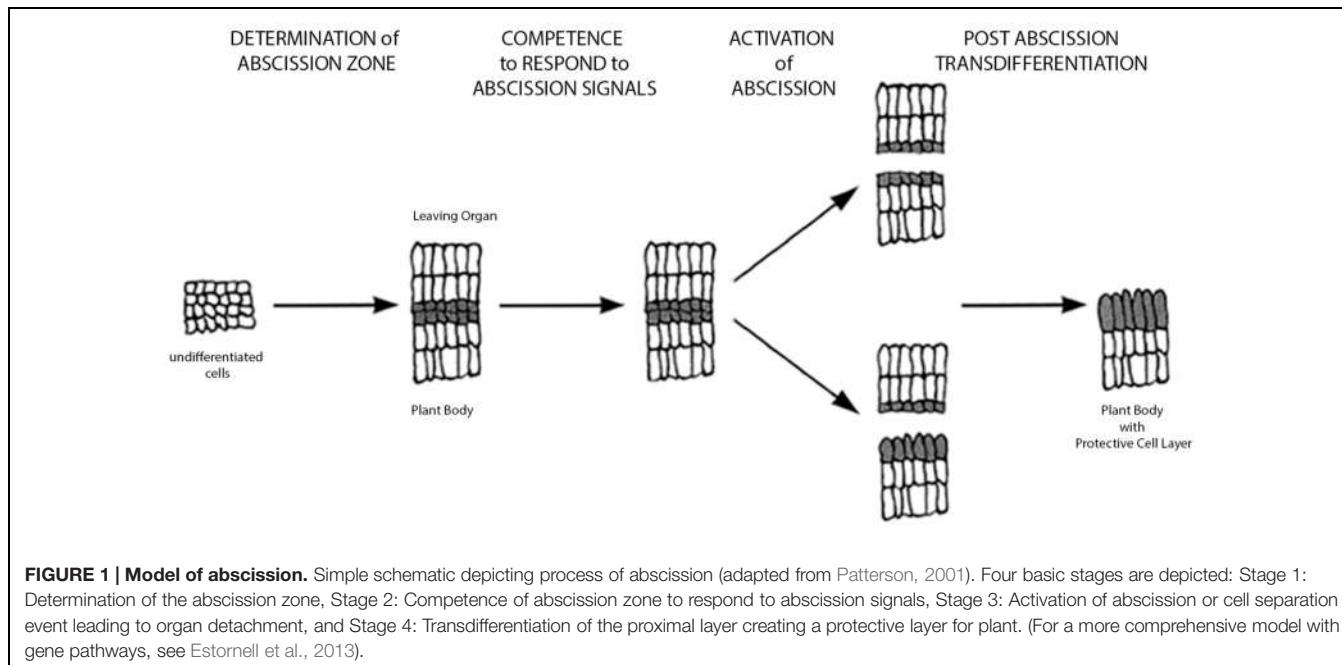
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As the world population grows and resources and climate conditions change, crop improvement continues to be one of the most important challenges for agriculturalists. The yield and quality of many crops is affected by abscission or shattering, and environmental stresses often hasten or alter the abscission process. Understanding this process can not only lead to genetic improvement, but also changes in cultural practices and management that will contribute to higher yields, improved quality and greater sustainability. As plant scientists, we have learned significant amounts about this process through the study of model plants such as *Arabidopsis*, tomato, rice, and maize. While these model systems have provided significant valuable information, we are sometimes challenged to use this knowledge effectively as variables including the economic value of the crop, the uniformity of the crop, ploidy levels, flowering and crossing mechanisms, ethylene responses, cultural requirements, responses to changes in environment, and cellular and tissue specific morphological differences can significantly influence outcomes. The value of genomic resources for lesser-studied crops such as cranberries and grapes and the orphan crop fonio will also be considered.

Keywords: abscission, shedding, seed-shatter, grape, cranberry, fonio, tomato

INTRODUCTION

Historically, humans have selected crop plants with delayed abscission for generations, as early fruit drop or seed shatter limited effective collection of the fruits, grains, or legumes (Harlan, 1992; Plants and Society, 2006). In general, we know the process of abscission results in shedding of organs as a developmentally programmed event; however, abscission may also occur in response to pathogens, environmental cues or other stresses. Early studies on abscission focused on the anatomical and physiological characterization of the abscission zone (Addicott, 1982; Sexton and Roberts, 1982). These studies have shown that the abscission zone consists of a few to multiple cell layers and is distinguished by small densely cytoplasmic cells. During the abscission process, there is breakdown of the middle lamella of cells within the separation layers. Although historically, there have been several proposed models for genes regulating abscission, scientists are still unclear



as what are the key players and how plant hormones, like ethylene, jasmonic acid (JA), abscisic acid, and auxin affect the regulation of gene expression during the process (Figure 1). There is strong evidence for interplay between the plant hormones ethylene and auxin in regulating abscission timing, where the former enhances the process and the latter inhibits (Abeles and Rubinstein, 1964; Jensen and Valdovinos, 1967; Addicott, 1982; Osborne, 1989). In addition, early researchers also focused on elucidating the role of cell degrading enzymes including the polygalacturonases and cellulases during the abscission process (Abeles, 1969; del Campillo and Bennett, 1996; del Campillo, 1999). Model systems including rice, maize, *Arabidopsis*, and tomato have provided new valuable genetic information on abscission and shattering, and knowledge of these genes associated with abscission has the potential to radically change approaches to studying abscission (Lewis et al., 2006; Estornell et al., 2013; Niederhuth et al., 2013).

Abscission in Monocots

In the grasses (maize, sorghum, and rice), initially two transcription factors were identified as associated with the regulation of shattering, *qSH1* and *SH4* (Konishi et al., 2006; Li et al., 2006). *SHAT1*, an *APETELA2* transcription factor, has also been identified as a gene that also affects shattering (Zhou et al., 2012). These genes are key factors in eliminating most shattering in members of the Poaceae family. In addition, homeodomain-leucine zipper transcription factors (HD-Zip TFs) and genes associated with growth regulation including auxin and ethylene responses from sorghum and maize are expressed in floral abscission zones (Chew et al., 2013; Dwivedi et al., 2014). Most recently, BRITTLE RACHIS1 and 2 were identified in barley and shown to be responsible for seed shatter (Pourkheirandish et al., 2015). Especially interesting is the fact

that BRI1, 2 are hypothesized to act as receptor and ligand and BRI2 has been shown to have homology with the *Arabidopsis* protein IDA that is also hypothesized as a receptor ligand (Butenko et al., 2003; Pourkheirandish et al., 2015). Orphan grain crops such as *Digitaria exilis* (fonio), *Eragrostis tef* (teff), and *Eleusine coracana* (finger millet) often have major losses due to early or unregulated shattering, and thus could highly benefit from breeding for delayed abscission and abscission associated genes through introgression of favorable alleles. While genes such as *qSH1*, *SH4*, and *SHAT1* have been shown to regulate shattering in domesticated rice, recent studies also show that panicle structure may also be critical (Ishii et al., 2013). Thus, undue attention to only specific genes or a single trait rather than multiple gene traits, might result in less effective selection.

Abscission in Dicots: *Arabidopsis* as Model System

In dicots, *Arabidopsis* has served as the model system to study abscission, and researchers have gained significant insights concerning regulation of the abscission process. Genes regulating development of the abscission zone and responses to hormonal, environmental, and newly discovered endogenous signals regulating abscission have been extensively studied. There are many excellent reviews: (Roberts et al., 2000, 2002; Aalen et al., 2006, 2013; Binder and Patterson, 2009; Van Nocker, 2009; Liljegren, 2012; Estornell et al., 2013; Niederhuth et al., 2013). While considerable inroads have been made on understanding the genes involved in signaling, the exact pathways are still being defined (Liljegren, 2012; Niederhuth et al., 2013). These key players include IDA (Butenko et al., 2003, 2009; Stenvik et al., 2008), HAESA (Jinn et al., 2000), HAESA LIKE (Shi et al., 2011), NEVERSHED (Liljegren

et al., 2009), and EVERSHED (Leslie et al., 2010). Additional downstream signaling factors have also been identified and include SERK1, BREVIPEDICELLUS/KNOTTED-LIKE FROM ARABIDOPSIS THALIANA (BP/KNAT1; Wang et al., 2006; Shi et al., 2011), and MAP kinases (Meng et al., 2012). Genes that are critical for formation of the abscission zone in *Arabidopsis* include *BOP1* and *BOP2*, and the MADS BOX gene *AGL15* (Fernandez et al., 2000; Hepworth et al., 2005; McKim et al., 2008).

Additional transcription factors that have been identified include FOREVER YOUNG FLOWER (FYF; Chen et al., 2011) and the zinc finger protein *Arabidopsis ZINC ZINGER PROTEIN 2* (Cai and Lashbrook, 2008). While these genes have been shown to be involved in the abscission process, the actual function during the abscission process is quite undefined. Similarly, genes regulating organ boundary patterning and elasticity of boundaries have been identified and while many have no determined role, the F-box gene *HAWAIIAN SKIRT* has been shown to disrupt normal patterning leading to fusion of sepals, and consequently delayed abscission (Aida and Tasaka, 2006; Gonzalez-Carranza et al., 2007b; Rast and Simon, 2008). An additional F-box gene *COI1* also delays abscission; however, it has been determined that this delay is most likely due to altered regulation of ethylene and auxin responses during the process of abscission in response to the absence of JA signaling rather than formation of the abscission zone (Kim et al., 2013). And, while the role of JA during abscission was initially a surprise, the role of other hormones such as ethylene and auxin during abscission has been well characterized in *Arabidopsis* (Patterson and Bleecker, 2004; Ellis et al., 2005; Binder and Patterson, 2009; Ogawa et al., 2009; Basu et al., 2013; Kim, 2014). These include ethylene synthesis genes (*ALLENE OXIDE SYNTHESIS*), ethylene response genes (*ETR1*, *EIN2*, and *EIN3*) and auxin-associated genes (*ARF1*, *ARF2*, and *AUX1*).

Many genes regulating cell wall modifications have also been identified and studied for their role in abscission in *Arabidopsis* as well as other species. These include polygalacturonases (Gonzalez-Carranza et al., 2002, 2007a; Kim and Patterson, 2006; Kim et al., 2006), cellulases (del Campillo, 1999), expansins (Cho and Cosgrove, 2000; Lashbrook and Cai, 2008), pectate lyases, xyloglucans and glycosylase transferases (Lashbrook and Cai, 2008; Wei et al., 2010; Singh et al., 2011). In addition, determination of unique morphological characteristics of the abscission zone have been characterized by multiple research groups: cell number, scar formation, timing and the relationship to environmental stresses, and developmental processes such as pollination, fertility, and senescence (Sawicki et al., 2015). There are also new studies indicating that alkalization of the cytosol of cells within the abscission zone is particularly important (Sundaresan et al., 2015). Last, cell death markers including *LZ ribonuclease* and *BFN1 nuclease* have been characterized for their roles during the abscission process (Farage-Barhom et al., 2008; Bar-Dror et al., 2011). In summary, there are many genes identified in *Arabidopsis* that impact the process of abscission and additional research will be needed before all the key players are characterized.

Abscission in Dicots: Tomato as Model System

Abscission in tomato has also been studied quite extensively, as tomato has been considered a model crop that is relatively easy to work with: true breeding (self pollinated), moderate sized genome (900 Mb), excellent isogenic stock collections, well characterized genetics, excellent physiological research studies, and easily transformed. Genome information, gene expression, and information about isogenic genetic stocks are available through the tomato functional genomics database (TFGD), the Sol Genomics network (<http://solgenomics.net/>) and NCBI. While there is considerable knowledge about genes regulating abscission within the pedicel in jointed tomatoes *JOINTLESS* (Mao et al., 2000; Nakano et al., 2013; Guan et al., 2014; Ito and Nakano, 2015), there is still relatively little understood concerning regulation of abscission at the fruit pedicel junction. These distinctions are valuable in terms of marketing different types of tomatoes (cluster on the vine versus slicing) and in shipping. In jointed tomatoes (see **Figure 2**) abscission within the pedicel or at the knuckle has been shown to be regulated by numerous MADS Box genes, auxin associated genes and several novel transcription factors (Mao et al., 2000; Nakano et al., 2013; Guan et al., 2014; Ito and Nakano, 2015; Ma et al., 2015). Researchers have also extensively studied the role of cell wall hydrolytic enzymes and polysaccharides including cellulases, polygalacturonases, pectinases, xyloglucans, arabinans, and galactans during tomato fruit abscission (del Campillo and Bennett, 1996; Kalaitzis et al., 1997; Iwai et al., 2013). Many of the cell-wall associated genes are members of large gene families; and thus, efforts to alter the abscission process through modification of these genes have not yielded significant changes. In addition not all ethylene associated genes that have been shown to affect abscission in *Arabidopsis* delay abscission in tomato. While mutations in *ETR1*, *ETR2*, and *ETR3* all delay the process of abscission in *Arabidopsis*, only the mutated ortholog of *ETR1* (*NEVERRIPE*) delays abscission in tomato; and they all are primarily associated with fruit ripening (Lanahan et al., 1994; Klee, 2002). Historically, breeders have focused on fruit size, color, and flavor rather than abscission. Perhaps the marketing of increased tomato varieties in markets and the new emphasis on local produce, abscission in cluster tomatoes may warrant further study on abscission in tomatoes.

Approaches to Study Abscission in Both the Grasses and Dicots

Many of the genes found in maize, rice, and *Arabidopsis* are highly conserved and plant breeders have embraced this knowledge to direct research programs toward utilizing genomic approaches. With the tools and knowledge to identify and engineer alternative plant species, will these efforts prove productive? There is no doubt that as the world population grows and resources and climate conditions change, it is critical that we continue to increase crop production and develop more sustainable agricultural practices. While there are 100s of crops to consider, this manuscript will use examples such as tomato (an annual vegetable crop), grapes (a perennial fruit tree crop), cranberries

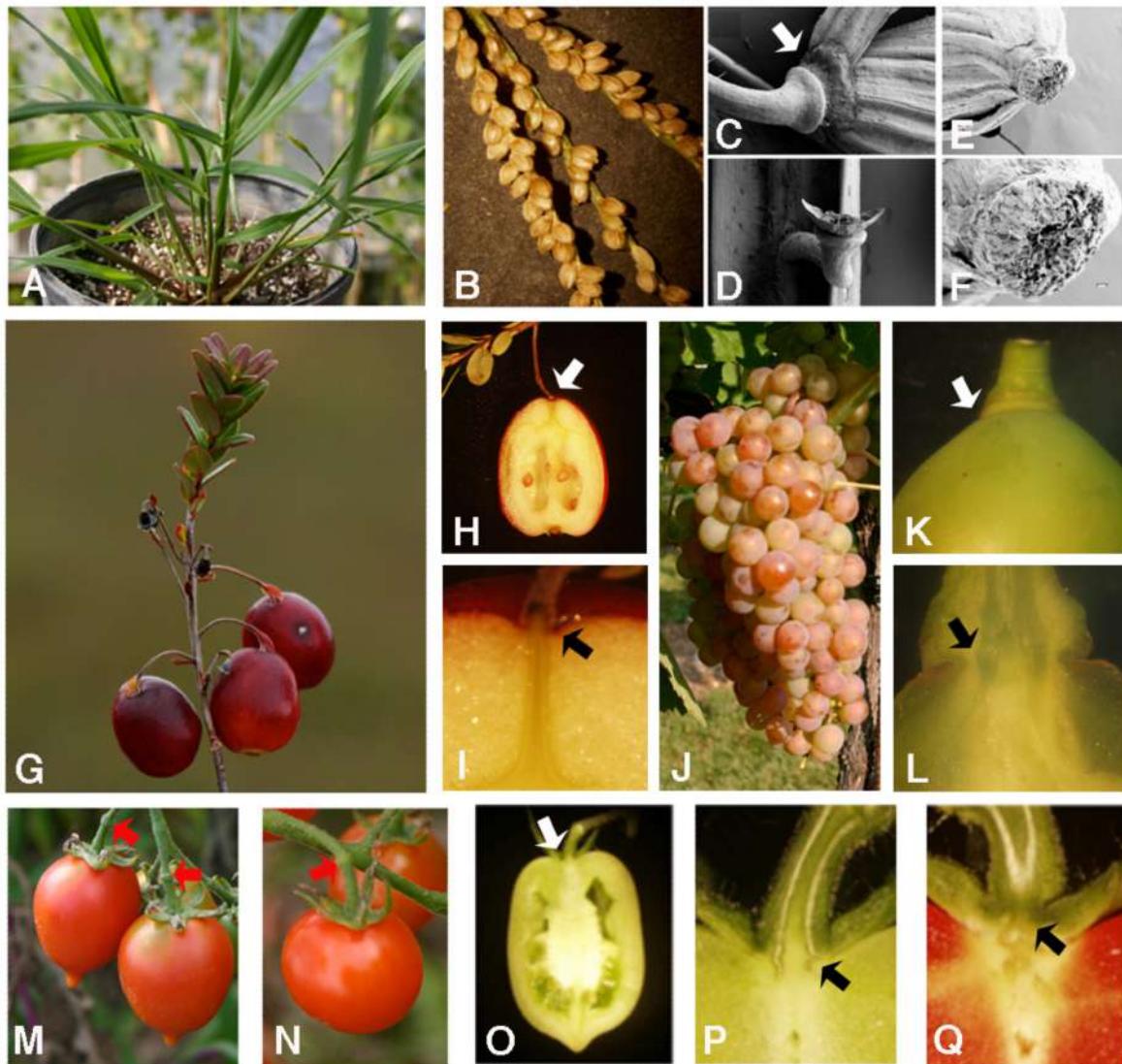


FIGURE 2 | Abscission in fonio, cranberry, grapes, and tomatoes. **(A–F)** Images of fonio illustrating the developing plant **(A)**, a mature inflorescence **(B)**, and scanning electron micrographs of the abscission zone of a mature seed **(C–F)**. **(G–I)** Images of cranberry illustrating the developing fruit **(G)** and sections revealing the fruit abscission zone **(H,I)**. **(J–L)** Images of grape illustrating a fruit cluster **(J)** and sections revealing the fruit abscission zone **(K,L)**. **(M–Q)** Images of tomatoes illustrating the abscission zones in tomatoes. A variety of cultivars were observed and pictured are two cluster varieties: Principe Borghese **(M)**, and Ladybug **(N)**. **(O–Q)** Illustrate freehand sections of the pedicel abscission zone attached directly to the developing fruit. Black and white arrows show fruit/pedicel abscission zones and red arrows show the “knuckle” abscission zone.

(a perennial fruit crop), and fonio (an annual grass native to West Africa). These crops have been selected to provide a perspective on both well-studied crops and less developed crops (Figure 2).

Crop Examples

Fonio – An Orphan Grass: Challenges and Success

Our major grain crops including maize, wheat, and barley have been selected for delayed abscission by early gatherers for thousands of years. In addition, some millet varieties that have been cultivated for centuries have reduced shatter (Zohary and Hopf, 2000; Brink and Belay, 2006). However, uncontrolled seed shatter in fonio *Digitaria exilis* and many of the orphan grain

crops is one of the growers' greatest concerns and a major limiting factor in their potential for expansion as food crops. These orphan grain crops are often drought and disease resistant and small plots are routinely grown in many communities. Addressing seed shattering in crops such as fonio could increase yields by more than a third, providing farmers significant additional income (Foltz, personal communication). Of major importance is that this could be done without adding fertilizer. In contrast to other commonly grown grains, fonio requires little or no inputs; thus not requiring additional investment by farmers. For example, in Mali, yields of common millet could be improved with fertilizer applications, yet this would require investment;

and, predicted profits from a 50% increase in yield due to these added resources would not exceed the predicted profits from improved fonio with reduced shattering. As the world population grows and climate conditions change, resource management is critical and thus attention to breeding and management practices for small orphan grains has become extremely important.

Currently, fonio is grown extensively in Guinea, Gambia, Senegal, Mali, Burkina Faso, Benin, Senegal, Togo, Nigeria, and the Dominican Republic. In most of West Africa, fonio is grown primarily by smallholders on plots typically of one hectare or less; and harvest must be performed within a couple days of full grain development due to seed shatter. Abscission, or seed shatter in fonio has not been studied in the past; and thus, one of the first challenges has been to characterize flowering and the abscission process. We have observed that flowering in fonio is tightly regulated, as initially reported by Kwon-Ndung and Misari (1999), Cruz (2004), Cruz et al. (2006), Tekete (2006), and Cruz and Béavogui (2011). This photoperiodic control of flowering and seed development results in the narrow window for harvest. We also believe that abscission in fonio is both environmentally and developmentally regulated, and grains abscise with the first heavy rainfall after reaching full maturity. These observations propelled us to identify genetic factors regulating abscission as modification of weather or flowering time was less achievable.

Since there was initially no sequence data on fonio, we performed a single Illumina sequence run to generate a snapshot of the transcriptome of a *Niatia* seedling (one of the most common commercially available lines). The run yielded 38 million reads that we mapped directly onto to the rice genome and the rice transcriptome, as well as assembled *de novo*. We aligned contigs from the transcript assembly with the sequence of several known shattering genes and their homologs in maize, rice, and sorghum, and found extensive fonio sequence similarities with the rice shattering gene *qSH1*, members of the Agamous-related family of genes such as *JOINTLESS 1-3*, *SH2*, and *SH3*, the free-threshing locus *Q* of wheat, as well as the *Arabidopsis* abscission-associated gene *NEVERSCHED* (Liljegren et al., 2009; Sang, 2009). Using this sequence information we have been able to PCR amplify portions of the *qSH1* homolog from fonio genomic DNA. While this provides proof of principle, it still needs to be determined if selection for mutants in *qSH1* would provide a delay in seed shatter. As fonio seed is quite small like *Arabidopsis*, a targeted approach to screen mutagenized seed similar to iTILLING could be utilized (Bush and Krysan, 2010).

Overall, there are gains to be made through targeted gene selection in fonio and other small orphan grains; but the challenges still remain. Selection of cultivars/lines to study is one of the first questions, as historically each community has their own local lines. Some lines are diploid and others thought to be tetraploid. In addition, fonio is self-pollinated and potentially apomictic; and thus, crosses between lines are challenging. Techniques for pollination of finger millet have been developed by the Devos and Bennetzen labs at the University of Georgia and are being applied to crossing fonio. Communication with remote villages and farmers as well as distribution of new seed and management practices will also be challenging. However,

despite these concerns, crops like fonio have significant potential for increases in yield by understanding the abscission process and breeding for genes regulating seed shatter.

Woody Perennial Dicots: Cranberries and Grapes-Challenges and Successes

Historically berry drop or abscission has not been a trait that either grape or cranberry growers have focused on improving. However, with the recent focus on conservation of water and resources and the introduction of new cold-hardy hybrid grapes (*Vitis vinifera* × *Vitis riparia* and *Vitis vinifera* × *Vitis labrusca*) berry drop has become an issue in both crops. With wine grapes, early abscission results in lower sugar content and higher acidity, and thus a poorer quality wine in general. Alternatively, in table grapes it is even more important to retain high quality full clusters after harvest. Uncontrolled berry drop decreases both value and quality. Curiously, increased berry drop in some cultivars such as "Sunpreme" may prove fruitful for raisin producers, as some growers are now taking advantage of berry abscission to reduce harvest costs (Romero, 2015). In cranberry, there has been minimal research regarding abscission, but it is believed that cultural and environmental factors such as limited nutrient availability and extreme heat conditions can cause fruit drop. Growers have placed a new emphasis on management of water and sustainable production in response to climate change and new environmental stresses. Consequently, the loss of fruit is an important issue as fruit growth and abscission is most likely dependent on transport of water, nutrients and other factors across this zone (Sawicki et al., 2015).

We searched available databases for both grapes and cranberries for orthologs to 15 genes previously identified in *Arabidopsis* that have been characterized as regulating the abscission process or associated with unique stages of development in the abscission process (Supplementary Table S1). In grapes (*Vitis vinifera*), we selected orthologs for ten genes (NCBI); and are currently looking at gene expression during the abscission process in four hybrid cultivars of cold-hardy Wisconsin grapes. In cranberry, joint efforts across the United States have recently yielded a transcriptome and nuclear genome assembly (Polashock et al., 2014) and a nuclear genome assembly (Fajardo et al., 2014). We used both of these cranberry generated databases to search for abscission genes. While matches for all fifteen of the genes we searched for were identified in both grape and cranberry genomes, too many close matches to identify a single ortholog was frequently an issue (Supplementary Table S1). In general, the high homology of both cranberry and grape genes to known abscission-related genes from *Arabidopsis* suggests that there may be shared functions and similar signaling pathways regulating the abscission process. Identification of the best candidate ortholog, transcription factors and unique aspects of development associated with each species may make altering abscission more challenging. Ultimately, improving our understanding of both early and late fruit abscission in these fruit crops using molecular tools combined with traditional breeding, morphological and physiological studies will lead to better management practices and improved quality and greater yields.

SUMMARY: WHY THE SUCCESS IN SOME CROPS AND NOT OTHERS?

Building our knowledge on abscission in crop plants continues to be an important challenge not only not to prevent unwanted abscission but also to promote early abscission, as in many cases early bud removal and accelerated fruit abscission promotes improved root development, a more vigorous plant, and higher quality fruit and flowers. The rapid advances in molecular techniques and availability of quality sequence information on most species has spurred interest and promoted new research on the cloning and engineering known genes. While many of these genes will definitely have similar functions in many crops, researchers must always pause and remember the developmental biology of their plant such as flowering time, pollination, fertility, fruit development, life cycle, and senescence. Crops may vary as to whether they abscise at the abscission zone associated directly with the fruit or at an independent zone within the pedicel; and thus knowing the biology and marketing traits of the crop must be considered. In addition, developmental programs may mask other traits; and thus, a gene altering the abscission process may have no effect in specific genetic backgrounds. This is the case in the recently discovered role of panicle structure in rice and the hidden role in abscission (Ishii et al., 2013). Clearly, it is essential that breeders and molecular biologists work together providing an understanding of the unique development of each species as well as the targeted genes or pathways of interest. Similarly, it will also be critical to consider genome size, ploidy, and genetic relationships amongst lines as well as between species. Our progress may be

slow at times; but a concerted combined effort promises new insights.

AUTHOR CONTRIBUTIONS

AM, JB-M, JH, DN-M, TF, JZ, and SP designed the experiments. AM, JB-M, DN-M, TF, and SP performed the experiments. SP and JZ wrote and edited the manuscript. In addition, all authors contributed to editing the manuscript.

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SUPPLEMENTARY MATERIAL

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Allele-Specific Interactions between CAST AWAY and NEVERSHED Control Abscission in *Arabidopsis* Flowers

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An advantage of analyzing abscission in genetically tractable model plants is the ability to make use of classic genetic tools such as suppression analysis. We have investigated the regulation of organ abscission by carrying out suppression analysis in *Arabidopsis* flowers. Plants carrying mutations in the NEVERSHED (*NEV*) gene, which encodes an ADP-ribosylation factor GTPase-activating protein, retain their outer floral organs after fertilization. Mutant alleles of CAST AWAY (*CST*), which encodes a receptor-like cytoplasmic kinase, were found to restore organ abscission in *nev* flowers in an allele-specific manner. To further explore the basis of the interactions between *CST* and *NEV*, we tested whether the site of a *nev* mutation is predictive of its ability to be suppressed. Our results suggest instead that the strength of a *nev* allele influences whether organ abscission can be rescued by a specific allele of *CST*.

Keywords: abscission, cell separation, shedding, NEV, CST, ARF GAP, RLCK

INTRODUCTION

Plants have the astonishing ability to release their floral organs, leaves, fruit, and seeds at programmed points in their life cycle or in response to signals from their environment. Within *Arabidopsis* flowers, the series of events leading to organ abscission is genetically tractable. Analysis using this model system has revealed the influence of organ boundary genes in establishing the placement of abscission zones (Wang et al., 2006; González-Carranza et al., 2007; Gómez-Mena and Sablowski, 2008; McKim et al., 2008; Gubert et al., 2014), the critical roles played by hormones such as jasmonic acid (Kim et al., 2013) and managers of membrane traffic (Liljegren et al., 2009; Liu et al., 2013), and a signaling module that regulates the cell separation phase of organ abscission (Fang and Fernandez, 2002; Cho et al., 2008; Stenvik et al., 2008; Shi et al., 2011; Gubert and Liljegren, 2014; Patharkar and Walker, 2015; Santiago et al., 2016; Taylor et al., 2016). Central components in this module include a secreted peptide, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and redundant leucine-rich repeat receptor-like kinases, HAESA (HAE) and HAESA-like2 (HSL2), that activate a MAP kinase cascade leading to organ abscission.

We have used suppression analysis as a genetic tool to identify additional genes that control the abscission process in *Arabidopsis* flowers. Starting with the *nevershed* (*nev*) mutant which blocks organ shedding due to defects in membrane traffic (Liljegren et al., 2009), we screened for second-site mutations that would restore organ abscission in the presence of the original mutation. The *nev-3* allele chosen for this screen (Figure 1A) changes an invariant arginine in the encoded protein known to be essential for ADP-ribosylation factor GTPase-activating activity (Luo et al., 2007).

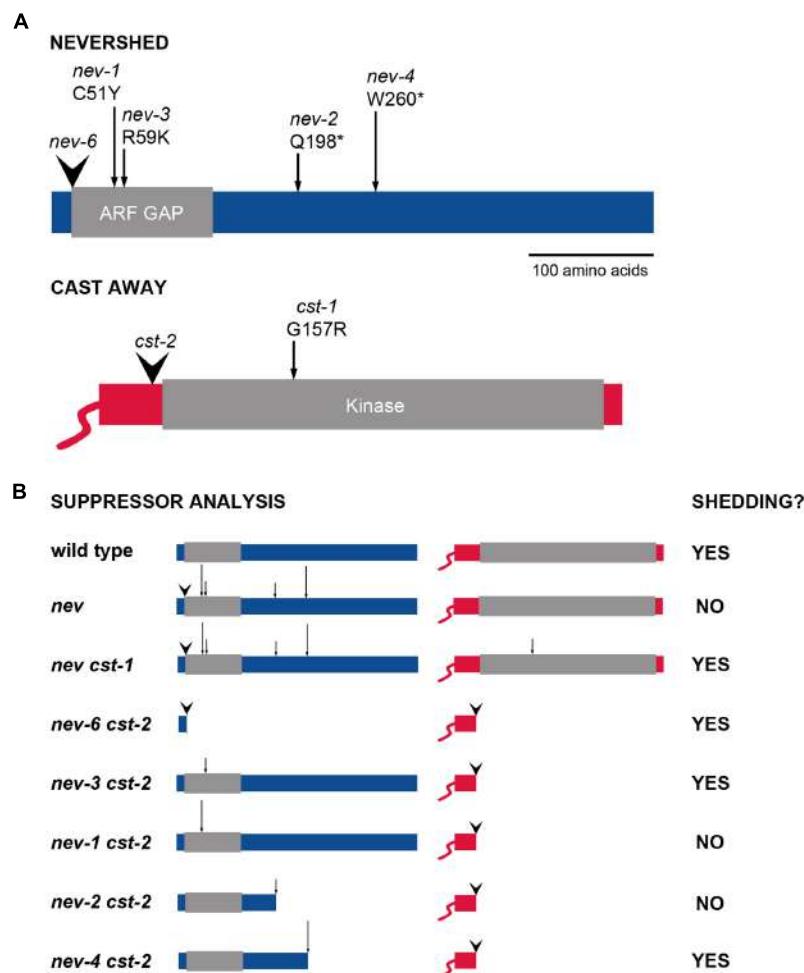


FIGURE 1 | Alleles of NEV and CST used in suppressor analysis of organ abscission. (A) The sites of the mutations analyzed are indicated within the encoded NEV and CST proteins (Liljegren et al., 2009; Burr et al., 2011). T-DNA insertions are marked by arrowheads and point mutations by arrows. **(B)** Diagram of the *nev cst* allele combinations tested for rescue of organ shedding (Burr et al., 2011; this study).

Multiple alleles of genes encoding three receptor-like kinases—EVERSHED (EVR), SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 (SERK1), and CAST AWAY (CST)—were found to rescue abscission in *nev* flowers (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). Mutations in these receptor-like kinases are also able to reverse *nev*-mediated alterations in the structure of the Golgi apparatus and associated *trans*-Golgi network. Additional analyses suggest that activation of organ abscission is modulated by inhibitory interactions between CST and EVR with HAE/HSL2 (Burr et al., 2011; Gubert and Liljegren, 2014). Recent studies have demonstrated that SERK1 and two related receptor-like kinases act as co-receptors of HAE/HSL2 (Meng et al., 2016; Santiago et al., 2016). We have proposed that CST and EVR may prevent the signaling that leads to organ abscission by sequestering HAE/HSL2 at the cell surface and promoting their internalization prior to activation by IDA (Burr et al., 2011). As NEV is thought to function in the cycling

of HAE/HSL2 to the plasma membrane, disruption of CST or EVR activity may restore organ abscission in *nev* flowers by shifting the balance of stabilized HAE/HSL2 receptors at the cell surface from an excessive pool of internalized, inactive receptors in endosomal compartments (Burr et al., 2011; Bryan et al., 2012; Liljegren, 2012).

Contrasting behaviors are shown by the pair of *cst* mutant alleles we identified with regard to their ability to rescue abscission in *nev* flowers (Burr et al., 2011). The *cst-1* allele introduces a missense mutation (G157R) near the ATP-binding site within the CST kinase domain (Figure 1A), abolishing the kinase activity of the mutant protein. Organ shedding in *nev-3*, *nev-2*, and *nev-6* flowers is recessively rescued by two copies of the *cst-1* allele (Figure 1B; Burr et al., 2011). The *cst-2* allele contains a T-DNA insertion immediately upstream of the kinase domain, and is predicted to encode a truncated protein (Figure 1A). One copy of *cst-2* dominantly restores organ abscission in *nev-3* and

nev-6 flowers, but *nev-2* flowers retain their organs even if both copies of *cst-2* are present (**Figure 1B**; Burr et al., 2011).

As these results were partially consistent with the allele-specific mechanism of conformational suppression, in which a suppressor mutation restores a physical interaction between two proteins, we designed a study to determine whether the location of a *nev* mutation would be predictive of its ability to be rescued by the *cst* alleles. Specifically, we tested whether alleles that independently affect either the ARF GAP domain or the C-terminal region of NEV would mimic the distinct interactions of *nev-3* and *nev-2* with *cst-2*.

MATERIALS AND METHODS

Plants

The mutant alleles used in this study and methods for genotyping *cst-1* and *cst-2* have been described previously (Liljegren et al., 2009; Burr et al., 2011). *nev-1* and *nev-4* were genotyped as described in Supplementary Table S1. The *nev-1*, *nev-4*, and *cst-1* mutants were isolated from the Ler ecotype; the *cst-2* mutant was isolated from the Col ecotype. Since the *nev cst-2* double mutants would be analyzed in a mixed Ler/Col background, a *cst-1* stock backcrossed once into the Col ecotype was used to generate the *nev cst-1* double mutants. Plants were grown at 21°C with 50% humidity and a 16-h photoperiod.

Imaging

Digital images were taken with a PowerShot SX160 IS (Canon, Melville, NY, USA) or Alpha Innotech gel documentation system (ProteinSimple, San Jose, CA, USA). Image brightness and contrast were adjusted with Photoshop CS6 (Adobe, Mountain View, CA, USA).

RT/PCR

Wild-type and mutant inflorescences with flowers through stage 15 (Smyth et al., 1990) were ground in liquid nitrogen, and RNA was extracted using the RNeasy Plant Mini Kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. Specific regions of wild-type and mutant cDNAs were synthesized using gene-specific primers (described in Supplementary Table S2) and SuperScript III reverse transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. A subset of the RNA samples were pre-treated with DNase using the Ambion DNA-free Kit (Thermo Fisher Scientific, Waltham, MA, USA) prior to cDNA synthesis. To confirm the presence of the *cst-2* transcript, a second round of amplification was performed with a nested primer. In addition, replicates were carried out with and without reverse transcriptase.

RESULTS

Allele-Specific Suppression of *nev*-Mediated Abscission Defects

Previously, we discovered that while one copy of the *cst-2* allele is sufficient to rescue organ shedding in *nev-3* (R59K) flowers,

abscission in *nev-2* (Q198*) flowers cannot be restored by either one or two copies of *cst-2* (**Figure 1B**; Burr et al., 2011). Whereas the *nev-3* mutation affects an arginine residue essential for the enzymatic activity of the ARF GAP domain, the protein encoded by *nev-2* is predicted to be truncated downstream of the ARF GAP domain (**Figure 1A**; Luo et al., 2007; Liljegren et al., 2009). Both copies of the *cst-1* allele are required to suppress the abscission defects of *nev-3* and *nev-2* flowers (**Figure 1B**; Burr et al., 2011). Based on these results, we hypothesized that if CST and NEV function in a complex, the ARF GAP domain of NEV might facilitate this interaction (Burr et al., 2011).

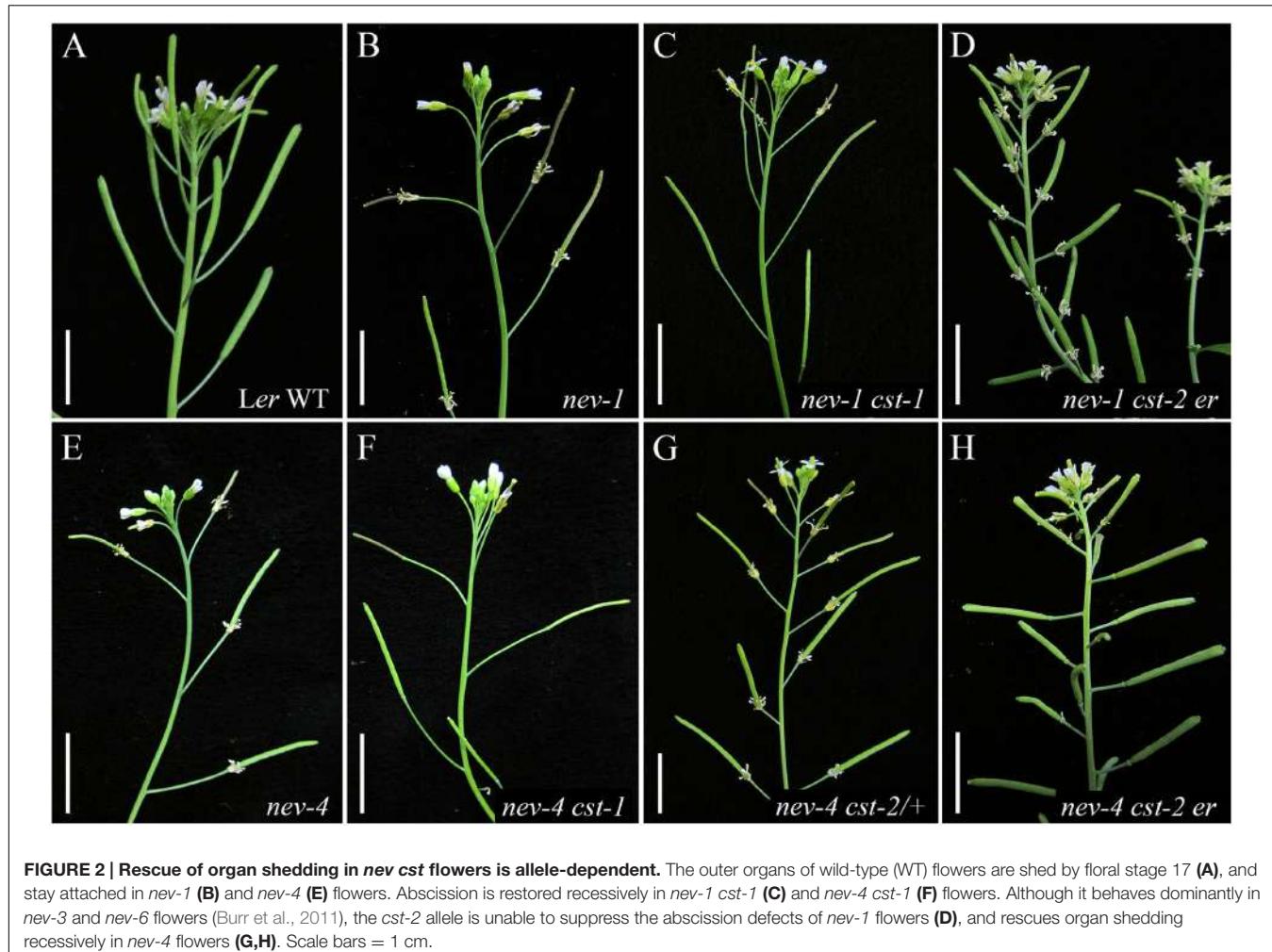
To investigate whether other *nev* alleles that alter critical residues in the ARF GAP domain show similar interactions with the *cst* alleles, we analyzed *nev-1 cst-1* and *nev-1 cst-2* double mutants (**Figure 2**). *nev-1* is a missense allele (C51Y) that alters the third essential cysteine within the Cys-x2-Cys-x(16,17)-Cys-x2-Cys zinc finger motif of the ARF GAP domain (**Figure 1A**; Liljegren et al., 2009). While *cst-1* is able to recessively suppress the shedding defects of *nev-1* flowers, the floral organs of the *nev-1 cst-2* double mutant remain firmly attached (**Figures 2A–D**). These results indicate that despite their close proximity within the ARF GAP domain and indistinguishable single mutant phenotypes, the *nev-1* and *nev-3* alleles do not behave equivalently when interacting with *cst-2* (**Figures 1A,B**).

We also tested whether another *nev* allele that introduces a stop codon downstream of the ARF-GAP domain exhibits similar interactions with the *cst* alleles. Like *nev-2*, *nev-4* is a nonsense allele (W260*) predicted to encode an abbreviated protein with an intact ARF-GAP domain (**Figure 1A**; Liljegren et al., 2009). As with all *nev* alleles tested, *cst-1* recessively rescues organ abscission in *nev-4* flowers (**Figures 2E,F**). However, unlike *nev-2 cst-2* flowers (**Figure 1B**), the shedding defects of *nev-4* flowers can also be suppressed with two copies of *cst-2* (**Figures 2G,H**). Therefore, despite the shared features of the *nev-2* and *nev-4* alleles, they interact with *cst-2* in distinct modes (**Figures 1A,B**).

Analysis of *nev* and *cst* Transcripts

To examine whether there are qualitative differences in expression of the *nev* and *cst* mutant transcripts compared to wild-type, RT/PCR experiments were carried out on total RNA isolated from the inflorescences of wild-type and mutant plants. Oligos located in exon 11 of *NEV* and exon 6 of *CST* were used to synthesize the first strand of the cDNAs, and specific regions of the transcripts were subsequently amplified (see Supplementary Table S2). Substantial differences were not observed for the transcript levels of either missense (*nev-3*, *nev-1*, *cst-1*) or nonsense (*nev-4*, *nev-2*) alleles of *NEV* and *CST* compared to wild-type (Supplementary Figures S1A and S2A). In contrast, comparable levels of correctly spliced transcripts were not apparent in either of the insertional alleles (*nev-6*, *cst-2*) analyzed (Supplementary Figures S1A and S2A).

To test for the presence of altered transcripts in *nev-6* and *cst-2* flowers, oligos located upstream of the T-DNA insertion sites were used to synthesize cDNA fragments from DNase-treated RNA samples (Supplementary Table S2; Supplementary Figures S1B and S2B). Similar levels of a *NEV* cDNA product including



part of exon 1 were observed in all *nev* mutants and wild-type plants examined (Supplementary Figure S1B). Since the *nev-6* T-DNA insertion is located in intron 1, these results indicate that an altered mutant transcript is produced in *nev-6* flowers that may encode an abbreviated protein. Reduced levels of nested products including parts of exon 1 and 2 of *CST* were observed in *cst-2* flowers compared to *cst-1* and wild-type (Supplementary Table S2; Supplementary Figure S2B). These results are consistent with the production of a truncated *cst-2* protein.

DISCUSSION

Here we report further evidence that the *cst-1* and *cst-2* alleles differ in their ability to restore organ shedding in *nev* flowers. While *cst-1* recessively suppresses each of the five *nev* alleles tested, *cst-2* suppresses *nev-3* and *nev-6* dominantly, *nev-4* recessively, and fails to rescue *nev-1* and *nev-2* (Burr et al., 2011; this study).

These results highlight the complexity of interpreting the mechanisms of allelic suppression. Although the ultimate goal for many geneticists carrying out suppression analysis is to

find instances of conformational suppression, whereby allele-specific rescue reflects a restored physical interaction between two mutant proteins, this scenario is actually rare in practice (Manson, 2000). Indeed, our selection of the *nev-3* missense allele (Figure 1A) as the genetic background for this screen was driven by an interest in identifying a mutant version of an unknown protein that might interact with and restore the ARF GAP enzymatic activity of the *nev* mutant protein.

Instead, our results suggest that the *cst-1* and *cst-2* alleles restore organ abscission in *nev* flowers through distinct suppression mechanisms. We have found that the kinase-dead CST protein encoded by *cst-1* recessively suppresses the abscission defects of all *nev* alleles tested, including *nev-6*, which is predicted to produce an abbreviated protein missing the ARF GAP domain (Figure 1A; Supplementary Figure S1B). Suppression of a deletion (or null) allele of the original gene by an extragenic suppressor is considered strong evidence of bypass suppression (Prelich, 1999). Bypass suppression occurs when a second site mutation creates an alternate opportunity to cover the function disabled by the first mutation (Manson, 2000; Michels, 2002). Another hallmark of bypass suppression is that it is not allele-specific (Manson, 2000), which fits

with the observed behavior of the *nev cst-1* double mutants (**Figure 1B**). Considering that interactions between CST and HAE were detected in subdomains of the plasma membrane via biomolecular fluorescence complementation assays (Burr et al., 2011), the *cst-1* allele may consistently rescue organ shedding in *nev* flowers due to the failure of the kinase-deficient *cst-1* protein to promote the internalization of the HAE/HSL2 receptors from the cell surface. The recessive nature of the *cst-1* suppression suggests that the reduced amount of the functional CST kinase in *nev* flowers heterozygous for *cst-1* is sufficient to remove enough of the HAE/HSL2 receptors from this plasma membrane pool to prevent activation of the MAP kinase module leading to organ abscission. Redelivery of HAE/HSL2 to the cell surface after internalization is predicted to be disrupted in each of the *nev* mutant alleles tested (**Figure 1A**).

We have found that *cst-2*, which may produce reduced levels of an abbreviated, membrane-associated protein without a kinase domain (**Figure 1A**; Supplementary Figure S2B), rescues organ abscission in *nev-3*, *nev-4*, and *nev-6* flowers but not in *nev-1* or *nev-2* flowers (**Figure 1B**). This allele-specific outcome may result from a dominant-negative mutation enacting a gradient of suppression (Manson, 2000; Burr et al., 2011). Under this scenario, the predicted strength of the *nev* alleles tested would range from *nev-6* and *nev-3* (relatively weak; rescued by one copy of *cst-2*) to *nev-4* (intermediate, rescued by two copies of *cst-2*) to *nev-1* and *nev-2* (strong, not rescued by *cst-2*). Relative differences in the activities of *nev* mutant proteins may impact the ratio of HAE/HSL2 receptors trapped in the endosomal compartments and thereby influence the ease of *cst-2* mediated suppression (**Figure 1B**). While a truncated *nev-6* mutant protein without an ARF GAP domain would not be expected to retain more function than the *nev-2* mutant protein (**Figure 1**), intronic T-DNA insertions can be spliced out in a fraction of the transcripts produced, leading to synthesis of functional protein (Chehab et al., 2011; Rodriguez et al., 2014). Although we did not detect notable levels of correctly spliced *NEV* transcripts in *nev-6* flowers (Supplementary Figure S1A), it is likely that even a small amount of functional protein is sufficient to promote abscission. Indeed, it has been previously observed that the petals of *nev-7* flowers detach more readily than those of *nev-3* flowers (Liu et al., 2013). Like *nev-6*, the *nev-7* allele contains a T-DNA insertion in the first intron (Liljegren et al., 2009).

It is striking that the *nev-1* and *nev-2* alleles can be recessively rescued by *cst-1* but not by *cst-2*. These results, in addition to the dominant suppression of *nev-3* and *nev-6* by *cst-2*, suggest that the truncated *cst-2* protein may exhibit an altered set of interactions with receptor-like kinase complexes than the *cst-1* protein. Future analysis of the expression, localization, and ability of the *cst-2* and *cst-1* mutant proteins to form heteromeric complexes with EVR and HAE may reveal additional clues to the unique mechanisms underlying their restoration of the signaling leading to organ abscission in *nev* flowers.

With the growing accessibility of approaches to identify the transcriptomes of abscission zone cells in model as well as crop plants using laser capture microdissection (Cai and Lashbrook, 2006, 2008; Agustí et al., 2009) and RNA sequencing (Niederhuth et al., 2013; Kim et al., 2016; Sundaresan et al., 2016), the

agronomic value of using model plants to study abscission is under debate (Patterson et al., 2015). The recent discovery that drought-triggered leaf abscission is dependent on the activities of IDA, HAE/HSL2, and NEV (Patharkar and Walker, 2016) significantly enhances the usefulness of *Arabidopsis* as a model system. Furthermore, until analysis of gene function is feasible in crop plants, parallel approaches to investigate the functions of abscission zone-enriched genes in model plants with reverse genetic approaches will be crucial.

Forward genetic screens, when carefully designed, are also expected to provide novel insights regarding the regulation of organ abscission. In addition to our discovery of a set of receptor-like kinases that modulate organ abscission via proposed interactions with HAE and HSL2, the homeodomain transcription factor BREVIPEDICELLUS (BP) was found to act downstream of the IDA-HAE/HSL2 signaling module through suppression analysis of *ida* flowers (Shi et al., 2011). While *bp* mutants display enlarged abscission zones (Wang et al., 2006), it is noteworthy that the *cst*, *evr*, and *serk1* mutants do not present phenotypes on their own, yet alleles of each are able to rescue organ shedding in the context of *nev* flowers (Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011). Suppression analysis of a weak *hae hsl2* mutant has revealed that mutations in either of two mannosyltransferases that normally mediate degradation of the mutant *hsl2* protein in the ER may restore abscission by allowing this partially functioning receptor to escape to the cell surface (Baer et al., 2016). Understanding the threshold levels at which organs are released in sensitized mutants like *nev*, *ida*, and *hae hsl2* may inform the future design of nuanced solutions to control abscission in crop plants.

AUTHOR CONTRIBUTIONS

SL designed the experiments. All authors performed the experiments and contributed in preparing the figures.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01588>

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Examination of the Abscission-Associated Transcriptomes for Soybean, Tomato, and Arabidopsis Highlights the Conserved Biosynthesis of an Extensible Extracellular Matrix and Boundary Layer

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Abscission zone (AZ) development and the progression of abscission (detachment of plant organs) have been roughly separated into four stages: first, AZ differentiation; second, competence to respond to abscission signals; third, activation of abscission; and fourth, formation of a protective layer and post-abscission trans-differentiation. Stage three, activation of abscission, is when changes in the cell wall and extracellular matrix occur to support successful organ separation. Most abscission research has focused on gene expression for enzymes that disassemble the cell wall within the AZ and changes in phytohormones and other signaling events that regulate their expression. Here, transcriptome data for soybean, tomato and Arabidopsis were examined and compared with a focus not only on genes associated with disassembly of the cell wall but also on gene expression linked to the biosynthesis of a new extracellular matrix. AZ-specific up-regulation of genes associated with cell wall disassembly including cellulases (beta-1,4-endoglucanases, CELs), polygalacturonases (PGs), and expansins (EXPs) were much as expected; however, curiously, changes in expression of xyloglucan endotransglucosylase/hydrolases (XTHs) were not AZ-specific in soybean. Unexpectedly, we identified an early increase in the expression of genes underlying the synthesis of a waxy-like cuticle. Based on the expression data, we propose that the early up-regulation of an abundance of small pathogenesis-related (PR) genes is more closely linked to structural changes in the extracellular matrix of separating cells than an enzymatic role in pathogen resistance. Furthermore, these observations led us to propose that, in addition to cell wall loosening enzymes, abscission requires (or is enhanced by) biosynthesis and secretion of small proteins (15–25 kDa) and waxes that form an extensible extracellular matrix and boundary layer on the surface of separating cells. The synthesis

of the boundary layer precedes what is typically associated with the post-abscission synthesis of a protective scar over the fracture plane. This modification in the abscission model is discussed in regard to how it influences our interpretation of the role of multiple abscission signals.

Keywords: **abscission, Arabidopsis, ethylene, IDA, soybean, tomato, transcriptome, cuticle biosynthesis**

INTRODUCTION

The process of abscission is roughly divided into four sequential stages with slight modifications by different authors (Patterson, 2001; Estornell et al., 2013): first, abscission zone (AZ) differentiation; second, competence to respond to abscission signals; third, activation of abscission; and fourth, formation of a protective layer and post-abscission trans-differentiation. It was recognized many years ago, based on light and electron microscopy, that during the activation of abscission (i.e., abscission stage 3) AZ cells expand at the fracture plane and the cells separate along the middle lamella (Hall and Sexton, 1974). It has been assumed that the swelling of these cells helps to create the forces necessary to break vascular connections (i.e., rigid xylem vessels), which then allows the organs to separate. Many genes and proteins linked to cell wall loosening and degradation of the middle lamella including cellulases (beta-1,4-endoglucanases, CELs), polygalacturonases (PGs), xyloglucan endotransglucosylase/hydrolases (XTHs), and expansins (EXPs) have been identified and characterized from AZ of several different plant species (Roberts et al., 2002). Also commonly observed at this stage in organ abscission is an up-regulation of defense genes, which includes pathogenesis-related (*PR*) genes (Del Campillo and Lewis, 1992; Meir et al., 2011; Gonzalez-Carranza et al., 2012; Estornell et al., 2013). The role of *PR* gene expression during abscission is often assumed to protect vulnerable abscising cells from opportunistic pathogen invasion (Del Campillo and Lewis, 1992).

In addition to cell wall loosening and *PR* genes, a great deal of attention has been directed toward the identification and characterization of signals that initiate abscission (Taylor and Whitelaw, 2001; Roberts et al., 2002; Liljegren, 2012). Phytohormones and other signals that have been shown to modulate abscission are many-fold: ethylene, auxin, abscisic acid, jasmonic acid, and IDA (a small secreted peptide named INFLORESCENCE DEFICIENT IN ABSCISSION) (Taylor and Whitelaw, 2001; Roberts et al., 2002; Liljegren, 2012; Kim et al., 2013). These abscission signals, however, may not all act in synchrony but sequentially or independently (Patterson and Bleeker, 2004). It is not our intention here to evaluate the role of a multitude of abscission signals, but rather focus on the cellular processes that these signals initiate and regulate. A more complete understanding of the processes and mechanisms utilized for successful organ separation will help to clarify the interactions and interdependencies of the various signals that regulate these processes.

Technological advances have made it possible to now obtain expression results for the entire transcriptome of any desired tissue or developmental program (Zhong et al., 2011; Grassi et al., 2013). In the present work we performed RNA-seq of soybean leaf AZs (LAZ) and the petioles after the AZs were removed (non-AZ petiole, NAZ-pet) harvested from explants exposed to ethylene for 0, 12, 24, 48, and 72 h, and compared the results to microarray data for RNA collected from tomato flower pedicel AZ (FAZ) and proximal non-AZ pedicel (NAZ) at 0, 4, 8, 12, 16, and 20 h after removal of the flowers. In addition, we compared the soybean and tomato results to RNA-seq results for the transcriptomes of *Arabidopsis* flower receptacles from wild type (WT) and the *hae-hsl2* double mutant (Niederhuth et al., 2013). If left untouched, the petals, sepals and stamens of the *hae-hsl2* mutant do not abscise (Niederhuth et al., 2013). The *Arabidopsis* data is not a time-course study of differential gene expression during floral organ abscission; nevertheless, the AZ tissues (receptacles) were collected at an early stage of abscission (stage 15) when changes in gene expression for cell separation were first evident in the WT AZ, and, therefore, useful for comparing to early stages of soybean and tomato abscission. Inclusion of the *Arabidopsis* results in comparison to the soybean and tomato results further highlights shared processes associated with abscission and also the independence and interdependence of regulatory pathways controlling abscission.

We focus here on genes associated with the disassembly and modification of the primary cell wall of the AZ cells, and also the synthesis of a new and different extracellular matrix on the AZ cells as abscission progressed. As expected, we found many homologous and orthologous genes annotated as cell wall loosening and PR proteins that were previously reported to be up-regulated during abscission in many species (Tucker et al., 1988; Kalaitzis et al., 1997; Roberts et al., 2002; Lashbrook and Cai, 2008; Meir et al., 2010). Of special interest here was the early and abundant expression in all three transcriptomes of genes encoding small (15–25 kDa) secreted proteins and genes associated with the deposition of a wax-like cuticle. The expression profiles for many of these genes were very similar to those for cell wall disassembly, i.e., cellulase, PGs, and EXPs. These observations led us to propose that secretion of small proteins and a wax-like cuticle might play an important role in restructuring the extracellular matrix to facilitate organ separation independent of the deposition of a protective scar after separation is complete. The need to synthesize a boundary layer during separation is discussed in regard to abscission signals and their regulation of gene expression required for successful organ separation.

METHODS

Plant Material

Soybean (*Glycine max*, cv. Williams82) plants were grown in the greenhouse and harvested when the primary leaves were fully expanded (19–24 days). Stem-petiole explants were prepared by cutting the stem approximately 4 cm below the leaf node and removing all but approximately a 5-mm triangular portion of the primary leaf blade (**Supplemental Figure S1**). Explants were placed in Erlenmeyer flasks with water and put into a darkened chamber wherein 25 µL/L ethylene in air saturated with water was passed through at a rate of 2 L/min at 25°C. In soybean, there is an AZ at the base of the petiole at the juncture with the stem (lower AZ) and another AZ at the distal end of the petiole approximately 1 mm below the leaf blade (upper AZ) (**Supplemental Figure S1**). In our system with ethylene treatment, the petiole separated from the stem at the lower AZ at approximately 48 h from just the weight of the petiole, but the distal portion of the upper AZ at the top of the petiole, which is rather small and light, did not sometimes fall away from the petiole even after 72 h of ethylene. To assess the extent of separation at the upper AZ, the distal part of the AZ was gently touched with forceps and, if it fell away from the petiole, the AZ was recorded as having fully abscised. Nonetheless, with high humidity, the petiole and AZ continue to senesce after separation from the parent plant, and did not dry out. Approximately 2 mm, which included approximately 1 mm of proximal and distal sides of the upper, leaf AZ (LAZ), was collected from 20 explants (2 upper AZs per explant or 40 AZs total) and flash frozen in liquid nitrogen. After excising the AZ, the petiole ends were again trimmed to avoid incidental collection of any AZ tissue and the petioles (NAZ-pet) were flash frozen. The lower AZ was not collected, because at this stage of growth the petioles are relatively small and we wanted to avoid incidental collection of a portion of the lateral bud that is very close to the lower AZ. Collection of a small part of a lateral bud with a meristem in it would compromise the interpretation of AZ-specific gene expression. AZs and petioles (NAZ-pet) were collected at 0 h (immediately prior to ethylene treatment) and at 12, 24, 48, and 72 h after the ethylene treatment had begun. Plants were grown, explants prepared and samples collected three separate times to give three independent experimental replicates.

Flower clusters (inflorescences) from tomato (*Solanum lycopersicum*) cv. New Yorker were harvested from 4-month-old greenhouse-grown plants between 08:00 and 10:00 a.m. Explants were prepared as previously described (Meir et al., 2010). Briefly, inflorescences bearing at least 2–4 freshly open flowers were brought to the laboratory under high humidity conditions. Closed young flower buds and senesced flowers including the entire pedicel and AZ joint were removed so that only freshly opened flowers and pedicels remained on the inflorescence. The stem ends were trimmed, and groups of 2–3 inflorescence explants were placed in vials containing double distilled water (DDW). Abscission was induced by cutting the flowers off the inflorescence at the base of the receptacle. Cutting off the flowers removes a source of auxin that inhibits abscission (Meir et al., 2010). Tissue samples for RNA extraction were taken from the

flower AZ (FAZ) and proximal pedicel (NAZ) of 30 segments. The 2 mm FAZ collection included approximately 1 mm on either side of the AZ joint or fracture once it appeared. Samples were collected at 0 h (immediately before flower removal) and 4, 8, 12, 16, and 20 h after flower removal. Explants were harvested and tissue samples collected twice for two independent experimental replicates.

RNA Sequencing and Microarrays

RNA was isolated from soybean LAZ and NAZ-pet after 0, 12, 24, 48, and 72 h of exposure to ethylene using a Qiagen RNeasy Mini Kit following the standard protocol (Qiagen, Germantown, MD, USA). Each experiment produced 10 RNA samples, which resulted in 30 RNA samples for the 3 replicate experiments. Further RNA purification, cDNA synthesis and sequencing on an Illumina GAII sequencer were performed at Cornell University, Ithaca, NY, USA as previously described (Zhong et al., 2011; Grassi et al., 2013). The 30 RNA samples were processed, barcoded and run together on the GAII sequencer. The raw sequence files have been submitted to the NCBI SRA databases with the study accession SRP050050. On average, each RNA sample produced approximately 4 million reads (**Supplemental File S2**). Raw sequences were trimmed to remove ambiguous ends. Using Bowtie (Langmead et al., 2009), approximately 40,000 (1%) of the reads mapped to ribosomal RNA (rRNA) and were removed from the data set. Using TopHat (Trapnell et al., 2009), approximately 90% of the remaining RNA mapped to a predicted soybean transcriptome (cds) (*G. max* 189 genome assembly). Multiple versions (splice variants) were not taken into account. A single version (usually the last version) was used for alignment. A total of 54,175 transcripts were used for the alignment. The number of reads that aligned to a gene was normalized as Reads Per Kilobase per Million mapped reads (RPKM) (Mortazavi et al., 2008).

For a gene to be counted as expressed in the LAZ or NAZ-pet, we required that the mean RPKM for the three replicates be at least 1.0 or greater in at least one of the treatments. Using a cutoff of 1.0 RPKM resulted in the selection of 37,572 genes as being expressed in the soybean LAZ or NAZ-pet between 0 and 72 h of exposure to ethylene (**Supplemental File S2**). Then, to avoid ratios with a zero in the numerator or denominator, any RPKM of less than 0.1 was given the minimal value of 0.1. QPCR was performed as previously described (Tucker et al., 2007) on a few selected genes to confirm that the RNA-seq and RPKM normalization produced the expected expression profile (results not shown).

The Meir lab at the Volcani Center, Bet-Dagan, Israel has prepared a tomato AZ-specific microarray chip in collaboration with Genotypic Technology Pvt. Ltd., Bangalore, India. Each chip includes 111,718 probe sets for more than 40,000 transcripts. Probe sets were designed using RNA-seq results for pooled RNA samples from non-induced and induced tomato flower pedicel and AZ tissue as described above. However, for the RNA sequencing, the tissues were collected from *Solanum lycopersicum* cv. "VF-36." Transcriptome libraries for sequencing were constructed according to the Illumina TruSeq RNA library protocol outlined in "TruSeq RNA Sample Preparation Guide"

(Part # 15008136; Rev. A, Illumina, USA). The DNA obtained from the prepared libraries was denatured and sequenced on the Illumina Genome Analyzer IIX, using the sequencing by synthesis method to read 72 bases per end. The raw sequencing data were then extracted from the server using the proprietary Illumina pipeline software to obtain a sequence data set in a Fastq format. Quality check of raw data was performed using SeqQC -V2.0 program. The raw sequence data and array information were submitted to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) with GEO IDs GSE45355 and GSE45356, and array ID AMADID:043310. Expression was validated by qPCR (results not shown).

For the tomato microarray results, a gene was considered to be expressed, if the minimum mean signal for two replicates was >10 in at least one time point in either the NAZ or FAZ. This selection criterion resulted in the selection of 26,527 genes as being expressed in the FAZ or NAZ. Then, to avoid ratios with a zero in the numerator or denominator, any signal of less than 1.0 was given the minimal value of 1.0.

The RNA-seq results reported here for Arabidopsis WT and the *hae-hsl2* mutant are a reformulation of data from Additional file 2 in Niederhuth et al. (2013). Arabidopsis receptacles, which included the floral organ AZs, were collected as described (Niederhuth et al., 2013). To conform to the analysis of the soybean and tomato transcriptomes, the log₂ fold change was reversed to WT over *hae-hsl2*. Similar to the other data sets, a minimum of >10 reads per gene was required to consider the gene as being expressed in either WT or the mutant, and any reads per gene of less than 1.0 was given a minimal value of 1.0 to avoid complications arising from having a zero in the numerator or denominator (**Supplemental File S4**).

RESULTS AND DISCUSSION

Differences in Experimental Design

The objective of this study was to identify processes directly linked to successful organ separation that are common to a variety of abscission systems. However, how abscission data is collected is not done the same by all researchers. Therefore, what first needs to be addressed and fully understood are the differences in experimental approaches used to study abscission in soybean, tomato and Arabidopsis. The explant system used to study abscission in soybean and tomato was established many years ago (Addicott, 1982; Sexton and Roberts, 1982). It is a versatile system in which a variety of hormones and chemicals can be exogenously applied. An important part of the explant system is the removal of the source of auxin, i.e., soybean leaves or tomato flowers. Moreover, in soybean and tomato, ethylene is essential for leaf and flower abscission, respectively (Lanahan et al., 1994; Meir et al., 2010; Tucker and Yang, 2012). In the soybean experiments, the explants were continuously exposed to a high concentration of ethylene (25 μ L/L), which synchronizes and accelerates abscission, but also induces senescence and ethylene-regulated defense gene expression (Abeles et al., 1992). After a 72 h exposure to ethylene, both the petiole and AZ turned

yellow. Thus, treating the entire explant with ethylene removes the variable of none uniform synthesis of ethylene between the AZ and petiole, and reduces differential gene expression linked solely to ethylene.

In the tomato experiments, the inflorescences were not treated with ethylene. However, because ethylene is essential for tomato flower abscission (Lanahan et al., 1994; Meir et al., 2010), it is likely that upon removal of the auxin source ethylene was synthesized in the inflorescence but the synthesis of ethylene may not have been equal in the FAZ and NAZ (discussed later). Nonetheless, the tomato system reflects a more natural process that would occur during flower abscission on the intact plant.

The leaves and flowers of Arabidopsis do not normally abscise and, therefore, Arabidopsis abscission scientists study the abscission of floral organs, petals, stamens, and sepals (Bleecker and Patterson, 1997; Patterson, 2001; Butenko et al., 2003). The Arabidopsis data we used here was collected and published by Niederhuth et al. (2013). They collected flower receptacles from WT and the double mutant *hae-hsl2* at developmental stage 15 flowers. HAE and HSL2 are redundant receptor-like kinases that bind the IDA (INFLORESCENCE DEFICIENT IN ABSCISSION) signaling peptide (Cho et al., 2008; Stenvik et al., 2008; Butenko et al., 2014). In the *ida* mutant, as in the *hae-hsl2* double mutant, the floral organs do not abscise if they are left untouched (Butenko et al., 2003). At flower stage 15 both WT and mutant flowers are fully open but the petals have not abscised (Niederhuth et al., 2013). Stage 15 flowers correspond to approximately the same developmental stage as flowers at positions 3 and 4 used in other studies of Arabidopsis floral organ abscission (Patterson and Bleecker, 2004; Cai and Lashbrook, 2008; Cho et al., 2008; Basu et al., 2013; Liu et al., 2013). In wild-type flowers at positions 3–4 abscission-associated gene expression has already begun and the break-strength of the petals has started to decline but the petals have not fallen off under their own weight (Kim and Patterson, 2006; Cai and Lashbrook, 2008; Niederhuth et al., 2013). The levels of auxin and ethylene in this system are unknown. Nonetheless, ethylene can accelerate abscission of Arabidopsis floral organs (Gonzalez-Carranza et al., 2007) but it does not appear to be essential because floral organ abscission is only delayed in ethylene-insensitive mutants (Patterson and Bleecker, 2004). Moreover, auxin also plays a role in Arabidopsis floral organ abscission (Basu et al., 2013). If auxin levels are genetically up-regulated or down-regulated in the AZ of Arabidopsis floral organs, abscission is delayed or accelerated, respectively (Basu et al., 2013). Thus, although the experimental approach to study Arabidopsis abscission is different from soybean and tomato and ethylene appears not to be essential to Arabidopsis floral organ abscission, comparison of differential gene expression in the transcriptomes of WT and the *hae-hsl2* mutant to soybean and tomato provides additional information and confirmation of conserved molecular and metabolic processes that contribute to organ separation.

Overview of Differential Gene Expression

We plotted the overall change in expression for those genes that changed markedly over time in the AZ and NAZ of soybean

and tomato and between WT and *hae-hsl2* of Arabidopsis. For soybean and tomato we used an arbitrary threshold for a change in gene expression of >8 -fold ($\log_2 > 3$ or <-3) (Figure 1). In soybean where the auxin source has been removed and the explants treated with ethylene, approximately 5% of all the expressed genes displayed an increase in expression of >8 -fold in both the AZ and petiole; however, the expression of a much greater number of genes, 30%, decreased >8 -fold (Figure 1A). Although 5% of all the genes increased and 30% decreased, only about 1% of the genes were expressed in an AZ-specific manner. Interestingly, at 72 h the 5% of the genes that increased in expression accounted for 50% of the transcripts in the transcriptome of both the AZ and petiole (Figure 1A). There was also a significant increase in transcripts that were AZ-specific but still much less than the total. In tomato, there was a similar but much smaller decline in gene expression that is common to both AZ and NAZ (Figure 1B). In tomato flowers, which abscise much more rapidly than the soybean leaves, approximately 2% of the genes changed expression >8 -fold by 16 h, which accounted for almost 9% of the transcripts in the transcriptome; however, 90% of the gene expression was AZ-specific (Figure 1B). A possible explanation for why most of the differential expression >8 -fold was AZ-specific in tomato may be that ethylene synthesis was greater in the AZ than the NAZ (discussed later).

In Arabidopsis, the magnitude of differential gene expression between WT and the mutant was relatively small. Less than 2% of the genes increased in expression more than 2-fold, $\log_2 > 1$, which accounted for only a 0.5% change in the total transcriptome (Figure 1C). There are at least two explanations for the small difference in gene expression between the WT and *hae-hsl2* receptacles. First, the receptacle collection was an early stage of abscission, and, second, the IDA signaling path is one of several signals that regulate gene expression in Arabidopsis abscission (discussed later).

The leaves and flowers were removed from the soybean and tomato explants, respectively, and, as expected, the removal of the auxin source is reflected in a general decline in auxin associated gene expression in both the AZ and NAZ (Figures 2, 3). Nonetheless, there were a few auxin-associated genes that increased in an AZ-specific manner in both soybean and tomato (Figures 2, 3) and many of these were linked to auxin movement (e.g., PIN) or auxin conjugation (e.g., GH3) (Supplemental Files S2, S3).

The soybean explants were treated with ethylene and because of this there was an expected non AZ-specific increase in gene expression for ethylene-associated genes linked to senescence and programmed cell death (PCD) (Figures 2A, 3A). There was also an expected increase in ethylene-induced defense gene expression (PR gene expression) (Figures 2A, 3A); nonetheless, several PR genes were expressed AZ-specifically (see below). In tomato, the increase in ethylene-associated genes (senescence, PCD and PR genes) was mostly AZ-specific (Figures 2B, 3B). In both soybean and tomato there was an AZ-specific increase in expression of genes for ethylene synthesis (i.e., ACS and ACO) (Supplemental Files S2, S3). This would suggest that in tomato ethylene synthesis was AZ specific whereas in soybean, because the explants were treated with a high

concentration of ethylene, the AZ-specific synthesis of ethylene was inconsequential. Thus, in tomato, ethylene-induced gene expression was AZ-specific due to the synthesis of ethylene in the AZ tissue.

Changes in the Transcriptome Linked to Cell Wall Disassembly

Genes within the categories for cellulases (CELS), pectinases (PGs) and pectin lyase-like (PGs and PLs), EXPs, XTHs were selected based primarily on their annotation, which is most often derived from the most similar gene sequence in Arabidopsis. The category labeled cellulases included any gene annotated as cellulase or glycoside hydrolase (GH) family 9, but genes in the subfamily GH9A1 were placed into the cellulose biosynthesis category, which is more appropriate for *KORRIGAN*-like cellulase genes (Doblin et al., 2002).

As expected, AZ-specific gene expression for cell wall disassembly proteins increased markedly in soybean, tomato and Arabidopsis (Figures 1, 2). Of interest in regard to cell wall disassembly was an unexpected result for XTH expression in soybean leaf abscission. Transmission electron micrographs indicate that cellulose microfibrils (striations) are untethered during abscission but not degraded (Hall and Sexton, 1974). It is generally accepted that a xyloglucan network plays an important role in tethering cellulose microfibrils and, along with pectins distributed within the primary cell wall, may influence the free movement of proteins and other large compounds across the primary cell wall out to the middle lamella (Carpita and Gibeaut, 1993; Cosgrove, 2001). It was surprising to find that in soybean no XTHs were up-regulated in an AZ-specific manner and most XTHs declined (Figure 2). However, in tomato and Arabidopsis there was an AZ-specific increase in expression of XTH. Ethylene treatment of soybean may be part of the reason for this difference. It is plausible that XTHs also play a role in ethylene-induced senescence and this is why XTH was not LAZ-specific. However, there is another possibility. Cellulase (beta-1,4-endoglucanase) activity is assayed using carboxymethyl cellulose, which is a soluble cellulose derivative (Urbanowicz et al., 2007). *In vitro* assays with purified plant cellulases by themselves do not degrade crystalline cellulose microfibrils. The *in vivo* substrates for cellulases are not known. Therefore, it is possible that some cellulases may cleave chains of beta-1,4-glucans within xyloglucan polymers that fix the cellulose microfibrils in place (Hayashi and Kaida, 2011; Eklöf et al., 2012), or they cleave beta-1,4-glucans at the surface of the microfibrils that play a role in tethering and crosslinking the microfibril network. Transcription of *GmCEL01* increased earlier than other cell wall loosening enzymes during soybean abscission (Figure 3A). It is possible that the role of the *GmCEL1* enzyme is to loosen the hemicellulosic fraction (xyloglucans, etc.) or their tethering to the cellulose microfibrils, which then opens up the cell wall for movement of protein and other compounds out to the middle lamella.

A heat map (Figure 2) is useful to display a general perspective on the expression patterns within each category of genes, but it does not take into account transcript abundance. For example, *GmCel01*, (Glyma11g02350, accession U34755) accounted for

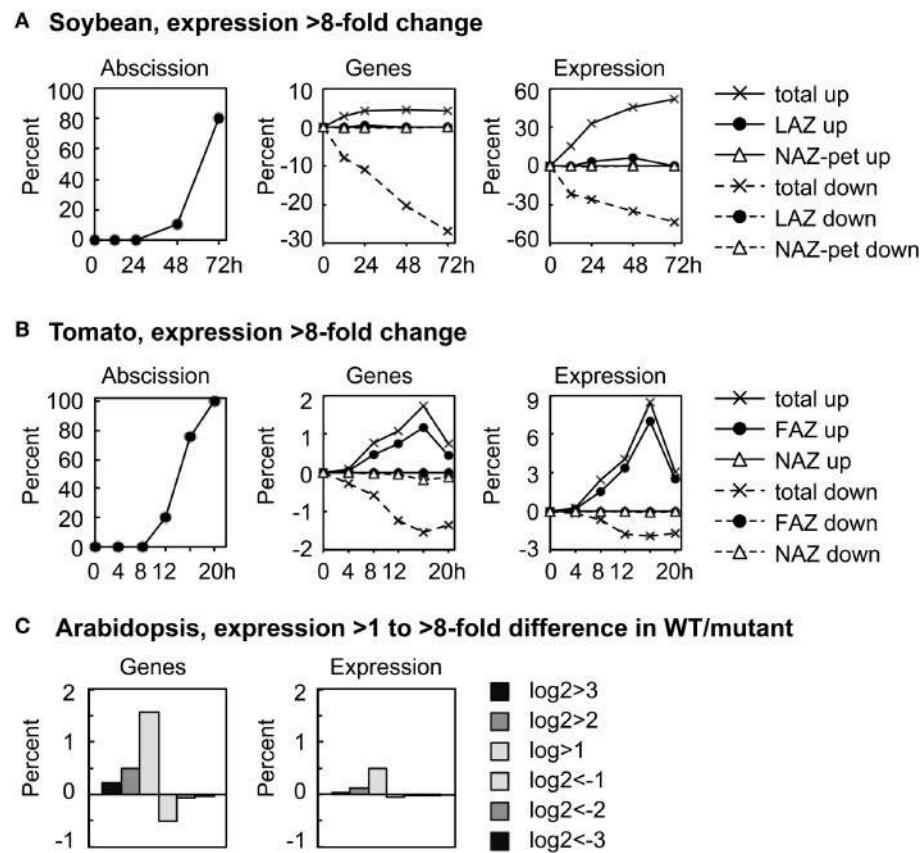


FIGURE 1 | Overall perspective on major changes in gene expression during abscission of soybean leaves, tomato flowers and Arabidopsis floral organs. **(A)** The percent of soybean pulvinal leaf abscission and the percent of genes with greater than 8-fold change ($\log_2 > 3$ or < -3) in expression relative to the total number of genes expressed (37,572) and the differential expression of the same genes relative to the average total RPKM for each RNA sample (666,213 RPKM). **(B)** Similar to **(A)** except that tomato flower abscission had 26,572 expressed genes with a total average microarray signal of 64,696,620. **(C)** The percent of expressed genes in wild-type Arabidopsis receptacles relative to genes in the double mutant *hae-hsl2* and the differential expression of the same genes (Niederhuth et al., 2013). In the Arabidopsis data, 20,883 genes were counted as expressed with a total mean of 38,770,114 reads. Because differential expression was much less in the Arabidopsis data, 2-, 4-, and 8-fold differences are plotted as a bar graph.

approximately 75% of the RNA for the 27 CELs expressed in the soybean LAZ (Figure 3A). The same was true for tomato where *SICEL1* accounted for 75% of the transcript for 17 CELs (Figure 3B). Similarly, one or just a few PGs accounted for most of the transcript in both soybean and tomato AZ (Figures 3A,B). This was not necessarily the case for other gene families (Figure 3 and Supplemental Files S2–S4).

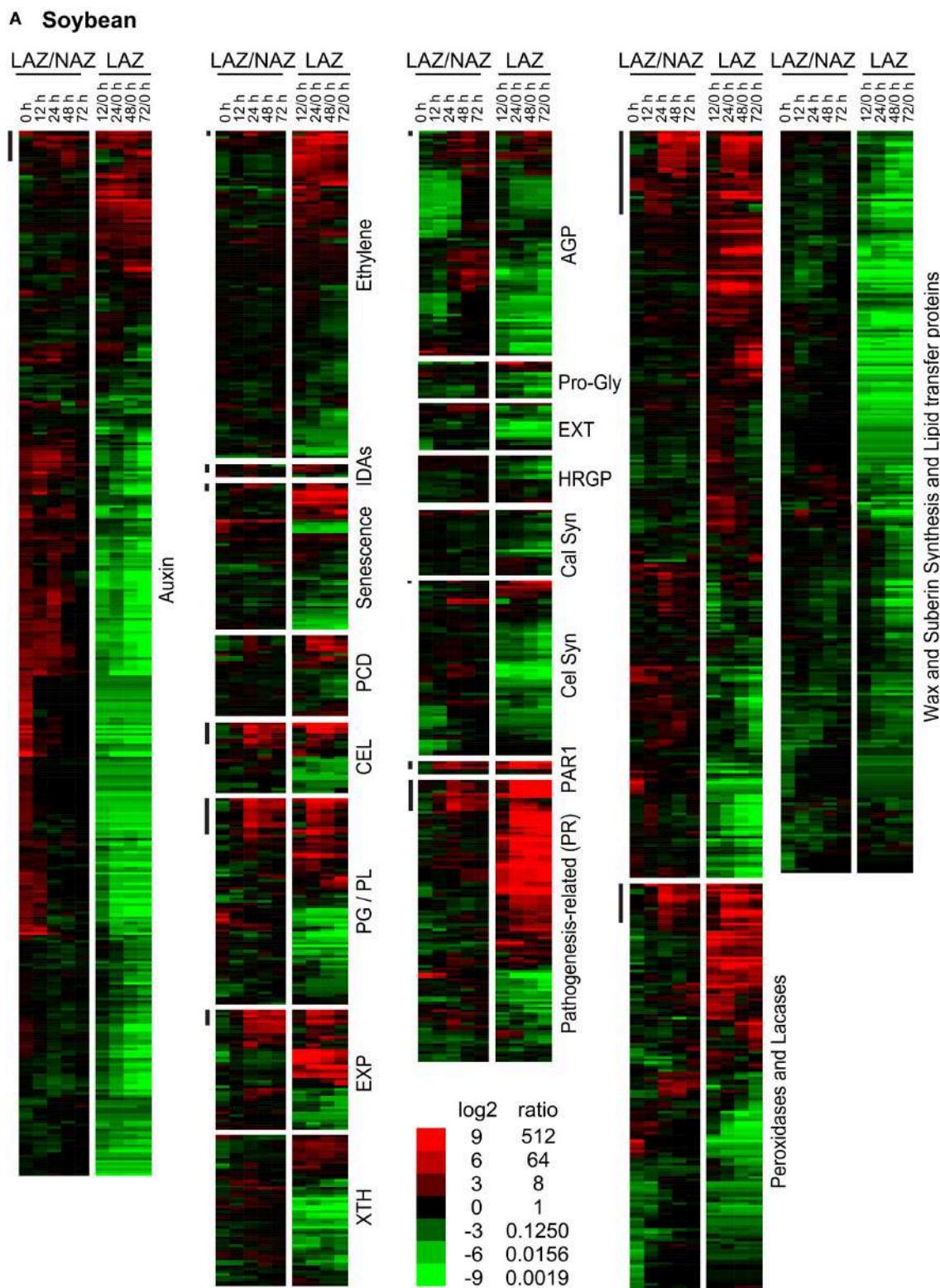
Expression of EXPs in abscission was more complex. In soybean, EXPs increased markedly in both the LAZ and petiole but only alpha EXPs were AZ-specific, but the AZ-specific expression of alpha EXPs was less than 10% of the overall expression for EXPs (Figure 3A). EXP expression in tomato had an AZ-specific component to it but the level of EXP gene expression was already relatively high at 0h (Figure 3B). Based on our results, the role of EXPs in abscission is not easily interpreted.

Gene expression for cell wall disassembly in Arabidopsis abscission will be discussed in greater detail later. Nonetheless, the expression of CELs, PGs, EXPs, and XTHs were

significantly higher in WT than the mutant (Figure 3C and Supplemental File S4). What was most surprising in contrast to soybean and tomato abscission was that the level of gene expression for all of the cell wall disassembly genes in both the WT and the *hæ-hsl2* mutant started at fairly high levels (Supplemental File S4).

Expression of Cellulose Synthases and Typical Primary Cell Wall Proteins

We considered the possibility that the cell wall and middle lamella of fracture plane cells would be degraded but once separation was complete the same cells would synthesize a new cell wall much like the previous one that included new cellulose microfibrils, hemicelluloses and protein. The primary wall of a plant cell consists of approximately 20% protein (Carpita and Gibeaut, 1993). To assess whether or not a typical cell wall was synthesized we examined the expression of cellulose synthesis genes and typical primary cell wall proteins, extensins

**FIGURE 2 | Continued**

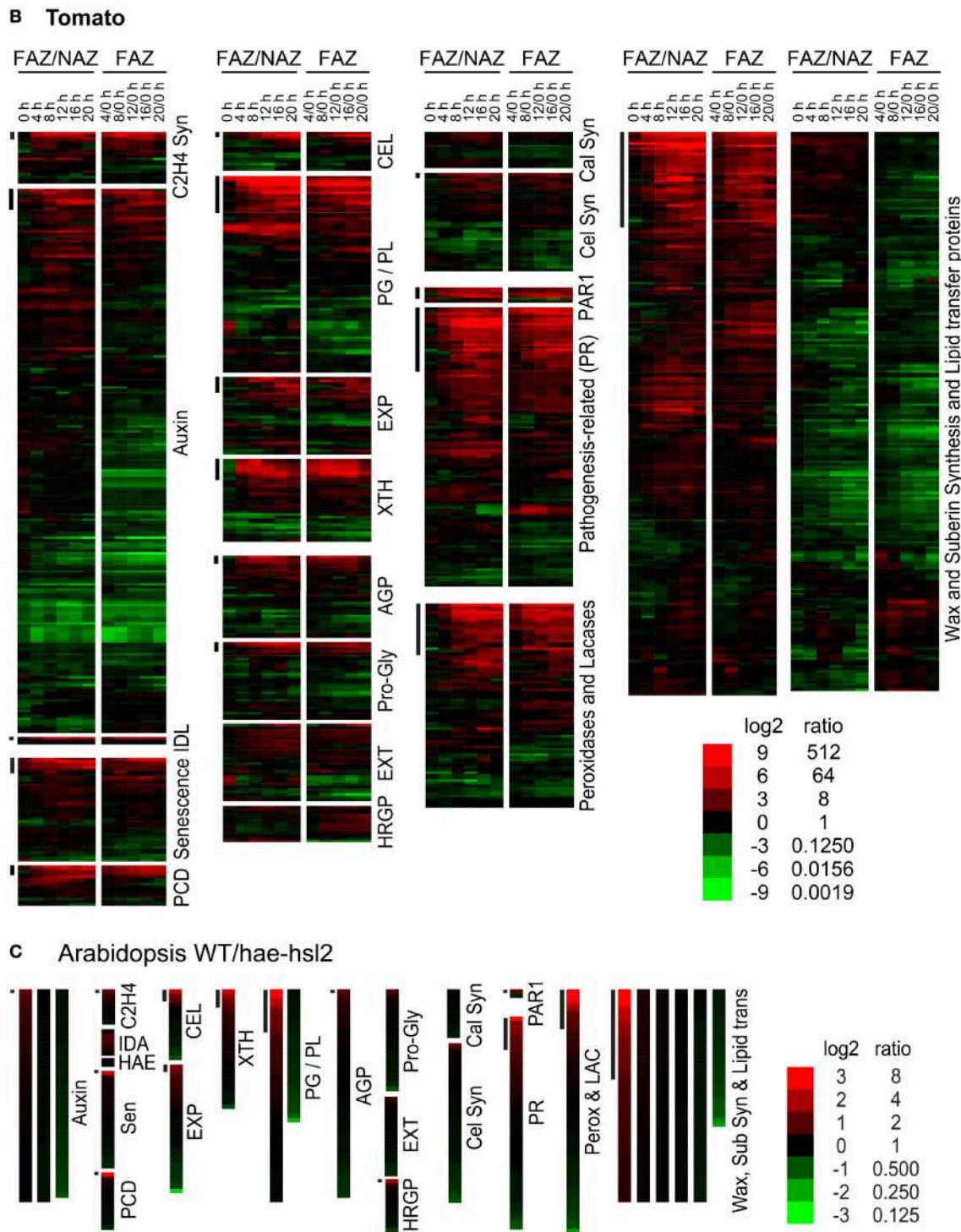
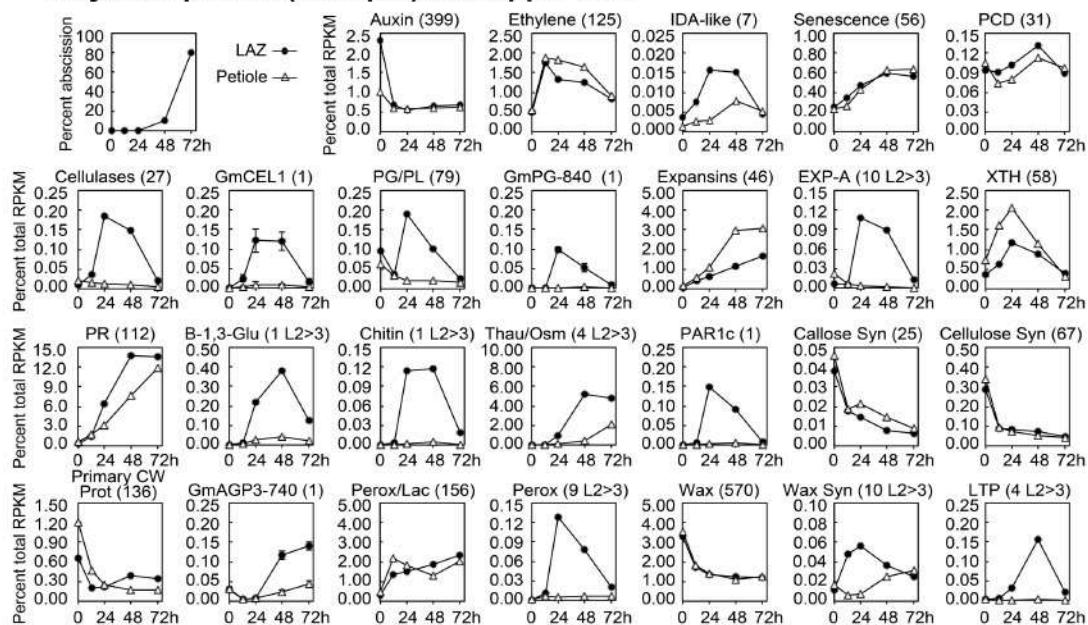
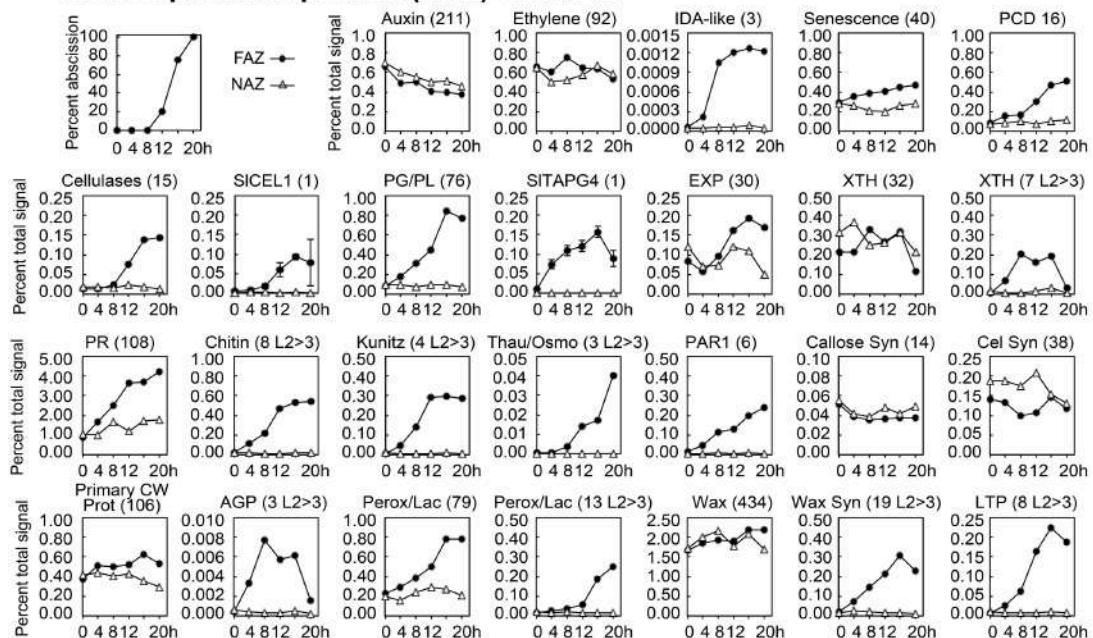


FIGURE 2 | Heat map display of expression profiles for genes in soybean leaf pulvinus AZs (LAZ) and petioles with the AZs removed (NAZ-pet), tomato flower AZs (FAZ) and proximal pedicel (NAZ) and Arabidopsis wild type (WT) and *hae-hsl2* mutant receptacle AZs. (A) Soybean, **(B)** tomato, and **(C)** Arabidopsis gene expression. The expression profiles are presented as ratios for AZ-specificity (AZ/NAZ), and as ratios for AZ expression at different time intervals relative to 0 h (start of ethylene treatment of soybean or removal of auxin source for tomato), e.g., 12/0, 24/0, 48/0, or 72/0 h. The bars at the top left of each category mark genes whose expression is >8-fold AZ-specific (AZ/NAZ) and >8-fold up-regulated (time interval/0 h). The scale at the bottom indicates the color representing the log2 ratio and ratio listed next to it. The color scale for Arabidopsis is different than soybean and tomato because differential expression was less in the Arabidopsis tissues. The genes in each category, their nomenclature, log2 ratios, and annotations are given in **Supplemental Files S2–S4** for soybean, tomato and Arabidopsis, respectively.

A Soybean petiole (NAZ-pet) and upper LAZ



B Tomato proximal pedicel (NAZ) and FAZ



C Arabidopsis floral organ abscission (stage 15), Niederhuth et al. 2013

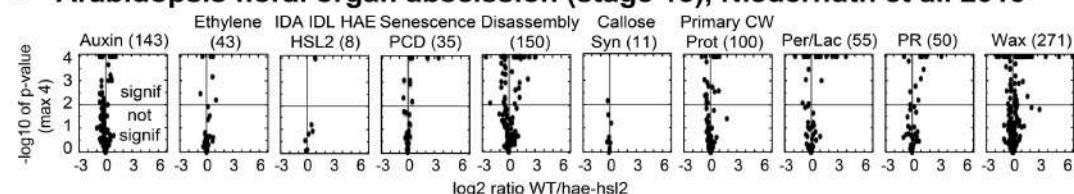


FIGURE 3 | Line graphs (soybean and tomato) and volcano plots (Arabidopsis) showing the change in gene expression for selected groupings of genes and some individual genes. The percent of abscission is indicated in the graph at the top left corner the sections for (A) soybean and (B) tomato as a reference. (A) The Y-axes indicate the percent expression relative to total RPKM for all 37,572 expressed soybean genes (transcriptome). The X-axes indicate the length of

(Continued)

FIGURE 3 | Continued

exposure to ethylene. Solid circles represent expression in the LAZ and the open triangles in petioles (NAZ-pet). (B) Similar to (A) but The Y-axes indicate the percent expression relative to total microarray signal for 26,527 expressed tomato genes. The X-axes indicate the hours after removal of the flowers, auxin source. Solid circles represent expression in the FAZ and the open triangles in NAZ. The number within the parenthesis to the right of each category or gene name is the number of genes included for that particular graph. Plots with an L2>3 after the number in parentheses show the cumulative expression for only those genes with a significant 8-fold increase in expression ($p < 0.015$ and $\log_2 > 3$ for any time relative to 0 h). Standard error bars cannot be calculated for plots of multiple genes. (C) Volcano plots of differential expression in WT/*hae-hsl2* receptacles at developmental stage 15. Y-axes are the $-\log_{10}$ of the p -values. A $p < 1\%$ (<0.015) is a p -value of $-\log_{10} > 2$, which is indicated by the horizontal line through the middle. Any expression with a p -value (significance) of less than 0.0001 ($-\log_{10} > 4$) was set to a minimum of 0.0001, which is why many points align across the top. The X-axes are the log₂ ratios for expression (reads) of WT/*hae-hsl2*. The genes in each category, their nomenclature, log₂ ratios, and annotations are given in **Supplemental Files S2–S4** for soybean, tomato, and Arabidopsis, respectively.

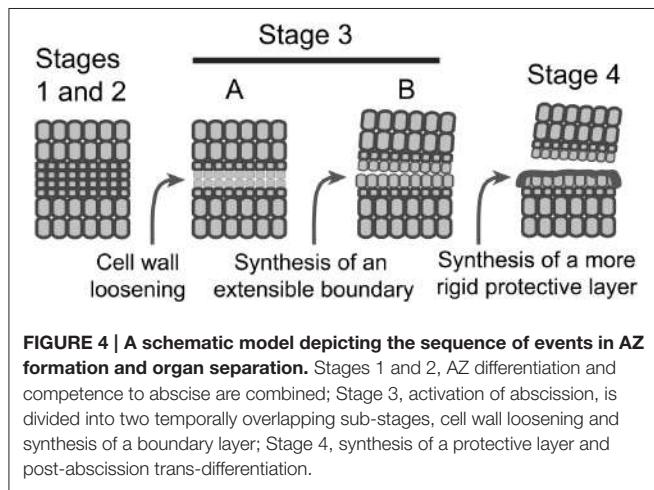


FIGURE 4 | A schematic model depicting the sequence of events in AZ formation and organ separation. Stages 1 and 2, AZ differentiation and competence to abscise are combined; Stage 3, activation of abscission, is divided into two temporally overlapping sub-stages, cell wall loosening and synthesis of a boundary layer; Stage 4, synthesis of a protective layer and post-abscission trans-differentiation.

(EXTs), arabinogalactan proteins (AGPs), proline and glycine-rich proteins, and hydroxyproline-rich glycoproteins (HRGPs). In soybean, the expression of almost all of these genes declined (Figures 2A, 3A); however, in tomato and Arabidopsis, this grouping of genes were mostly unchanged or increased slightly (Figures 2B,C, 3B,C). Based on these observations, we conclude that cellulose synthesis and synthesis of typical primary cell wall proteins are not a significant part of abscission or formation of a protective layer.

Synthesis of a More Proteinaceous Extracellular Matrix

Overall there was a strong up-regulation of *PR* genes. It might seem odd to include *PR* gene expression under the sub-heading of synthesis of a new and different extracellular matrix because the role of *PR* gene expression during abscission is customarily assumed to protect the vulnerable abscising cells from opportunistic pathogen invasion (Del Campillo and Lewis, 1992). However, the first and possibly the most important defense against pathogens is a structural barrier, which includes the cell wall and cuticle (Hamann, 2012). However, at the site of a microbial infection, the cell wall is further reinforced by the synthesis of papillae (Voigt, 2014). Callose, a 1-3 linked beta glucan polymer, is major component of papillae. Because gene expression for callose synthesis is either down-regulated or unchanged in all three abscission systems, we conclude that callose is not a primary component in the new extracellular

matrix (Figures 2, 3). Nonetheless, what is important to successful organ separation is the creation of a flexible barrier to pathogens that both inhibits infection and allows cell expansion. We argue that in addition to an enzymatic role in the defense against pathogens, a remodeled extracellular matrix containing an abundance of small PR proteins is important to the actual separation process. An extracellular matrix made up of cross-linked protein rather than long chains of polysaccharides, i.e., callose, cellulose or hemicelluloses, might better allow for cell enlargement that creates the physical stress across the fracture plane, but still provide enough structure to prevent the fracture plane cells from rupturing.

What is the PR protein composition of the extracellular matrix? It does not appear to be the same in each of the three systems. In soybean, *thaumatin* begins to increase early and by 48 h expression of *thaumatin* accounted for 5% of the AZ transcriptome (Figure 3A and Supplemental File S2). Expression of *Chitinase* and *beta-1,3-glucanase* is also greatly up-regulated in an AZ-specific manner. In tomato, *chitinase* and *kunitz trypsin inhibitor* are abundantly expressed in the AZ but *thaumatin* is not as strongly expressed (Figure 3B). In Arabidopsis, *thaumatin*, *kunitz trypsin inhibitor*, and *chitinase* are all more highly expressed in WT than the mutant (Supplemental File S4).

PAR1 (*photoassimilate-responsive-1*) is also considered to be a *PR* gene (Herbers et al., 1995). We have separated *PAR1s* from the others because they exemplify our view that synthesis of a new protein-rich extracellular matrix is important to the separation process. *PAR1s* are expressed in an AZ-specific manner in all three systems with an expression profile much like the AZ-specific *CELs* and *PGs*. *PAR1s* are small proteins (approximately 18 kDa) with no known enzymatic function but have several cysteines that might support protein cross-linking in the cell wall. Also, *PAR1s* are highly conserved in both dicots and monocots (Supplemental File S5).

Synthesis of a Waxy Cuticle

Now, to detail and discuss our observation that was somewhat unexpected for us because it is not commonly discussed in the abscission literature as being a part of the separation process but rather a part of the synthesis of a protective layer after separation has occurred. We found an early AZ-specific increase in gene expression of several genes that are best linked to the synthesis and secretion of a waxy cuticle. Because both wax and suberin biosynthesis require protein for lipid modification and transport and phenylpropanoid metabolism,

we have also included suberin-associated genes in this category. Genes included in the wax-suberin category were selected based on the cuticle synthesis genes listed by Suh et al. (2005), suberin biosynthesis genes listed by Soler et al. (2007) and genes whose gene ontology (GO) annotation included terms for lipid or fatty acid synthesis or modification. Peroxidases are also a part of wax and suberin synthesis. Gene expression for several peroxidases and laccases increased markedly during abscission and some quite early. Although peroxidases and laccases may be involved in the synthesis of waxes and suberin, they may also be tied to cross-linking of protein and pathogen defense (Mattheis and Whitaker, 1984; Almagro et al., 2009). For this reason, we have put *peroxidase* and *laccase* genes into their own category. Nonetheless, collectively, we conclude that independent of the category they are included in peroxidases and laccases are a part of creating a new and different extracellular matrix.

In soybean, there was more than a 50% decline in the expression of the 570 genes in the wax-suberin category (**Figure 2A**). This is probably because cuticle synthesis is not generally required for senescing tissue. However, there was a subset of 10 wax synthesis genes that were significantly up-regulated in the AZ more than 8-fold within the first 12 h (**Figure 2A**). This was significantly earlier than the increase for *CELS* or *PGs*. Three of the 10 wax synthesis genes encoded GDSL-like lipases, which are important to cuticle synthesis (Yeats and Rose, 2013), and the most AZ-specific of the 10 was a *CER4*, Jojoba acyl CoA reductase, which is also important to the synthesis of a wax cuticle (Rowland et al., 2006) (**Supplemental File S2**). In addition to fatty acid and wax synthesis, there was a strong AZ-specific increase in four lipid transfer proteins (LTP) (**Figure 3A**). In tomato, we found 19 wax synthesis and 8 *LTP* genes that increased specifically in the AZ more than 8-fold (**Figure 3B**). Like soybean, the most AZ-specific wax synthesis gene in tomato was a *CER4*, Jojoba acyl CoA reductase (**Supplemental File S3**).

Gene expression for wax synthesis and lipid transfer is particularly interesting in the *Arabidopsis* data. This category of genes (wax-suberin) had the greatest difference between the WT and the *hae-hsl2* mutant (**Figure 3C** and **Supplemental File S4**). Moreover, if one sorts the original published Additional file 2 (fold-change calculated for the opposite ratio, i.e., mutant/WT) the gene showing the greatest difference between WT and the mutant was a *bifunctional inhibitor/lipid-transfer protein* (AT4G22485.1) and the third gene in the sorted file was a *GDSL-like lipase* (AT5G03810.1) (Niederhuth et al., 2013). Both of these cuticle-associated gene families are strongly up-regulated and AZ-specific in soybean and tomato (**Supplemental Files S2, S3**). When the original *Arabidopsis* Additional file 1 was sorted this way, the first cell wall disassembly gene, *PGAZAT* (AT2G41850), was not even in the top 50 differentially expressed genes. The large differential expression of putative cuticle synthesis genes in the *Arabidopsis* data may be significant to an interpretation of the role of IDA signaling in abscission (discussed below).

It should be noted that expression of *LTPs* in abscission has been previously reported (Agusti et al., 2009; Nakano et al., 2013). Here we put *LTPs* expression in context with additional

observations and propose that synthesis of a waxy-like cuticle is important to successful organ separation.

Comparison to Separation During Organogenesis

Based on our abscission transcriptome results, it appears that an early up-regulation of gene expression for synthesis and secretion of a waxy cuticle-like substance during abscission is a common feature for abscission in many species. One possible explanation for this is that the separating cells need to be protected from water loss (Samuels et al., 2008). Also, synthesis of a cuticle could be part of a defense mechanism (Samuels et al., 2008). However, there is another possible role for synthesis of a waxy cuticle. *Arabidopsis* has proven to be an excellent model system for the study of cuticle synthesis (Samuels et al., 2008; Shi et al., 2011; Yeats and Rose, 2013). Interestingly, a common phenotype for knockout mutants for transcription factors and other genes related to the synthesis of the cuticle is that organs remain fused or display structural anomalies (Shi et al., 2011; Yeats and Rose, 2013). Fused floral organs were also observed in a wax-deficient mutant in tomato (Smirnova et al., 2013). The composition of the extracellular matrix around boundary cells that separate organs in the meristem is not well understood (Žádníková and Simon, 2014). Nevertheless, based on the genetic results, it seems likely that the synthesis of a cuticle like substance plays a role in the separation of organs in the meristem. It seems plausible that a similar process might be implemented during abscission of leaves, flowers, fruit, or floral organs. Moreover, expression of meristem-associated developmental genes in AZ seems to be consistent with this hypothesis (Wang et al., 2013). It should also be noted that modification of pectin structure was found to play a role in organogenesis (Peaucelle et al., 2011). Organogenesis appears to have many features in common with abscission.

Synthesis of a Boundary Layer and Hormonal and IDA-Like Signaling

If the synthesis of a waxy, cuticle boundary is important to successful organ separation, you might assume someone would have identified *Arabidopsis* mutants that were linked to this function. We suggest that the *ida*, *hae-hsl2* mutants are more closely linked to the synthesis of a cuticle-like boundary layer than expression of genes for cell wall disassembly. To explain, first, although we do not have a time course comparison of WT and *hae-hsl2*, differential expression of cuticle synthesis and peroxidases genes was greater in this data than for cell wall disassembly genes (**Figure 3C** and **Supplemental File S4**) (Niederhuth et al., 2013). Moreover, break-strength measurements for the *ida* and *hae-hsl2* mutants indicate that IDA-signaling affects only a part of the separation process (Cho et al., 2008; Liu et al., 2013). Break-strength is a measure of the force needed to pull the distal organ, e.g., petal, away from the proximal organ, e.g., receptacle (Craker and Abeles, 1969; Del Campillo and Bennett, 1996). In WT flowers, the petal break-strength begins to decline at flower position 3 soon after the flowers open (Patterson and Bleecker,

2004). Break-strength of WT petals continues to decline until positions 6–8 when the petals, stamens and sepals fall off from their own weight. In the *ida* mutant (Butenko et al., 2003), which has an abscission phenotype very similar to the *hae-hsl2* mutant (Cho et al., 2008), the petal break-strength also begins to decline at position 3 but declines at a slightly slower rate than WT; however, between flower positions 8–12 the petals begin to re-adhere to the receptacle so that by position 22 the petals are senescent but have regained a break-strength approximately equal to that of a flower at position 2 (Butenko et al., 2003). Scanning electron micrographs (SEM) of the fracture plane cells of WT petals on flowers at positions 10 show nicely rounded and enlarged cells. SEM micrographs of the petal fracture plane of *ida* at flower position 10, when break-strength was at its lowest, displayed partially enlarged and rounded cells (Butenko et al., 2003). However, at flower position 22 of the *ida* mutant, after the petals had re-adhered to the receptacle and were forcibly pulled off, the micrographs of the fracture plane displayed cells that were broken and torn (Butenko et al., 2003). Clearly, part of the abscission process is implemented in *ida* but the process is incomplete. The *nevershed* (*nev*) mutant, that has a defect in the cellular secretion process through the golgi apparatus, also displays a V-shaped break-strength profile; however, the cells in the fracture plane at the lowest break-strength appear to enlarge even more than in the WT cells (Liu et al., 2013). The phenotypes of *ida*, *hae-hsl2*, and *nev* are similar but crosses between them suggest that *NEVERSHERD* expression is not solely dependent on IDA signaling (Liu et al., 2013). Nevertheless, the *nev* mutant may also affect secretion of components needed for synthesis of a waxy cuticle more than cell wall disassembly.

It is possible based on the results and interpretations we have presented here that IDA signaling in *Arabidopsis* plays a greater role in the synthesis of a new boundary layer than cell wall disassembly and that this boundary layer appears to aid in successful floral organ separation. Can we propose a similar role for IDA in soybean, tomato and other abscission processes? IDA-like genes are highly conserved in dicots and also found in some monocots (Vie et al., 2015). In soybean leaf abscission and tomato flower abscission there is an AZ-specific up-regulation of IDA-like gene expression (Figures 2, 3 and Supplemental Files S2, S3). However, nobody to the best of our knowledge has demonstrated that IDA signaling is necessary for successful organ separation in any species other than *Arabidopsis*. It is worth mentioning here that we have used virus-induced gene silencing (VIGS) to suppress IDA-like expression in soybean and did not observe any effect on leaf abscission (results not shown). Regulation of abscission is a nexus of signaling events including ethylene, auxin, IDA, and others (Taylor and Whitelaw, 2001; Gonzalez-Carranza et al., 2012; Liljegren, 2012; Aalen et al., 2013; Kim, 2014; Tucker and Kim, 2015). It is possible that, even if IDA-signaling plays a more prominent role in regulating the synthesis of a boundary layer, a separation phenotype in IDA-like suppressed mutants will not be obvious in many abscission systems because disassembly of the cell wall and middle lamella is sufficient when the distal organ has a mass greater than that of a flower

petal, stamen, or sepal. Nevertheless, we propose a modification to the abscission model to include synthesis of an extensible boundary layer early in the abscission process that is different from the deposition of a protective layer (Figure 4). However, the waxy cuticle synthesized early during separation may become a part of a more rigid protective layer deposited after separation. Moreover, as suggested much earlier by Patterson and Bleeker (2004), we reassert that multiple hormonal and peptide signals regulate the rate of abscission and successful organ separation and these different signals do not necessarily regulate the same set of genes. Of interest in this regard was that differential gene expression of ethylene (e.g., ERFs) and auxin-associated genes (e.g., SAUR, AUX.IAA) was not greatly changed between WT and *hae-hsl2* *Arabidopsis* receptacles at flower stage 15 (Figures 2C, 3C). This further supports a model where IDA signaling does not directly affect ethylene or auxin signaling in *Arabidopsis* floral organ abscission and that multiple signals influence abscission. What is necessary is discovering how these signals work together and independently to bring about a successful separation process.

CONCLUDING REMARKS

Fruit softening includes expression of cell wall and middle lamella degrading enzymes (Tucker, 2014). The fruit ripening genes that affect cell wall modifications are not necessarily the same genes as those expressed in abscission, but they likely have similar functions. However, cell separation is not the same in a ripe fruit as it is in the fracture plane. Evaluation of the transcriptome of the pericarp of a wild-type ripening tomato (Osorio et al., 2011) indicated strong up-regulation of *CELS*, *PGs*, *EXPs*, and *XTHs* but not a marked increase in wax synthesis genes or lipid transfer proteins (Supplemental File S6). Could it be that an important difference between fruit ripening and abscission is the synthesis of a cuticle-like boundary on the abscission cells? Our proposal that a waxy-like cuticle is important to the abscission process is further supported by observations for separation during organogenesis. It seems to make evolutionary sense that abscission of plant organs would be an adaptation of a primal process occurring in the meristem. Although we do not know the exact composition of the abscission boundary layer, our proposal can be tested genetically and biochemically.

SEQUENCE SUBMISSION

The raw soybean sequence files have been submitted to the National Center for Biotechnology Information (NCBI) SRA databases with the study accession SRP050050. The soybean results including RPKM and annotations for all 54,175 genes can be downloaded at <http://sgil.ba.ars.usda.gov/mtucker/Public/Tucker.html>. The raw tomato sequence data and array information were submitted to the Gene Expression Omnibus (GEO) at the NCBI with GEO IDs GSE45355 and GSE45356, and array ID AMADID:043310.

AUTHOR CONTRIBUTIONS

Conceived, designed experiments and interpreted data: JK and MT. Performed experiments and prepared and analyzed data: SS and RY. Analyzed and interpreted data: SH and SM.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01109>

Supplemental Figure S1 | Soybean explant preparation.

Supplemental File S2 | Excel file for gene names, expression, annotation and graphs for all the soybean genes included in Figures 2A, 3A.

Supplemental File S3 | Excel file for gene names, expression, annotation and graphs for all the tomato genes included in Figures 2B, 3B.

Supplemental File S4 | Excel file for gene names, expression, annotation and graphs for all the *Arabidopsis* genes included in Figures 2C, 3C.

Supplemental File S5 | Phylogenetic tree of dicot, monocot, pine and moss PAR1-like protein sequences. Alignment was performed with the signal peptides removed.

Supplemental File S6 | Excel file for gene names, expression and annotation for tomato fruit genes.

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Conservation of the abscission signaling peptide IDA during Angiosperm evolution: withstanding genome duplications and gain and loss of the receptors HAE/HSL2

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The peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA), which signals through the leucine-rich repeat receptor-like kinases HAESA (HAE) and HAESA-LIKE2 (HSL2), controls different cell separation events in *Arabidopsis thaliana*. We hypothesize the involvement of this signaling module in abscission processes in other plant species even though they may shed other organs than *A. thaliana*. As the first step toward testing this hypothesis from an evolutionarily perspective we have identified genes encoding putative orthologs of IDA and its receptors by BLAST searches of publically available protein, nucleotide and genome databases for angiosperms. Genes encoding IDA or IDA-LIKE (IDL) peptides and HSL proteins were found in all investigated species, which were selected as to represent each angiosperm order with available genomic sequences. The 12 amino acids representing the bioactive peptide in *A. thaliana* have virtually been unchanged throughout the evolution of the angiosperms; however, the number of IDL and HSL genes varies between different orders and species. The phylogenetic analyses suggest that IDA, HSL2, and the related HSL1 gene, were present in the species that gave rise to the angiosperms. HAE has arisen from HSL1 after a genome duplication that took place after the monocot–eudicots split. HSL1 has also independently been duplicated in the monocots, while HSL2 has been lost in gingers (Zingiberales) and grasses (Poales). IDA has been duplicated in eudicots to give rise to functionally divergent IDL peptides. We postulate that the high number of IDL homologs present in the core eudicots is a result of multiple whole genome duplications (WGD). We substantiate the involvement of IDA and HAE/HSL2 homologs in abscission by providing gene expression data of different organ separation events from various species.

Keywords: LRR-RLK, phylogeny, genome duplication, peptide signaling, leaf abscission, fruit abscission, *Populus*, oil palm

INTRODUCTION

Plant architecture is dependent on the balance between cell division and cell elongation, proliferation and differentiation, as well as formation and abscission of organs. Plants are sessile organisms that over evolutionary time have adapted to their environment. They have developed reproductive strategies involving mechanisms that in various ways facilitate fruit and seed dispersal. The shedding of organs that have served their purpose is furthermore part of normal development, but detachment can also be a response to injury or environmental changes. The model plant *A. thaliana* shed individual floral organs (petals, sepals, and stamen) shortly after pollination (Bleecker and Patterson, 1997), and displays opening of the valves of the mature siliques (so called dehiscence), and thereafter seed dropping. Other species may shed leaves (e.g., deciduous trees like aspen, *Populus tremula*) and whole flowers (e.g., Citrus), or fruits (e.g., tomato, palms).

Despite variation in the sites of abscission in different species of flowering plants, various abscission events are similar at the cellular level in that they take place in specialized abscission zones (AZs). AZs are either generated already during the development of the organ, or can be induced in response to hormonal or environmental cues (Patterson, 2001; Roberts et al., 2002; Lewis et al., 2006; Aalen, 2011). Organ detachment is a cell separation process that involves dissolution of the middle lamella between adjacent AZ cell files presumably through the action of a number of different cell wall remodeling and degrading proteins, e.g., polygalacturonases, expansins, XTHs, and endoglucanases (Cho and Cosgrove, 2000; González-Carranza et al., 2007; Lashbrook and Cai, 2008; Meir et al., 2010; Liu et al., 2013; Niederhuth et al., 2013; Tsuchiya et al., 2015). One can assume that there is a need for tight regulation and coordination of expression of genes involved in cell separation processes, since cell wall weakening and breakdown could render the plant more susceptible to pathogen attack, and premature organ loss would reduce reproductive success.

In *A. thaliana* a peptide ligand—receptor system responsible for such tight regulation of floral organ abscission has been identified (Butenko et al., 2003; Stenvik et al., 2008). This signaling module consists of the secreted peptide IDA encoded by the gene *INFLORESCENCE DEFICIENT IN ABSCISSION* (*At1g68765*), and the two leucine-rich repeat (LRR) receptor-like kinases (RLK) HAESA (HAE) and HAESA-LIKE2 (HSL2) (Cho et al., 2008; Stenvik et al., 2008). In both *ida* and *hae hsl2* mutants floral organs are retained indefinitely. IDA belongs to a small gene family in *A. thaliana* with five additional *IDA-LIKE* (*AtIDL*) members that to a varying degree can substitute for *IDA* function (Butenko et al., 2003; Stenvik et al., 2008). IDA and the IDL proteins share a short C-terminal sequence encompassing the bioactive peptide (Stenvik et al., 2008), which for IDA has been delineated to a 12 amino acids (aa) long proline-rich peptide (hereafter called mature IDA, mIDA) using genetic and biochemical tools (Stenvik et al., 2008; Butenko et al., 2014). Overexpression either of *IDA* or *AtIDL* genes, results in early abscission and enlarged abscission zones (AZ) at the base of the floral organs, but when expressed using IDA's own promoter,

only *IDA* and *AtIDL1* can fully complement the *ida* mutant phenotype. Together this may suggest that AtIDL2-5 peptides have lower affinity to HAE and HSL2, and possibly signal through (a) different, but related receptor(s) (Stenvik et al., 2008). There are more than 600 genes in *A. thaliana* encoding receptor-like kinases, and of these more than 200 encode extracellular domains with LRRs of varying length assumed to bind small peptides (Shiu and Bleecker, 2001). Only about a dozen of these have been matched with a ligand (Butenko et al., 2009). The majority of these, including HAE and HSL2, belong to the RLK subclass XI, which has more than 20 LRRs.

Interestingly, ectopic expression of IDA in *A. thaliana* leads to abscission of fruit, flowers, branches, and cauline leaves at vestigial AZ at the base of these organs, which are sites of cell separation in a number of other species, but normally not in *A. thaliana* (Stenvik et al., 2006). Additionally, the IDA-HAE/HSL2 signaling module is involved in a quite different cell separation process, namely lateral root emergence (Kumpf et al., 2013). IDA and the receptors are expressed in the endodermal, cortical, and epidermal cells overlaying lateral root primordia, induce expression of cell wall remodeling genes and facilitate emergence without cellular disruption (Aalen et al., 2013; Kumpf et al., 2013).

The objective of the presented work has been to investigate to what extent orthologs of *IDA*, *HAE*, and *HSL2* are present in other angiosperms irrespective of which organs they shed, and whether gene expression data could substantiate a role of these orthologs in cell separation events.

MATERIALS AND METHODS

Phylogenetic Analyses

Sequences used in this study were obtained from various databases [NCBI-protein, NCBI-Assembly, Phytozome, Ancestral Angiosperm Genome Project (AAGP), Comparative Genomics (CoGe)] using either tBLASTN or BLASTp (as of 3.2015) with an *A. thaliana* IDL or HSL query. Outgroup taxa for both monocot and eudicot receptors were closely related *A. thaliana* representations within the subclass XI LRR-RLKs. IDA was inferred without an outgroup. An aa alignment (~59 aa) of angiosperm IDA without the N-terminal secretion signal and LRR-RLK group (full length 1926 aa) was constructed using MAFFT v6 E- INS-I model under default settings (Katoh and Toh, 2008). The resulting alignments were checked with Gblocks v0.91b (Castresana, 2000), under the least stringent parameters (small final block, gap positions in final block and less strict flanking), to exclude poorly aligned positions and divergent regions from subsequent phylogenetic inferences. Multiple homologous copies of HSL1/HSL2/HAESA (*At1g28440/At5g65710/At4g28490*) for each species were removed to reduce phylogenetic noise; in all cases the copy with most sequence data and thereafter shortest branch length was retained for further analysis. ProtTest v2.4 (Abascal et al., 2005) determined LG as the optimal evolutionary model. Maximum Likelihood (ML) analyses were performed with RAxML-VI-HPCv8.0.26, using the PROTCATLG model with 25

rate categories (Stamatakis, 2006). The most likely topology was established from 100 separate searches and bootstrap analyses were performed with 500 pseudoreplicates.

All model estimation and phylogenetic analyses were done using either Lifeportal (<https://lifeportal.uio.no>) or the abel server at the University of Oslo. All alignments constructed as part of this study are available as Supplementary Data sets, and will in addition be made freely available as Supplementary resources through the authors' Research Gate pages (https://www.researchgate.net/profile/Reidunn_Aalen) and (https://www.researchgate.net/profile/Russell_Orr2).

Plant Material

A. thaliana has been transformed with *HAE* and *HSL2* promoter::GUS constructs in the vector pMDC162 made using Gateway Cloning Technology (Curtis and Grossniklaus, 2003) with the same promoter region upstream of the coding sequence (1601 bp and 2300 bp, respectively) as previously reported for *HAE* and *HSL2* constructs with YFP reporter (Kumpf et al., 2013).

Fully expanded leaf blades of hybrid aspen, *Populus tremula* L. X *P. tremuloides* Michx.; clone T89, were shaded in aluminum foil to induce abscission and total RNA was extracted from 3 mm-thick leaf axils 6 days after shading started using RNeasy Plant Mini Kit (Qiagen). Two micrograms of total RNA was used as a template for reverse transcription with the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative real-time PCR (qPCR) was performed using SYBR Green I Master in combination with a LC4800 (Roche Diagnostics) qPCR machine. Primers are specified in Supplementary Table S1. Expression was normalized to *PtACTIN1* expression.

mRNA was isolated from AZs of unripe and ripe of oil palm (*Elaeis guineensis*) fruit, and AZs of fruit treated with ethylene as described previously (Roongsattham et al., 2012). Primers were designed to fit the various *EgIDA* genes, *EgHSL2*, and *EgHSL1* (Supplementary Table S1). qPCR was performed as previously described (Roongsattham et al., 2012). All expression was normalized to the *EgEfa1* (accession number: AY550990) expression. No change of *EgEfa1* transcript accumulation was found in the fruit tissues treated or not treated with ethylene. Controls were conducted to validate the absence of DNA in each sample.

RESULTS

HSL2 has been Lost in the Gingers and the Grasses, and HAE is Present only in Eudicots

The *A. thaliana* HAE and HSL2 receptors are closely related (60% similar and 45 % identical aa) but HAE is even more closely related to HSL1 (73% similarity, 58% identical aa), an RLK so far with unknown function. BLASTp and tBLASTn were used to identify putative HSL1 angiosperm orthologs in addition to orthologs of HAE and HSL2. Other related receptors from the LRR-RLK subclass XI were used as outliers. Protein sequences from 60 species belonging to 24 orders covering basal

angiosperms, monocots, basal eudicots, and core eudicot clades were used to determine the phylogenetic relationship.

The LRR-RLK ingroup is monophyletic, separated from the outgroup with high support (98% Bootstrap, BP) (Figure 1, Supplementary Figure S1). HSL2 and HSL1/HAE represent two fully supported distinct clades (100% BP). In the HSL2 clade, the basal angiosperm copy is first to diverge within a fully supported grouping, leaving a highly supported (92% BP) monocot/eudicot clade. Subsequently, a moderately supported (71% BP) monocot clade diverges, leaving a poorly supported (54% BP) eudicot monophly. HSL2 is however missing in the taxa from the orders Poales and Zingiberales (Supplementary Figure S1).

The HSL1/HAE clade is fully supported with the basal angiosperm copy being first to diverge leaving a moderately supported (84% BP) monocot/eudicot clade (Figure 1). The monocot HSL1 copy diverges next forming a highly supported clade (96% BP). Two groupings are found within the monocot clade; the first—constituting all monocot orders (HSL1A)—is unsupported (40% BP), but excluded from the second, longer-branching HSL1B. This duplicate HSL1 clade for the orders Arecales (palms), Poales, and Zingiberales, has moderate support (72% BP) (Figure 1, Supplementary Figure S1). The eudicot HSL1/HAE grouping is moderately supported (70% BP). The HAE clade, representing Vitales in addition to all Rosids and Asterids, is moderately supported (80% BP) and excludes the basal eudicot orders Ranunculales, Proteales, and Caryophyllales which have a closer affinity to the moderately supported (82% BP) eudicot HSL1 grouping. The fully supported Caryophyllales clade forms a moderately supported (80% BP) monophly with the eudicot HSL1 grouping.

Our analyses revealed that all angiosperms have *HSL1* genes, in in basal angiosperms and some monocots, and almost all eudicots in combination with a *HSL2* gene. Only the core eudicots have both a *HAE* and a *HSL2* gene. In *A. thaliana* *HAE* and *HSL2* are redundant in function with respect to floral organ abscission, as both genes must be mutated to give the abscission-deficient phenotype (Cho et al., 2008; Stenvik et al., 2008).

HAE and HSL1 Differ from HSL2 Especially in their Kinase Domains

LRR-RLK proteins consist of three domains with different functions: the intracellular kinase domain that confers the signaling output upon ligand binding, the transmembrane domain that anchors the receptors in the plasma membrane, and the extracellular LRR to which the ligand will bind (called the ectodomain). As these three domains have different functions, the selective pressure for rejecting or preserving new mutations might differ.

The intracellular kinase domain has typically two regions: the N-terminal lobe with several β strands and a conserved ATP binding site, and the larger C-terminal part with a number of α -helices, and an activation loop with serine/threonine [Ser(S)/Thr(T)] target residues for phosphorylation. In the BRASSINOSTEROID INSENSITIVE1 (BRI1) LRR-RLK several such residues are phosphorylated (Wang et al., 2005). These residues are well conserved in the *A. thaliana*

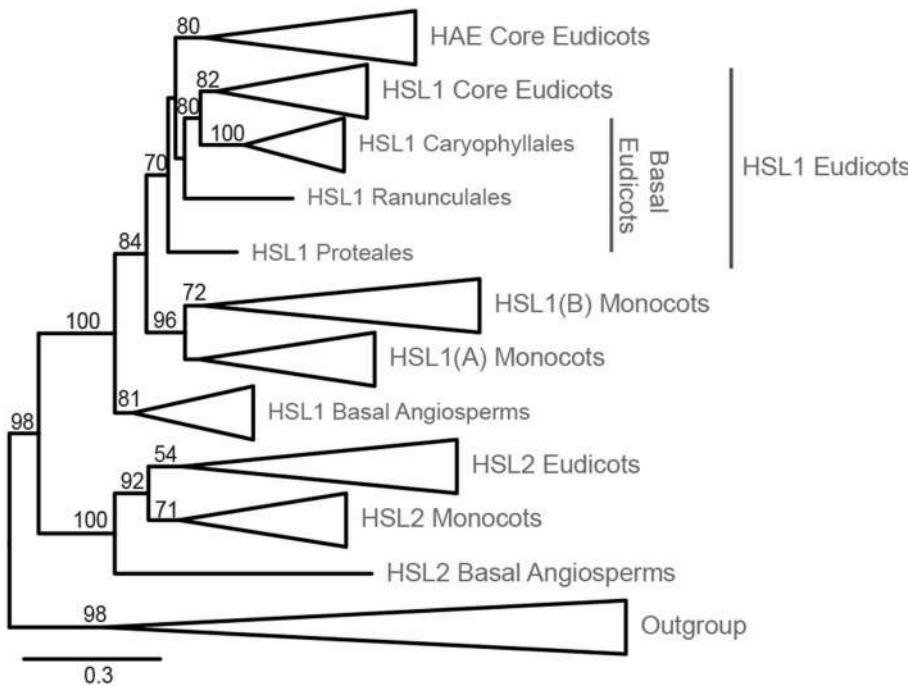


FIGURE 1 | Phylogeny of HSL LRR-RLK evolution within Angiosperms. Phylogeny inferred from ML with 145 ingroup taxa and 784 amino acid characters. The phylogeny has been collapsed at different taxonomic levels and shows only bootstrap values >50. The expanded tree is presented in Supplementary Figure S1, which includes an overview of species comprising each order. The alignments used for the construction of this phylogeny are available as Supplementary Data Sheet 1 and 2.

HAE, HSL1 and HSL2 receptors and their orthologs in other species (Supplementary Figure S2A). This includes the residue corresponding to S861 of HAE, which is subject to autophosphorylation (Taylor et al., 2013), and several residues predicted to be targets of Ser/Thr kinases (e.g., by using PhosPhAt <http://phosphat.uni-hohenheim.de/>) (Durek et al., 2010). However, in loops between helices positions of putative phosphorylation sites differ in HSL2 receptors compared to HAE/HSL1. Furthermore, HSL2 receptors lack a C-terminal extension with putative phosphorylation sites (Supplementary Figure S2A). The predicted secondary structures of the transmembrane domains of HAE, HSL1, and HSL2 are similar, with a membrane-spanning, hydrophobic α -helix flanked by conserved aa (Supplementary Figure S2B). HSL2 sequences differ from HAE and HSL1 sequences by a shorter α -helix. HAE sequences can be distinguished from HSL1 by a two aa deletion near the end of the transmembrane domain (Supplementary Figure S2B).

So far, the exact location of the *A. thaliana* mIDA peptide when binding HAE or HSL2 is not known, however, in other cases small peptides have been shown to bind consecutive repeats on the inside pocket of the LRR (Sun et al., 2013). Each repeat has typically 24 residues with six evenly distributed conserved leucine residues (Leu, L), which together with asparagine (Asn, N) and glycine (Gly, G) in conserved positions build the structural framework of the ectodomain (Figure 2). These residues are almost invariable and have therefore limited informative value in a phylogenetic perspective. In contrast, conserved non-structural

residues are interesting since selection pressure conserving residues involved in ligand binding is likely. To identify such residues we employed the Repeat Conservation Mapping (RCM) (<http://144.92.198.58/main/main.php>), which relies on a set of algorithms to identify predicted functional sites of LRR domains. This is accomplished by identifying the extent of conservation of different aa patches on the predicted surface of LRR and generating a colored heat map, displaying how conserved an aa is in a given position in a set of orthologs (Helft et al., 2011). For all the HSL receptors a region with highly conserved aa was stretching from the seventh LRR with the common signature sequence QIEL[Y,F], to the fourteenth LRR (Figure 2). The signatures were more similar between the HAE and HSL1, than HSL2.

The Bioactive IDA Peptide is Conserved in all Flowering Plants

IDA and IDL are secreted peptides generated from prepropeptides (Figure 3A), where the hydrophobic N-terminus is a signal directing the protein to the secretory pathway. The variable middle part and the C-terminus are assumed to be cleaved off in the apoplastic space to release a mature 12 aa peptide named PIP after the three first residues, PIPPSAPSKRHN (Butenko et al., 2003). Synthetic mIDA peptide with hydroxylation on the central proline (Pro, P) can bind and activate HSL2 efficiently, and HAE at a higher concentration (Butenko et al., 2014). The Pro residues in positions 2, 3, and 7, serine (Ser, S) in positions 5 and 10, and histidine

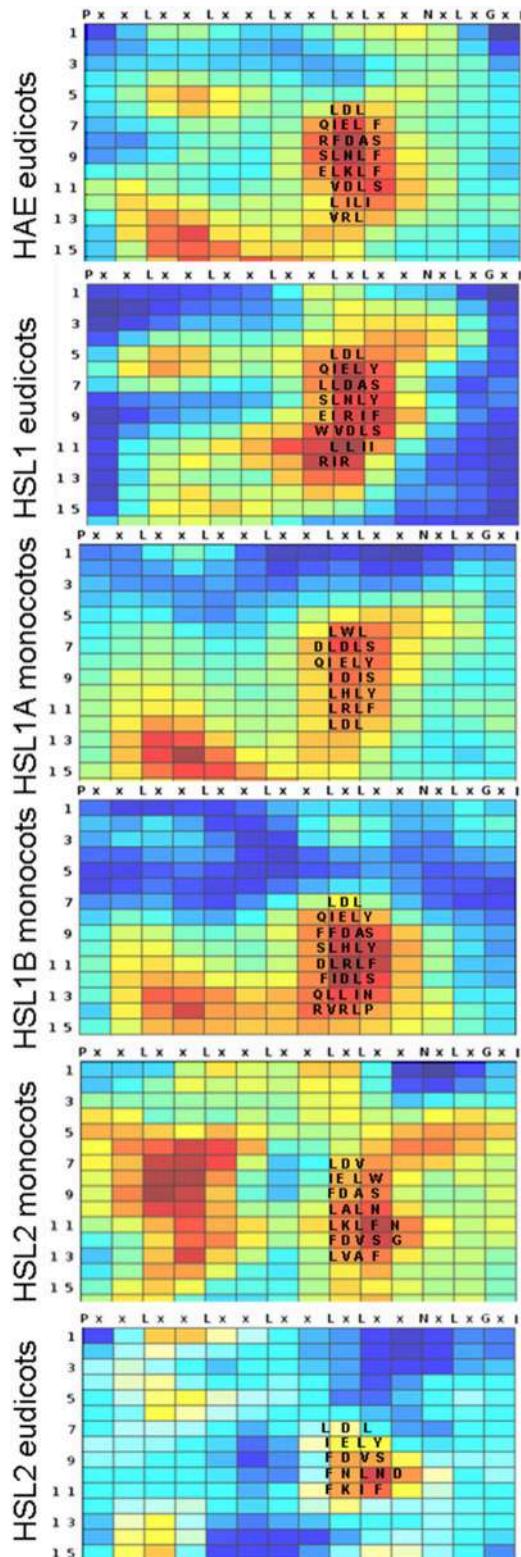


FIGURE 2 | Conserved amino acids in the LRR of HSL proteins.
Heatmaps generated using Repeat Conservation Mapping (RCM) of LRR domains (<http://144.92.198.58/main/main.php>) reflecting degree of identity
(Continued)

FIGURE 2 | Continued

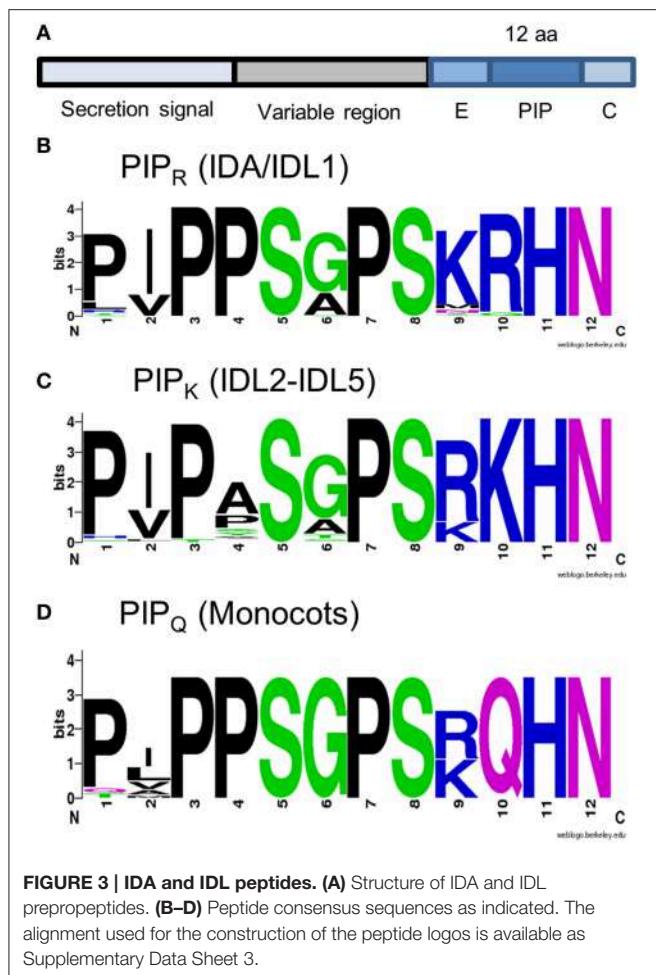
and similarity of aa residues in a given position (X axis) in a given repeat (Y-axis) on the surface of the LRR domain of HAE orthologs from the eudicots, HSL from eudicots, HSL1A and B from monocots, HSL2 from eudicot and from monocots. The heatmaps are generated using the same ortholog sequences as used for the phylogenetic analysis (Supplementary Figure S1). The alignments used for heatmap construction are available as Supplementary Data Sheet 2.

(His, H) asparagine (Asn, N) at the end are found in all *A. thaliana* IDA/IDL peptide sequences. Experiments where the part of the *AtIDA* gene encoding PIP was swapped with the corresponding *AtIDL* sequence and the recombinant *AtIDA-IDL* gene introduced in the *ida* mutant, indicate the importance of different aa residues (Stenvik et al., 2008). The AtIDL1 peptide (LVPPSGPSMRHN) complements the mutation, suggesting that the initial Pro is of little importance, that the hydrophobic isoleucine (Ile, I) and the small central Ala residues can be exchanged with the hydrophobic valine (Val, V) and the small Gly, respectively, without affecting the biological activity, and furthermore that a positively charged aa in position 9 is no absolute requirement. In contrast, *AtIDL2*, *AtIDL3*, *AtIDL4*, and *AtIDL5* cannot fully complement the *ida* mutation (Stenvik et al., 2008). Their PIP motifs are characterized by arginine-lysine (ArgLys, RK) in position 9 and 10, in contrast to the LysArg (KR) found in mIDA.

BLAST and tBLASTn searches were executed using the C-terminal sequences of *A. thaliana* IDA and IDL (i.e., PIP extended with eight aa N-terminally, and the C-terminal aa, **Figure 3A**). BLAST hits were curated for length of the coding region (<120 aa), presence of hydrophobic secretion signal, the C-terminal position of the conserved PIP motif, and the presence of the conserved Pro, Ser, and His/Asn residues. IDA and/or IDL sequences were identified in all groups in our data sets.

We were unable to infer a phylogeny over the evolution of IDA, due to the short sequence of the conserved peptide. IDA lacks phylogenetic information and is also present in a high number of copies in many species; any possible pattern was unclear, with no clear clades, branching patterns, and a general lack of stability. Despite this we attempted an inference based on multiple datasets (PIP, EPIP, and EPIP-C, **Figure 3A**), considering species that have undergone fewer genome duplications (Vanneste et al., 2014), however, without success.

As an alternative approach to understand the evolution of IDA/IDL peptides, we grouped the different PIP motifs of the BLAST-identified proteins based on their amino acid composition. In all eudicots, peptides could be classified either as IDA/IDL1 ([P,L][V,I]PPS[A,G]PSKRHN) or like IDL2-5 (PIP[A,T,H,P]S[A,G]PSRKHN), named PIP_R and PIP_K respectively based on the residue in position 10 (**Figures 3B,C**). Both motifs were found in every eudicot species tested. In the monocot dataset PIP_R was found in the orders Arecales and Zingiberales. Additionally, we identified a version in all monocots with close resemblance to PIP_R, but containing a glutamine (Gln, Q) in position 10 (PIP_Q, **Figure 3D**).



The *IDL* Gene Family has Expanded in Many Taxa of Core Eudicots

About 65 million years (Myr) ago after the split of the core eudicots into Rosids and Asterids, whole genome duplication (WGD) took place in a number of eudicot lineages and expanded both the number of species and the gene number of each species (Van de Peer et al., 2009). In line with this, detailed inspection suggests independent evolution especially of the *IDL2-5* genes in these two clades. Soybean (*Glycine max*), common bean (*Phaseolus vulgaris*) and *A. thaliana* belong to the Rosids, the beans representing the families of legumes (*Fabales*), and *A. thaliana* belonging to the *Brassicaceae*. Soybean has six pairs of *IDL* genes, each pair represented only once in common bean, consistent with a late genome duplication in soybean after the separation from bean (Tucker and Yang, 2012). Tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), and tobacco (*Nicotiana* spp.) belong to the family *Solanaceae* in the Asterid clade, and are considered diploid species. However, analyses of inter- and intrachromosomal duplications in the tomato and potato genomes suggest a second more recent WGD in the common ancestor of *Petunioideae*, *Nicotianoideae*, and *Solanoideae* (Song et al., 2012). We have found pairs of almost identical *IDL* genes both in *N.*

benthamiana and tomato that are more similar within than between the species (Supplementary Figure S3A), suggesting that additional independent duplications have taken place after the divergence of *Nicotianoideae* and *Solanoideae*. Similarly, we have previously suggested that the *AtIDL2* and *AtIDL3* genes in *A. thaliana* result from a recent duplication event (Stenvik et al., 2008).

IDA Ligands and HSL Receptors are Expressed in a Variety of AZs and Species

Having identified orthologs of IDA and its receptors in a variety of species, we examined in the literature and experimentally whether IDA/IDL1 and the relevant receptors were likely to be involved in cell separation events.

Floral Organ and Flower Abscission

Abscission plays an important role during plant reproduction. It is common among eudicots (including *A. thaliana*) to abscise turgid floral organs after pollination, when they no longer are functional (van Doorn, 2001). In some species the function of organs change during development, exemplified by tepals in *Eriophyllum* spp. (Asterales) and *Polygonum* spp. (Caryophyllales) that first act as protection of reproductive organs and later as organs involved in seed dispersal. In monocots floral organs may wither without abscission (van Doorn, 2001, 2002), or the perianth is retained and will cover the fruits during seed maturation. Hence, in such settings IDA and its receptors are not expected to be active. We postulate that the signaling system is intact in monocots in species where abscission of individual organs takes place when the tepals are free, like in tulips (*Tulipa* spp., Liliales). Unfortunately genomic and transcriptomic data are scarce for many monocots, especially the Liliales which have gigantic genomes (Shahin et al., 2012).

Normally *A. thaliana* does not shed cauline leaves, whole flowers or fruits. However, ectopic expression of IDA and IDL peptides in *A. thaliana* leads to induction of abscission in a HAE/HSL2-dependent manner at the base of pedicels, cauline leaves, and inflorescence branches, rather than a general separation of cells in the plant (Figures 4A–D) (Stenvik et al., 2006). These are sites of abscission in other species. Interestingly, *A. thaliana* lines transformed with promoter:GUS constructs for *HAE* or *HSL2* show that the receptor genes are expressed in the vestigial AZs at these sites (Figures 4E–G).

Fall of whole flowers is less common than floral organ abscission (Figure 5A), but occurs in both monocot and eudicot species, when pollination or fertilization fail (van Doorn, 2002). Abscission of immature fruit is a normal event in several cultivated species. In *Citrus* spp. (Sapindales), abscission of flowers and young fruit results from cell separation at an AZ at the base of the floral pedicel. The *CicIDA3* gene, expressed in *Citrus clementina* AZs, encodes a protein with a PIP motif identical to mIDA with the exception of a Gly instead of an Ala in position 6 (Estornell et al., submitted). Overexpression of *CicIDA3* in *A. thaliana* induced the same phenotypic changes as have been shown for overexpression of IDA and *AtIDL1* (Figures 4A–D) (Estornell et al., submitted), suggesting that the



FIGURE 4 | Expression of HAE and HSL2 promoter: GUS constructs at sites of ectopic abscission. (A–C) Enlargement of AZ and premature abscission of whole flowers and immature fruits compared to wild type siliques (to the left in A) in *A. thaliana* plants overexpressing AtIDL1. (D) Enlarged vestigial AZ after abscission of a caulin leaf in *A. thaliana* overexpressing AtIDL1. (E,F) pHAE:GUS expression and (G) pHSL2:GUS expression in vestigial AZs at the bases of pedicels, branches, and caulin leaves.

citrus prepropeptide undergo the correct processing, that the *Citrus* peptide can activate IDA's receptors and thus provides experimental evidence supporting a function in citrus abscission events.

Leaf Abscission

AZs, with layers of rather undifferentiated small cells, are normally formed during organ development both in *A. thaliana* and other species. Additionally, ethylene may promote the formation of new AZs and thereby induce abscission (Roberts et al., 2000). A thorough investigation of IDA and HAE/HSL2 in connection with ethylene-induced leaf abscission has been performed in soybean (*Glycine max*), common bean and tomato (Tucker and Yang, 2012). Of the five genes encoding tomato IDA/IDL peptides, one with a PIP motif identical to that of mIDA was expressed in ethylene-induced AZs on leaf pedicels. Likewise, the GmIDA2a and 2b, which had the highest expression level in leaf AZ and the highest relative expression ratio between AZ and petiole tissue both in the presence and absence of ethylene, are the paralogs most similar to AtIDA, which also display ethylene independent expression. The GmHAE3b and GmHAE5a/b with the highest similarities to *A. thaliana* HAE and HSL2, respectively, were also adequately expressed in the petiole (Tucker and Yang, 2013). Thus, these expression patterns support the involvement of the IDA-HAE/HSL2 module in leaf abscission both in Rosids and Asterids.

One of the most visible of all shedding processes in angiosperms is the leaf fall (Figure 5B) at the beginning of the dormant (i.e., cold or dry) season in deciduous trees, triggered by seasonal reduction of the photoperiod and temperature (Taylor and Whitelaw, 2001; Keskitalo et al., 2005). Autumnal

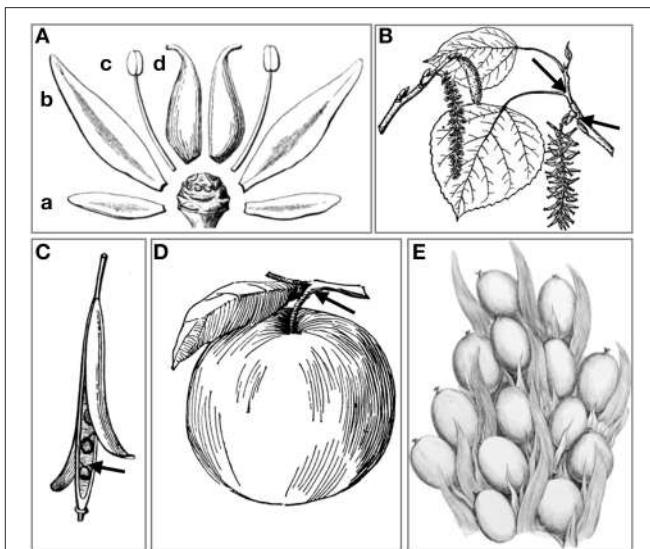


FIGURE 5 | Modes of abscission. (A) Abscission of (a) sepals, (b) petals, (c) stamen, and (d) carpels. (B) Abscission of leaves at the axil of the pedicel, and abscission of entire male inflorescence (catkin) in *Populus* spp. (C) Opening of valves in dehiscence zones of dry many-seeded capsules, and abscission of individual seeds. (D) Abscission of fleshy fruits at AZ on pedicel. (E) The oil palm drupe fruit are tightly arranged within spikelets and abscise one by one when ripe. (A–D) Image courtesy the private collection of Roy Winkelman. First published in Gray (1858) and Foster (1921). (E) Image courtesy Missouri Botanical Garden. <http://www.botanicus.org>.

leaf abscission provides deciduous trees with resistance against drought and freezing damage (Fischer and Polle, 2010; Zanne et al., 2014). Species with N-fixing symbionts like alder (*Alnus* spp., Fagales) can afford to lose green leaves, while other deciduous trees, like *Populus* spp. (Malpighiales), withdraw elements of valuable molecules (as chlorophyll) and shed yellowish or reddish leaves (Keskitalo et al., 2005; Fracheboud et al., 2009). Hence, the senescence process that allows trees to conserve resources and prepare for a dormant period terminates in a cell separation event in preformed AZs at the base of the pedicels.

Leaf abscission can be induced in hybrid aspen (*Populus tremula* X *P. tremuloides*) by depriving the leaves of light (Jin et al., 2015). In our experimental setup, 50% of the shaded leaves separated from the branch at the axil after 8 days, while non-shaded leaves were not shed. We designed gene-specific primers against the IDA, IDL1, and HAE orthologs of *Populus* spp., which seem to have lost the HSL2 ortholog. The two genes most similar to AtIDA were significantly upregulated in the axil upon induction of leaf abscission (Figure 6A). As in soybean, the expression levels of the receptors did not change in the course of this experiment, suggesting that the timing of abscission is rather dependent on the induction of IDA peptides than on the transcriptional regulation of the receptor.

Seed and Fruit Abscission

The fertilized ovule of Angiosperms (the seed) and surrounding maternal tissue that together constitutes the fruit, needs to be separated from the mother plant in one way or another when

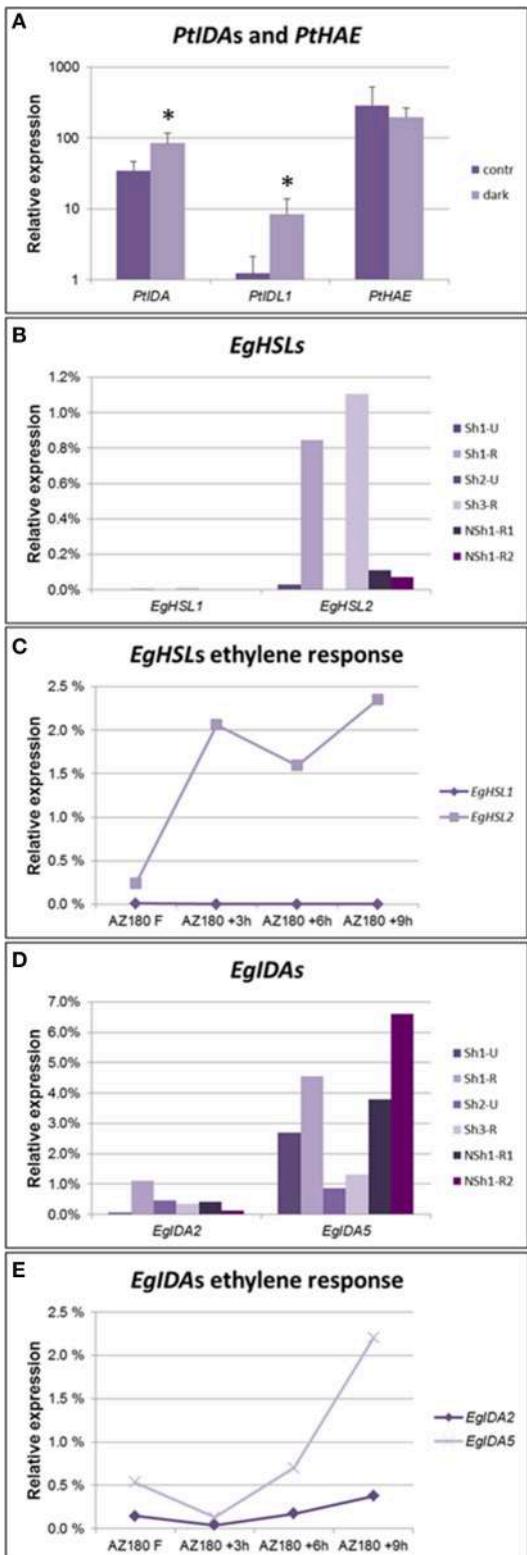


FIGURE 6 | Expression levels of genes encoding IDA ligands and HSL receptors in AZs. (A) qPCR detecting increased expression levels of *Populus* IDA genes, but not the *PtHAE* gene in axils of shade-treated leaves prone to (Continued)

FIGURE 6 | Continued

abscise compared to axils in non-shaded, non-abscission aspen leaves. qPCR, averages and standard deviations of three biological replicates. Normalized to *PtACTIN1* expression. * $p < 0.05$, t-test, non-shaded vs. shaded. (B) qPCR analysis of oil palm *EgHSL1* and *EgHSL2* expression in AZs from unripe (Sh1-U and Sh2-U) not actively abscising fruit and AZs of ripe (Sh1-R, Sh3-R) actively abscising fruit, as well as AZs from ripe fruit of a non-abscising tree (NSh1-R1, NSh1-R2). (C) qPCR analyses of oil palm *EgHSL* expression during ethylene-induced abscission in ripe 180 DAP fruits. Samples were taken after 0, 3, 6, and 9 h treatment with ethylene. Similar results were obtained when treating 145 DAP fruits (Supplementary Figure S3C). In both experiments fruit separated by 9 h of ethylene treatment. (D) qPCR analysis of oil palm *EgIDA2* and *EgIDA5* expression in the AZs of unripe (Sh1-U and Sh2-U) not actively abscising fruit and AZs of ripe (Sh1-R, Sh3-R) actively abscising fruit, as well as AZs from ripe fruit of a non-abscising tree (NSh1-R1, NSh1-R2). Expression levels of additional *EgIDA* genes are found in Supplementary Figure S3D. (E) qPCR analyses of oil palm *EgIDA2* and *EgIDA5* expression during ethylene-induced abscission in ripe 180 DAP fruits. Samples were taken after 0, 3, 6, and 9 h treatment with ethylene. Similar results were obtained when treating 145 DAP fruits (Supplementary Figure S3E). In both experiments fruit separated by 9 h of ethylene treatment.

ripe. In botanical terms there are roughly four main types of fruits: *berries* (fleshy most often many-seeded fruits), *drupes* (fleshy most often one-seeded fruits with hard exocarp), *capsules*, including siliques and pods (dry many-seeded fruits), and *nuts*, including caryopses of grasses (dry one-seeded fruits). Nuts are shed and abscised at the base of the carpels, while many-seeded capsules or pods open along dehiscence zones (Figure 5C). This is seen in a wide range of angiosperms such as orchids (Asparagales), poppies (*Papaver* spp., Ranunculales), and tobacco (*Nicotiana tabacum*, Solanales) as well as *A. thaliana*, before each single seed abscise where the funiculus is attached to the seed. We have earlier reported that *AtIDA* and *HAE* are expressed in *A. thaliana* dehiscence zones (Butenko et al., 2006).

For fleshy fruit eaten directly from the plant by birds or animals e.g., blueberries (*Vaccinium myrtillus*, Ericales), abscission is not necessitated for seed dispersal. Nevertheless, both larger fruits like apples (*Malus domestica*, Rosales) (Figure 5D) and drupes like olives (*Olea europaea*, Asteridae) (Gil-Amado and Gomez-Jimenez, 2013) may abscise at the apex or at the base of the pedicel (fruit stalk), so that animals may pick up the fruits from the ground. In line with this, *HAE* was recently shown to be strongly upregulated in abscising olives compared to the preabscising AZs (Gil-Amado and Gomez-Jimenez, 2013).

To test whether IDA-HAE/HSL2 is involved in control of fruit abscission also in monocots we chose to investigate abscission of the fruits of oil palm (*Elaeis guineensis*, Arecales). The flowers of the oil palm grow in large clusters giving rise to plumb-sized reddish fruits in large bunches (Figure 5E) that have large, preformed, multilayered AZs at the boundary of the mesocarp and the pedicel. Under natural conditions it takes about 160 days from pollination until the fruits are ripe and start separating from the bunches on the trees. mRNA was isolated from AZs of unripe and ripe fruit of trees that abscise their fruit (Shedding—Sh1, Sh2, and Sh3), and additionally from AZs of an unusual tree (Non-shedding, NSh1) that fails to abscise its fruit. We identified genes encoding HSL1 and HSL2

receptors; *EgHSL1* had a very low expression level that did not change over time (**Figure 6B**). *EgHSL2* on the other hand was low in unripe AZs of shedding trees, as well as AZs of ripe NSh1 fruit, but increased strongly in AZs of ripe fruit of abscising trees. From about 145 DAP, ethylene treatment induce fruit abscission after 9 h. This treatment also induces increased *EgHSL2* expression (**Figures 6C**, Supplementary Figure S3C). Thus, *EgHSL2* expression was consistently associated with active fruit abscission.

We identified 10 *IDA* oil palm genes that come in five pairs encoding almost identical preproteins (Supplementary Figure S3B), suggesting recent genome duplication. Expression of five of these genes (*EgIDA2* to *EgIDA6*) was detected in AZs of *E. guineensis* fruits using RT qPCR (Supplementary Figure S3D). In trees showing fruit abscission *EgIDA2*, *EgIDA4* and the most abundant, *EgIDA5*, all had their highest expression levels when fruit are separating (**Figure 6D**), and *EgIDA2* and *EgIDA5* could also be induced by ethylene (**Figure 6E**). These genes encode PIPs near to identical with mIDA; like CicIDA3, *EgIDA5* differs only by an exchange of the small Ala with the small Gly in position 6. Interestingly, the *EgIDA* genes were not strictly associated with separation; as transcripts were present also in ripe AZs of the non-shedding tree (**Figure 6C**). Hence, *EgIDA* are expressed and induced in NSh1 although the receptor gene *EgHSL2* has a very low level of expression.

DISCUSSION

Our phylogenetic analyses of the HSL LRR-RLKs have revealed that the evolution of this receptor family is congruent with that of angiosperm species evolution, and makes it likely that the common ancestor of angiosperms had both a *HSL1* and a *HSL2* gene (**Figure 7**). Both receptors are present already in the basal angiosperm (e.g., *Amborella*), where stamen abscission is a common feature that facilitates the access of pollinators (beetles and flies bringing pollen from other flowers) to the female organ (Endress, 2010). The *IDA* gene with *PIP_R* motif identified in *Amborella*, may play a role here, similar to IDA's role as a signaling ligand in sepal, petal, and stamen abscission in *A. thaliana* (Butenko et al., 2003, 2014).

While the receptors follow the general evolution of the angiosperms, the *PIP_R* motif likely to represent the peptide released from the propeptide upon secretion, has virtually been preserved without substantial changes throughout the evolution of flowering plants. In a short peptide that presumably shall bind to given aa side chains in the receptor, there are limited possibilities for aa changes that retain the relevant binding properties: Exchange of one small aa for another might pass, but exchange of an aa with different size or charge of the side chain (like a Lys or a Asn for an Arg), may affect the structure of the ligand and its binding properties (Czyziewicz et al., 2015).

The genome of a common ancestor of flowering plants has been suggested to harbor between 10,000 and 14,000 genes (Tang et al., 2008) while a genome size of 25,000–50,000 genes is common in modern angiosperms. This increase is due to a number of WGD, including a triplication in dicots before the separation of the *Vitis*. Two additional duplications and a

subsequent extensive loss of duplicated genes occurred in *A. thaliana* (Tang et al., 2008; Van de Peer et al., 2009). Relevant for our analyses is that *HSL1* has been duplicated in two separate events: Firstly, before the split of Vitales, Asterids, and Rosids, within eudicots to form *HAE*, and secondly, within monocots to form a more recent duplication before the split of Arecales. Still, multiple synteny investigations between five different and partly distantly related species (*Arabidopsis*, *Carica*, *Populus*, *Vitis*, and *Oryza*) showed that a total of 61% of the *A. thaliana* genes had preserved their ancestral locations (Tang et al., 2008). There are controversies regarding the placement of the smaller clades of Ceratophyllales and Caryophyllales, as well as Vitales, in relation to the eudicots (Zeng et al., 2014). Our analyses of the three receptor genes support Vitales and Caryophyllales as sister groups of the core eudicots. *HSL1/HAE* duplication is found on syntenic genome regions in the other eudicots, but not rice, supporting that *HAE* was generated by a WGD event (Tang et al., 2008). Specific genomic locations of *AtIDA* and *HSL2* and their neighboring genes in *A. thaliana* are preserved in *Populus* and *Vitis*, respectively (Woodhouse et al., 2011). The *IDL4* is positioned in synteny with other eudicots, but the other *IDL* genes may rather be a result of duplications known to have taken place after the divergence of Brassicaceae and Caricaceae families (Tang et al., 2008).

Duplications provide the opportunity for the new copy to evolve, like giving rise to the *PIP_K* or *PIP_Q* motif varieties. Transition from Arg to Lys or Gln may only require one nucleotide change. Based on available sequence data we hypothesize that *IDL4* with *PIP_K* evolved before the split of Proteales, however this variant may have a more basal history within angiosperm evolution. Likewise, the *PIP_Q* version has presumably developed after genome duplication in the monocot lineage, and we hypothesize that *PIP_Q* is present in all monocot orders (**Figure 7**). Sequence data for Acorales is needed to substantiate this. *PIP_R* may represent the original peptide gene that has been lost from some of the monocot orders: Alismatales and Poales are devoid of the gene, whilst it is present in Arecales and Zingiberales, suggesting independent loss for some lineages. This is more parsimonious than a basal loss within the monocots followed by multiple gains. A final conclusion must await genomic sequencing of species from orders where data at present are lacking.

We have evidence from *A. thaliana* that *IDA* can be substituted with *AtIDL1*, but the other *AtIDL* genes only to a limited extent can substitute for *IDA* function, indicating that their encoded peptides do not interact properly with the *HAE* and *HSL2* receptors (Stenvik et al., 2008). The high similarity between conserved aa in the ectodomain as well as the kinase domain of *HSL1* compared to *HAE*, might suggest *HSL1* to be their native receptor. This would require closely related expression patterns for *HSL1* and *IDL* genes. However, at least in *Arabidopsis*, the expression patterns of *HSL1* and these *IDL* genes do not suggest involvement in floral organ abscission (Cho et al., 2008; Stenvik et al., 2008), and mutant phenotypes are so far lacking. Different methods have lately been suggested for testing of peptide-ligand receptor interactions (Butenko et al., 2009, 2012, 2014). Interaction of the mIDA

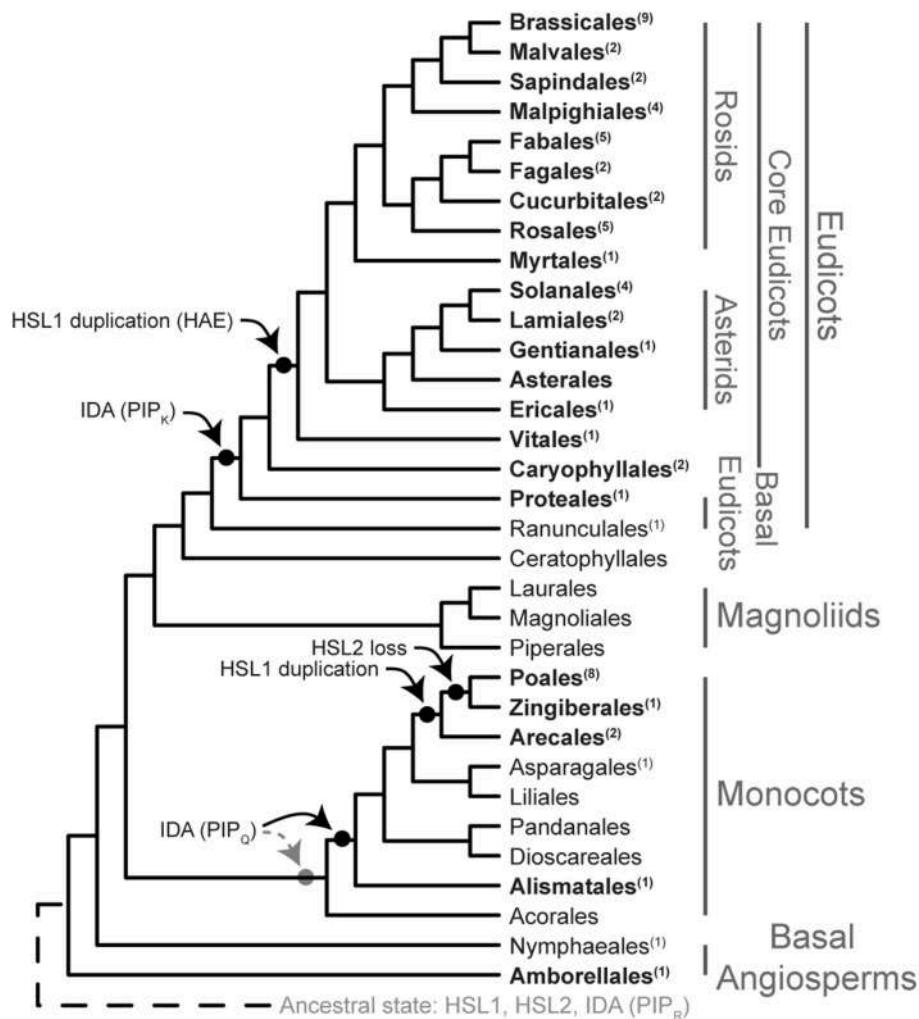


FIGURE 7 | Evolution of the IDA HAE/HSL2 signaling module. Phylogeny of Angiosperms adapted from Zhang et al. (2012), Vanneste et al. (2014), Zeng et al. (2014), and Dohm et al. (2014). Taxonomical levels are taken from Zeng et al. (2014). Numbers in superscript behind order names represent the number of species used in the analysis. Order names in bold represent those orders with at least one completely sequenced genome. The two possible evolutionary origins of IDA (PIP_Q) in monocots are illustrated.

peptide with HSL2 expressed in *N. benthamiana*, results in an immediate oxidative burst, similar to elicited LRR-RLKs involved in pathogen defense (Butenko et al., 2014). A thousand times more peptide is needed to provoke such a reaction from the HAE receptor under the same conditions, although the two receptors in genetic terms are functionally redundant with respect to control of floral organ abscission (Cho et al., 2008; Butenko et al., 2014). We have found distinct evolutionarily conserved differences between the kinase domains of HAE/HSL1 and that of HSL2. This may explain the differences in readout between HAE and HSL2 in the presence of IDA or IDL peptides (Butenko et al., 2014).

Interestingly, within monocots *HSL1* has been duplicated before the split-off of the Arecales, and *HSL2* has been lost after the split of Arecales, leaving the orders Poales, and Zingiberales devoid of the gene. Abscission still takes place in these orders, e.g., “finger drop” in banana (*Musa* spp.) and caryopsis shedding

(diminished in the domesticated species) in the grasses. We have demonstrated that oil palm of the Arecales use *HSL2* and *IDA* genes encoding PIP_R peptides in connection to fruit abscission. It is tempting to suggest that the PIP_Q version of the IDA peptide have developed to fit one or the other HSL1 receptors.

We have presented examples of the presence of IDA and its receptors in all the major groups of Angiosperms during abscission of floral organs, flowers, immature, and mature fruits and of leaves. The first Angiosperm developed more than 150 Myr ago, and IDA ligands may have been conserved most of this time. At the same time the number of IDA and receptor genes has increased and a variety of modes of organ detachment has developed. This lends support to the notion that it is not what you do, but where and when you do it that is important (Carroll, 2008). New gene copies have presumably made it possible to change place and time for cell separation events.

AUTHOR CONTRIBUTIONS

IS, RO, KF, XJ, JK, and IN performed the research; IS, RO, UF, TT, and RA analyzed the data; IN and RA designed the research, RA wrote the article with contributions from IS, RO, UF, TT, and IN.

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De novo Transcriptome Sequencing and Development of Abscission Zone-Specific Microarray as a New Molecular Tool for Analysis of Tomato Organ Abscission

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Abscission of flower pedicels and leaf petioles of tomato (*Solanum lycopersicum*) can be induced by flower removal or leaf deblading, respectively, which leads to auxin depletion, resulting in increased sensitivity of the abscission zone (AZ) to ethylene. However, the molecular mechanisms that drive the acquisition of abscission competence and its modulation by auxin gradients are not yet known. We used RNA-Sequencing (RNA-Seq) to obtain a comprehensive transcriptome of tomato flower AZ (FAZ) and leaf AZ (LAZ) during abscission. RNA-Seq was performed on a pool of total RNA extracted from tomato FAZ and LAZ, at different abscission stages, followed by *de novo* assembly. The assembled clusters contained transcripts that are already known in the Solanaceae (SOL) genomics and NCBI databases, and over 8823 identified novel tomato transcripts of varying sizes. An AZ-specific microarray, encompassing the novel transcripts identified in this study and all known transcripts from the SOL genomics and NCBI databases, was constructed to study the abscission process. Multiple probes for longer genes and key AZ-specific genes, including antisense probes for all transcripts, make this array a unique tool for studying abscission with a comprehensive set of transcripts, and for mining for naturally occurring antisense transcripts. We focused on comparing the global transcriptomes generated from the FAZ and the LAZ to establish the divergences and similarities in their transcriptional networks, and particularly to characterize the processes and transcriptional regulators enriched in gene clusters that are differentially regulated in these two AZs. This study is the first attempt to analyze the global gene expression in different AZs in tomato by combining the RNA-Seq technique with oligonucleotide microarrays. Our AZ-specific microarray chip provides a cost-effective approach for expression profiling and robust analysis of multiple samples in a rapid succession.

Keywords: auxin, ethylene, flower pedicel abscission, leaf petiole abscission, oligonucleotide microarray, RNA-Sequencing, tomato (*Solanum lycopersicum*), transcriptome

INTRODUCTION

Abscission is a systematically regulated process in plant development, by which subtended organs, leaves, flowers, fruit, and seed, separate from the parent plant in response to various physiological cues. This process is required to recycle nutrients for continuous growth, develop appropriate organs, survive diseases, and facilitate reproduction (Addicott, 1982; Sexton and Roberts, 1982; Roberts et al., 2002; Lewis et al., 2006). Since the domestication of crops was started, a great emphasis has been put forth on selection for abrupt abscission to improve crop quality and yield. For example, reduced seed shattering in rice (Li et al., 2006), wheat (Tanno and Willcox, 2006), maize (Doebley, 2004), and fruit tree species (Bangerth, 2000) was obtained. In an agricultural perspective, both enhanced and delayed abscission are highly relevant for growers.

In plants, the abscission process occurs at a predetermined region called abscission zone (AZ), composed of few layers of small and dense cytoplasmic cells, which lack large vacuoles and any maturation characteristics (Osborne and Morgan, 1989), resembling undifferentiated cells (Van Nocker, 2009). AZ cells belong to type II cells, in which extended growth can be enhanced by ethylene, but not by auxin (McManus, 2008), conferring their meristematic potential (Roberts et al., 2000). Physiological studies revealed that ethylene and auxin control the cell separation process. The abscission process is initiated or timed by changes in the auxin gradient across the AZ, and is accelerated by ethylene (Roberts et al., 2002; McManus, 2008; Meir et al., 2010). The four key steps in the abscission process are: differentiation of the AZ, acquisition of the competence to respond to abscission signals, execution of organ abscission, and formation of a protective layer (Meir et al., 2011; Wang et al., 2013). During the late abscission stages, the cell wall and middle lamella are the major targets for degradation, which is operated by many cell wall modifying enzymes, including polygalacturonases (PGs), xyloglucan endotransglucosylase/hydrolase (XTH), β -1,4-glucanase (cellulase, Cel), and expansins (EXP) (Lashbrook et al., 1994; del Campillo and Bennett, 1996; Cho and Cosgrove, 2000; Taylor and Whitelaw, 2001; Roberts et al., 2002; Ogawa et al., 2009; Meir et al., 2010).

Abbreviations: AREs, auxin-responsive elements; ARF, auxin response factor; Aux/IAA, auxin resistant/indole-3-acetic acid-inducible; AZ, abscission zone; bHLH, basic helix-loop-helix; Bl, blind; BLAST, basic local alignment search tool; bZIP, basic leucine zipper; Cel, cellulase; CTR, constitutive triple response; DEG, differentially expressed genes; EIL, ethylene insensitive-like; EIN, ethylene insensitive; ERF, ethylene responsive factor; EXP, expansin; FAZ, flower abscission zone; GH, Gretchen Hagen3; GO, gene ontology; IAA, indole-3-acetic acid; JA, jasmonic acid; KEGG, Kyoto encyclopedia of genes and genomes; LAX, auxin influx carrier (Like Aux); LAZ, leaf abscission zone; LBD1, lateral organ boundaries domain protein1; Ls, lateral suppressor; MAPKK, mitogen-activated protein kinase kinase; MAPKKK, MAPKK Kinase; 1-MCP, 1-methylcyclopropene; NAT, naturally occurring antisense transcripts; NAZ, non-AZ; NCBI, national center for biotechnology information; NGS, next generation sequencing; OFP, ovate family protein; PG, polygalacturonase; PIN, pin-formed; PE, paired end; QC, quality check; qRT-PCR, quantitative real time PCR; RNA-Seq, RNA sequencing; SAM, shoot apical meristem; SAUR, small auxin up-regulated RNA; TAGL, tomato AGAMOUS-like; TAPG, tomato abscission polygalacturonase; TF, transcription factor; WUS, WUSCHEL-related homeobox-containing protein; XTH, xyloglucan endotransglucosylase-hydrolase; ZF, zinc finger.

Tomato (*Solanum lycopersicum*) is one of the most important vegetable crops, whose genome sequence was recently published (The Tomato Genome Consortium, 2012). It serves as a model crop for studying fruit development (Klee and Giovannoni, 2011) and abscission, as it possesses a distinct joint-like structure in the AZ of the flower pedicels. The molecular mechanisms underlying the abscission progress in tomato are still evolving, even though the abscission physiology was studied long ago (Sexton and Roberts, 1982; Bleeker and Patterson, 1997; Roberts et al., 2000). The genes affecting AZ development have been identified by studying abscission-impaired mutants, such as *jointless*, *jointless2*, *macrocalyx*, *blind* (*bl*), and *lateral suppressor* (*ls*) (Butler, 1936; Rick, 1956; Schumacher et al., 1999; Mao et al., 2000; Szymkowiak and Irish, 2006; Shalit et al., 2009; Nakano et al., 2012; Liu et al., 2014). However, information regarding the expression of AZ-associated genes in tomato is still lacking.

Micro/oligo nucleotide arrays have been used until recently to study semi-global gene expression in AZs of citrus leaves (Agustí et al., 2008, 2009, 2012) and shoot tips (Zhang et al., 2014), tomato flowers (Meir et al., 2010, 2011; Nakano et al., 2012, 2013; Wang et al., 2013; Ma et al., 2015), *Arabidopsis* stamen (Cai and Lashbrook, 2008), and apple fruit and fruitlets (Botton et al., 2011; Zhu et al., 2011). The information on gene specific resources of tomato AZs during the abscission process obtained by microarrays is limited. The new era of high throughput next generation sequencing (NGS) technologies and bioinformatics tools to analyze and integrate the vast data, led to a significant rapid progress in the genomic research. RNA-Sequencing (RNA-Seq) involves direct sequencing of complementary DNAs (cDNAs), followed by mapping of reads to the reference genome or gene sets, to obtain a direct information from transcribed regions (Wang et al., 2009), gene expression profiles, and polymorphism detection in the genome. RNA-Seq provides a more comprehensive understanding of the transcriptome at a specific developmental stage of a tissue (Marioni et al., 2008; Wang et al., 2009; Parchman et al., 2010; Mäder et al., 2011), and an ability to detect novel transcripts, sense and antisense transcripts, single nucleotide polymorphism, small RNAs, alternate splice transcripts, and transcription initiation sites (Ozsolak and Milos, 2011). In comparison to other technologies, such as microarrays and Sanger-based sequencing technologies, RNA-Seq has additional advantages in terms of speed, depth, and accuracy. Recently, few RNA-Seq studies for studying the abscission process were carried out in various plant systems, such as olive (Gil-Amado and Gomez-Jimenez, 2013; Parra et al., 2013) and melon (Corbacho et al., 2013) fruit, *Arabidopsis* stamen AZ (Niederhuth et al., 2013), tomato flower AZ (FAZ) (Liu et al., 2014), and rose petals (Singh et al., 2013). However, a complete transcriptome study of the tomato FAZ and leaf AZ (LAZ) at various stages of the abscission process was not carried out, because of the higher cost of RNA-Seq analysis as compared to microarray. Considering the clear advantages of the RNA-Seq technology, the aim of the present research was to study the tomato FAZ and LAZ transcriptome, using RNA-Seq, followed by *de novo* transcriptome assembly and annotation.

In the last few years, customized-made expression arrays became more affordable to the scientific community due to the

number of total features, complete coverage of genomic regions, and automated data analysis of microarrays. The information regarding the new transcripts, such as unannotated transcripts, splice variants, naturally occurring antisense transcripts (NATs), and novel genes in particular tissues, is less emphasized on the traditional arrays (Bertone et al., 2005; Mockler et al., 2005). More complexity is added due to methylation sites on the 3' end that might interfere with transcription initiation and termination (Carninci et al., 2005, 2006). To overcome these problems, new customized microarray approaches for various needs, such as tilling arrays were developed, (Johnson et al., 2005). The custom-made arrays have a complete control of the number, expression, and distribution of probes specific to the studied system. In order to achieve higher hybridization and quality data, many considerations have to be taken while designing the customized microarrays, including probe length, density, melting temperatures, placement, level of cross hybridization, complexity, and mismatch levels to achieve the consensus property (Mei et al., 2003). The RNA-Seq information will be a useful tool to update the design of microarray probes for transcriptome analysis of large samples (Bellin et al., 2009).

In the present study, we used the Illumina sequencing technology to obtain a comprehensive transcriptome profile of the tomato FAZ and LAZ pooled samples taken at various time points during organ abscission induced by auxin depletion, thereby expanding the tomato transcript catalog. We focused on comparing the transcriptomes generated from the FAZ and the LAZ tissues to establish the divergences and similarities in their transcriptional networks, and particularly to characterize the biological processes and transcriptional regulators enriched in gene clusters that are differentially regulated in these two AZs. The RNA-Seq data were used as a major source to design an AZ-specific microarray for tomato abscission studies. Additionally, in this chip the probes were designed in both sense and antisense orientations for transcripts, which enable future analyses of expression profiles of NATs in the AZs. The unique design of this chip allowed us to accurately

quantify global changes in the transcriptome of the tomato AZs during the abscission process. Results from this study will help to identify target genes for further understanding and manipulating of abscission, as well as markers for breeding. We are currently using this chip to quantify molecular shifts in gene expression in transgenic plants that display altered abscission phenotypes.

MATERIALS AND METHODS

Plant Material and Treatments

Tomato (*S. lycopersicum*) cv. "VF-36" flower bunches were harvested from greenhouse grown 4-month-old plants between 08:00 and 10:00 a.m. Bunches bearing at least 2–4 freshly open flowers were brought to the laboratory under high humidity conditions. All procedures were performed in a controlled observation room maintained at 20°C, 12 h photoperiod, and 60% RH. Closed young flower buds and senesced flowers were removed, and the stem ends of the bunches were trimmed. Groups of 2–3 flower explants were placed in vials containing double distilled water. The pedicel abscission assays were performed after flower removal, as previously described (Meir et al., 2010). For RNA extraction from the FAZ, 30 sections, <2-mm-thick, were excised, about 1 mm from each side of the visible AZ fracture. The samples were collected at predefined time points: 0 h—before flower removal, and 2, 4, 8, and 14 h after flower removal (Figure 1A).

Shoots containing at least 5–6 expanded leaves were brought to the laboratory under high humidity conditions. The shoot cut ends were trimmed, and the shoots were placed in jars containing double distilled water and incubated for 3 h to avoid dehydration before starting leaf deblading. The fully expanded leaves were debladed using a sharp razor by leaving a subtended 2-cm long petiole. To accelerate petiole abscission, the debladed leaf explants were exposed to 10 $\mu\text{L L}^{-1}$ ethylene for 24 h in an airtight chamber at 23°C. The number of abscising petioles was then

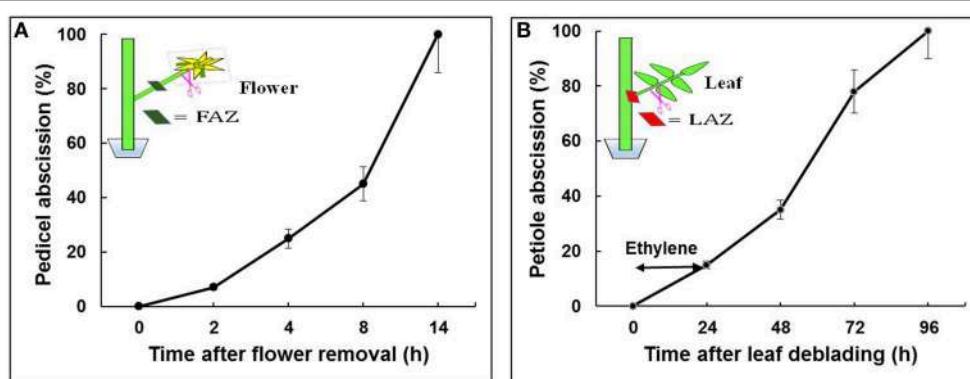


FIGURE 1 | Effect of flower removal (A) or leaf deblading and ethylene treatment (B) on the kinetics of pedicel and petiole abscission, respectively, in tomato explants. Flowers and leaves were excised as indicated in the schematic illustrations. The debladed-leaf explants held in vials with water were prepared as previously described for the flower explants (Meir et al., 2010), and exposed to ethylene (10 $\mu\text{L L}^{-1}$ for 24 h). The percentage of accumulated pedicel or petiole abscission were monitored at the indicated time intervals following organ removal. The results are means of four replicates ($n = 30$ explants) \pm SE.

recorded. The ethylene concentration was determined by a gas chromatograph (Varian 3300), equipped with an alumina column and a flame ionization detector, in a 5-mL air samples withdrawn from the chamber with a gas tight syringe. Tissue samples for RNA extraction were taken from 20 LAZ sections, as described above for the FAZ. The samples were collected at the following predefined time points: 0 h—before leaf deblading, and 24, 48, 72, and 96 h after leaf deblading and ethylene treatment (**Figure 1B**). All the collected FAZ and LAZ samples were placed in RNA-later solution for further analyses. Three biological replicates of equal tissue weight were taken from each sample for RNA extraction. Equal RNA quantities from all the time point samples of the FAZ or the LAZ were pooled to create one RNA sample of FAZ and one of the LAZ.

RNA Isolation and Quality Controls

Total RNA was extracted from the FAZ and the LAZ samples, using an Agilent plant RNA isolation mini kit (Agilent, USA). The concentration and purity of the RNA samples were examined by a Nanodrop instrument, and validated for quality by running an aliquot on a Bioanalyzer with RNA 6000 Nano Kit (Agilent Technologies, California, USA). Only samples having an RNA integrity number >8.0 were selected for cDNA preparation. RNA samples extracted from the FAZ and the LAZ at five specific time points were pooled to make individual transcriptome libraries for RNA-Seq study.

Illumina Sequencing and Quality Controls

Transcriptome libraries for sequencing were constructed according to the Illumina TruSeq RNA library protocol outlined in “TruSeq RNA Sample Preparation Guide” (Part # 15008136; Rev. A, Illumina, USA). Briefly, mRNA was purified from 1 µg of intact total RNA using oligodT beads (TruSeq RNA Sample Preparation Kit, Illumina, USA). The purified mRNA was fragmented at an elevated temperature in the presence of divalent cations, and reverse transcribed with Superscript II Reverse transcriptase (Invitrogen, USA) by priming with random hexamers. Second strand cDNA was synthesized in the presence of DNA polymerase I and RNase H enzymes. The cDNA was cleaned up using Agencourt Ampure XP Solid Phase Reversible Immobilization beads (Beckman Coulter, Switzerland). Illumina Adapters were ligated to the cDNA molecules after end repair and addition of A base. Solid Phase Reversible Immobilization cleanup was performed after ligation. The library was amplified using PCR for enrichment of adapter ligated fragments. The prepared libraries were quantified by a Nanodrop instrument and validated for quality by running an aliquot on High Sensitivity Bioanalyzer Chip (Agilent Technologies, California, USA).

The DNA obtained from the prepared libraries was denatured and sequenced by the Illumina Genome Analyzer IIx, using the sequencing by synthesis method to read 72 bases PE. The raw sequencing data were then extracted from the server using the proprietary Illumina software to obtain Fastq format. Quality check (QC) of raw data was performed using SeqQC-V2.0 program (NGS data QC).

De novo Transcriptome Assembly and Differentially Expressed Genes

The following methodology for transcriptome assembly was used for each of the pooled samples of the FAZ and the LAZ. Raw reads were assembled using the Velvet-1.1.05 software and transcripts were generated using Oases assembler (Schulz et al., 2012). The transcripts were then subjected to Basic Local Alignment Search Tool (BLAST) analysis against *S. lycopersicum* mRNA and protein sequences from the International Tomato Annotation Group 2 (ftp://ftp.sgn.cornell.edu/genomes/Solanum_lycopersicum/annotation/ITAG2.3_release/). The transcripts with more than 50% identity and 70% query coverage were used for further analysis. Raw reads of the FAZ and the LAZ were aligned to reference cDNA using the Bowtie-0.12.7 program (Langmead et al., 2009). Expression studies were performed based on enrichment calculation of each gene. Enrichment = average read depth × coverage. Genes were considered to be upregulated, down-regulated, or neutral based on the LAZ/FAZ enrichment ratio. The parameters definitions were as follows: Up, log fold change >1; Down, log fold change <-1; Neutral, log fold change >-1 to <1.

Design of the AZ-Specific Microarray (Pooled Data of the FAZ and the LAZ)

The following steps were performed: Raw reads of both the FAZ and the LAZ samples were pooled and assembled as described above for *de novo* assembly (steps 1 and 2); Unannotated transcripts were subjected to BLAST analysis against *Arabidopsis thaliana* and *Nicotiana tabacum* mRNA and protein sequences, with references derived from The National Center for Biotechnology Information (NCBI) and The Arabidopsis Information Resource (TAIR) databases (step 3); Transcripts with more than 50% identity and 70% query coverage were considered as annotated, and all the rest were categorized as novel transcripts (step 4); Pooled reads were further aligned to mRNA sequences of *S. lycopersicum* and unaligned reads were assembled to derive contigs. These contigs were further filtered by *E. coli*, *C. elegans*, *A. thaliana*, and *N. tabacum* mRNA sequences, with references derived from the NCBI database (step 5); Unannotated contigs from the above analysis (step 5) were added to the novel transcripts category (step 6); The unassembled reads from step 5 were further filtered by aligning them to *E. coli* and *C. elegans* mRNA sequences. Unaligned reads were assembled and added to the category of novel transcripts (step 7); Transcripts from steps 4, 6, and 7 were pooled together as novel transcripts, and transcripts which were annotated by *S. lycopersicum*, *A. thaliana*, and *N. tabacum* as performed in steps 2 and 3 were considered as known transcripts (step 8); To further filter the novel transcripts, BLAST was performed between novel and known transcripts, and only transcripts showing <10% query coverage with known transcripts were considered as novel transcripts (step 9). The novel (step 9) and known (step 8) transcripts were used for the design of the AZ-specific microarray. Probes were categorized as specific and cross hybridizing on the basis of their BLAST results. The criteria for a specific probe were as follows: a probe with a single hit

against the target, a probe alignment length of 60–31 bp, a probe with allowed mismatches <3 and gaps <2, and a probe with a minimum length of 28 bases. Thus, out of a total number of 176,026 designed probes, 88,445 were specific probes, and 5363 were cross hybridized probes. For 429 transcripts, no probes were designed as filtered due to repeats and vector masked.

Gene Ontology (GO) Term Enrichment and Biological Pathways

Distribution of transcripts into various biological pathways in Kyoto Encyclopedia of Genes and Genomes (KEGG) was done through the KEGG Automatic Annotation Server (<http://www.genome.jp/tools/kaas/>) to obtain the KEGG IDs for the transcriptome sequences, and to identify the genes involved in plant hormone signal transduction.

Validation of Gene Expression by Real Time PCR (qPCR)

Primers for qPCR analysis were designed manually using the Gene Runner V 3.05 software (Hastings Software Inc. Hastings, USA; <http://www.generunner.net>). The primers were validated using one of the samples, and the amplicon sizes were confirmed on a 2% agarose gel. The sequences, amplicon length, and melting temperature (Tm) of the primers used are detailed in Table S1. The RNA samples used for the qPCR assay were the same samples used for the microarray analysis. Samples of 400 ng of DNase-treated RNA were reverse transcribed to synthesize 20 ng/ μ l of cDNA, using an oligo (dT) primer with Affinity Script QPCR cDNA synthesis kit (Agilent Technologies, USA). Relative quantification by qPCR was then performed using Brilliant II SYBR Green qPCR Master mix (Agilent Technologies, USA) according to the manufacturer's instructions. The experiment was conducted using a Stratagene Mx3005P QPCR machine platform (Mx3005P system software, Stratagene, CA). The relative expression levels of the genes were determined after normalizing with *ACTIN* as the reference gene, using the Delta Ct method. The PCR program consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 1 min, and 72°C for 1 min. A melt curve was also performed after the assay to check for specificity of the reaction. Duplicates of each gene with 20 ng cDNA input per reaction from two independent experiments were used. The relative quantification of gene expression level was determined by the comparative Ct method $2^{-\Delta\Delta Ct}$.

RESULTS

Kinetics of Flower Pedicel and Leaf Petiole Abscission

The abscission of flower pedicels and leaf petioles was induced by removing the auxin sources, flowers or leaves, respectively. The selected two sets of time points analyzed, 0, 2, 4, 8, and 14 h for flower pedicels and 0, 24, 48, 72, and 96 h for leaf petioles, were based on the abscission kinetics of these organs after flower removal or leaf deblading followed by ethylene treatment, respectively. Flower pedicels completely abscised 14 h after flower

removal (Figure 1A), while leaf petioles completely abscised only 96 h after leaf deblading and exposure to ethylene (Figure 1B). The exposure of the leaf debladed explants to ethylene was required to enhance the abscission process, since the petioles abscised very slowly (8–12 days) without ethylene treatment, with a large variation between replicates (data not shown). It should be noted that a similar percentage of organ abscission was obtained in both systems, but on a different time scale (Figure 1). Based on these similar abscission percentages, we have chosen the indicated time points for RNA sampling from both AZs for the RNA-Seq experiments, which correspond to steps 2–4 of the abscission process, namely acquisition of the competence of AZ cells to respond to abscission signals, execution of organ abscission, and beginning of formation of a protective layer. The kinetics of tomato flower pedicel abscission in response to flower removal, without or with exogenous application of ethylene, is well documented (Meir et al., 2010; Wang et al., 2013).

Illumina Paired-End Sequencing and De novo Assembly

The RNA extracts of the FAZ and the LAZ sampled at specified time points after flower or leaf blade removal, respectively, were prepared as described in Figure 1, and sequenced using the Illumina sequencing platform. We used a pooled sequencing strategy by mixing RNA samples of the FAZ and the LAZ from various abscission stages. Individual cDNA libraries for *de novo* transcriptome sequencing were constructed for these pooled samples, in order to obtain a wide range of expressed transcript sequences and to reduce the sequencing costs (Sangwan et al., 2013; Xu et al., 2013). By PE sequencing of the tomato AZs in separate lanes, we obtained a total of 84,626,974 and 80,837,288 reads with 73 bp in length encompassing about 9.05 GB and 9.47 GB of sequence data for the LAZ and the FAZ, respectively (Table 1), in Fastq format after the initial quality filtering was performed with the default parameters. High quality reads were obtained by trimming primers/adapters and filtering by stringent parameters to increase the analysis reliability by trimming the reads with more than 70% of the bases having Phred quality score >20. This resulted in an average of 79,570,559 and 75,843,448 PE reads, which represent 94.02 and 93.82% of high quality read percentage for the LAZ and the FAZ, respectively (Table 1). In addition, this procedure enhanced the average quality score at each base position of the sequence reads by 94.61 and 94.71% (Table S2) of high quality base percentage for the FAZ and the LAZ, respectively (Figures S1, S2).

Initially, we performed a *de novo* assembly for the transcripts, and later on the reference-based assembly was performed using the tomato genome sequence (The Tomato Genome Consortium, 2012) as reference genome. Both the *de novo* and reference assemblies were merged to generate the final assembly, which was used for all further analyses. We also carried out an analysis of differentially expressed genes (DEG) for the FAZ and the LAZ. *De novo* assembly was optimized using different criteria, such as the numbers of used reads, total number of contigs, contigs length in bp (>100, 500, 1000 bp and so on), n50_Log, maximum, average, and minimum contigs length (100 bp), based on the function of K-mer length (Hash Length). The inverted relation between

TABLE 1 | Assembly statistics of pooled transcriptome of the tomato LAZ and FAZ.

	LAZ	FAZ
SEQUENCING		
Total number of reads	84,626,974	80,837,288
Total number of high quality reads	79,570,559 (94.02%)	75,843,448 (93.82%)
Mean read length (bp)	73	73
Number of reads assembled	65,043,974	61,796,756
Percentage of reads assembled	76.8596	76.4459
CONTIGS		
K-mer length used	47	51
Number of generated contigs	25,046	26,583
Maximum contig length (bp)	12,969	9756
Minimum contig length (bp)	100	101
Average contig length (bp)	1048.62	858.539
Total contigs length (bp)	26,263,627	22,822,531
Total number of non-ATGC characters	3784	6364
Percentage of non-ATGC characters	0.000144	0.000279
Number of contigs > 100 bp	25,005	26,583
Number of contigs > 500 bp	16,164	14,132
Number of contigs > 1 Kbp	10,451	8860
Number of contigs > 10 Kbp	3	0
Number of contigs > 1 Mbp	0	0
n50_Log	1581	1512

the k-mer and the number of contigs (Sangwan et al., 2013) led us to optimize the assembly with various k-mer values ranging from 33 to 65 (Table S2). At K-mer = 33 the highest number of contigs, 49,849 and 57,398, and a higher number of longer coting length (>10 Kb) was obtained in both the FAZ and the LAZ samples, respectively (Table S2). We assembled most of the high quality reads, 78.74 and 78.41%, into longer contigs at k-mer = 39 and 37 in the FAZ and the LAZ samples, respectively. These recorded the highest percentage of assembled reads (Table 1 and Table S2), thereby implying a high coverage for these sequencing data. The generated assembly had total sequences of 33,025 and 41,592, with an average sequence length of ~1233 and 1255 bp, and a minimum sequence length of 100 bp in the FAZ and the LAZ samples, respectively. Ultimately, FASTA files containing the assembled contigs were obtained for the FAZ and the LAZ, respectively. Table S2 presents a general view of the sequencing and assembly processes, which provides the length distribution for these high-quality reads. The statistics for the two assemblies is detailed in Table 1.

Differentially Expressed Gene Analysis for the FAZ and the LAZ

To investigate abscission distinctions, we compared the transcriptomes of tomato FAZ and LAZ using Illumina DEG analysis. Specifically, we analyzed variations in gene expression between the FAZ and the LAZ in pooled samples, which resulted

from two independent DEG libraries. Raw reads of the FAZ and the LAZ were aligned to reference cDNA using the Bowtie-0.12.7 program. The expression study was performed based on enrichment calculation of each gene, when enrichment = average read depth × coverage. The total contigs were classified into five groups, based on their differentially expression levels in the pooled LAZ and FAZ samples, expressed by the LAZ/FAZ enrichment ratio, as follows: Group A, contigs over-expressed in the LAZ; Group B, contigs over-expressed in the FAZ; Group C, contigs equally expressed in the FAZ and the LAZ; Group A1, contigs exclusively expressed in the LAZ; Group B1, contigs exclusively expressed in the FAZ. The parameters used for this classification included: Group A, genes which were up-regulated in the LAZ samples compared to FAZ samples with log fold change >1; Group B, genes which were down-regulated in the LAZ samples compared to the FAZ samples, with log fold change <-1; Group C, genes which were equally expressed in both AZs with log fold change ranging between -1 to +1. The results presented in Figure 2 show that out of 22,650 total genes, 4997 genes were classified in Group A, 2899 genes in Group B, 14,754 genes in Group C, 1349 genes in Group A1, and 1259 genes in Group B1. The number of genes expressed in the LAZ pooled sample was approximately twice the number of genes expressed in the FAZ pooled sample. Table S3 presents the detailed lists of genes expressed in each category.

Annotation and Gene Ontology Functional Enrichment Analysis

The tomato genome (The Tomato Genome Consortium, 2012) was recently published, and the functional annotation and assignment of GO by the ITAG enabled us to clearly designate the differences in gene expression between the two tomato AZs. However, the complete transcriptome information on the tomato FAZ and LAZ has not yet been determined. Annotation of assembled genes was performed using the Velvet-1.1.05 and Oases assembler programs and subjected to BLAST analysis against the *S. lycopersicum* mRNA and protein sequences from ITAG2.3 (ftp://ftp.sgn.cornell.edu/genomes/Solanum_lycopersicum/annotation/ITAG2.3_release/). Transcripts showing more than 50% identity and 70% query coverage were taken for further analysis. The average sequence length was 11,840 and 15,794 bp for the FAZ and the LAZ, respectively, with a minimum sequence length of 100 bp. The annotation description of mRNA and proteins is detailed in Table 2. The results indicate that out of 41,592 and 33,025 total contigs generated for the LAZ and the FAZ, respectively, 41 and 49% compared well with the annotated genes and proteins of tomato in the LAZ and the FAZ, respectively. On the other hand, we found that ~58.9 and 50.5% contigs of the LAZ and the FAZ, respectively, failed to map to ITAG2.3 identities of both mRNA and proteins (Table 2). This pool may serve as a good source for discovering new genes. The lists of mRNA-annotated, protein-annotated, mRNA-overlapping, and protein-overlapping contigs are detailed in Tables S4, S5. Once again the assembly was annotated with the recent available genome assembly (The Tomato Genome Consortium, 2012), which resulted in 32,949

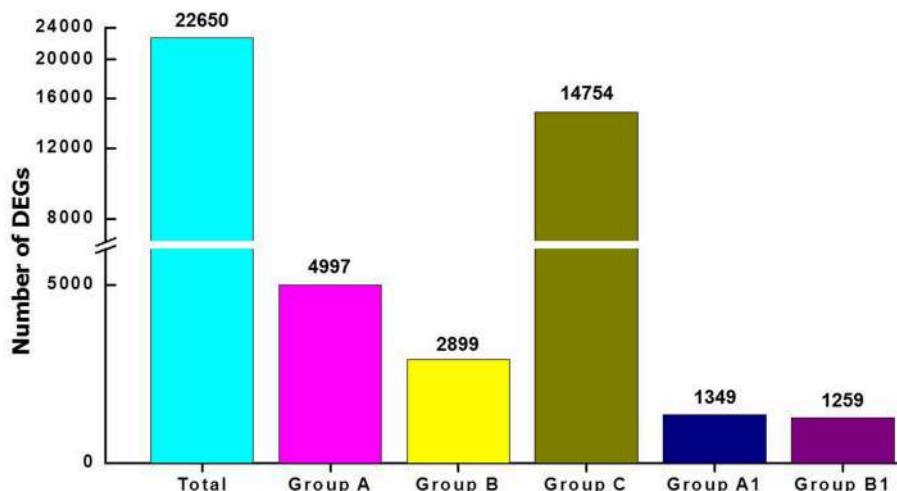


FIGURE 2 | Analysis of differentially expressed genes (DEG) in pooled samples of FAZ and LAZ. The number of transcripts indicated above the bars was obtained after performing the Illumina DEG analysis and dividing into groups. Total, transcripts expressed in both tissues regardless of the expression pattern; Group A, transcripts over-expressed in LAZ samples; Group B, transcripts over-expressed in FAZ samples; Group C, transcripts equally expressed in FAZ and LAZ samples; Group A1, transcripts exclusively expressed only in LAZ samples; Group B1, transcripts exclusively expressed only in FAZ samples. The detailed lists of genes in each category are summarized in Table S2.

TABLE 2 | Summary of the modules for contig annotation in the LAZ and FAZ.

Contig annotation summary	LAZ			FAZ		
	mRNA and proteins	mRNA	Proteins	mRNA and proteins	mRNA	Proteins
Total number of contigs	41,592	41,592	41,592	33,025	33,025	33,025
Total number of annotated contigs	17,088	15,232	15,741	16,343	14,717	14,773
Total number of unannotated contigs	24,504	26,360	25,851	16,682	18,308	18,252
Number of overlapping contigs in <i>Solanum lycopersicum</i>						
Number of mRNA showing annotation for more than one contig	4052				3988	
Number of proteins showing annotation for more than one contig		9272				9067

and 41,502 transcripts (FASTA file) for the FAZ and the LAZ, respectively (data not shown).

We have further carried out an enrichment GO analysis for the total annotated proteins (complete list), including 15,741 and 14,773 LAZ and FAZ proteins, respectively (Table 2). Among them, 12,409 FAZ and 13,106 LAZ proteins were designated with at least one GO term (Tables S6, S7). The GO terms of “protein binding,” “oxidation-reduction process,” and “membrane” were the most represented ones among the categories of molecular function, biological process, and cellular component, respectively, in both AZs (Figure 3). The analysis represents the top 10 GO in molecular function, biological process, and cellular component for the FAZ (Figure 3A) and the LAZ (Figure 3B). These data show that both the FAZ and the LAZ share a similar type of gene enrichments when samples of all-time points following abscission induction were pooled together.

In the search of cues for explanation of the different abscission rates of petioles and pedicels, we examined the overexpression

categories (Groups A and B) in both AZs, as the complete list resulted in similar types of gene enrichments (Figure 3). Out of 4997 and 2899 annotated contigs found in Groups A and B, respectively, 2066 and 1135 were designated with at least one GO term (Tables S8, S9) in the LAZ and the FAZ, respectively. The GO terms identified in Groups A and B (Figures 4–6) showed the enrichment of GO categories of highly represented contigs, as demonstrated in the complete annotation charts for both AZs (Figure 3).

The GO terms include indicators of the various biological processes operating in the two AZs during abscission. In the category of biological process, most of the DEG in Group B over-expressed in the FAZ were classified as associated with metabolic processes, oxidation-reduction, regulation of transcription, transmembrane transport, protein amino acid phosphorylation, and proteolysis. Interestingly, Group A (over-expressed in the LAZ) also showed enrichment in a similar list of genes (Figure 4), indicating that the same biological processes might require the operation of the same gene sets

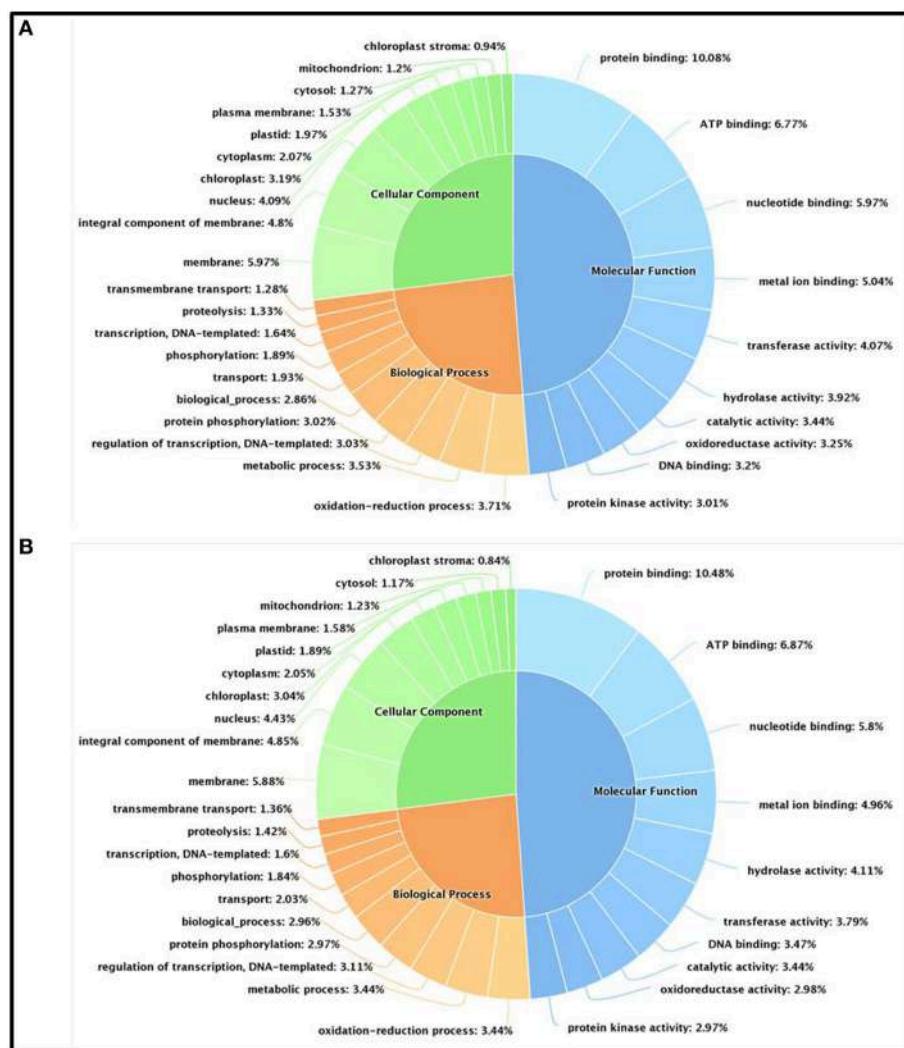


FIGURE 3 | Enrichment Gene Ontology (GO) terms in the FAZ (A) and LAZ (B). The enrichment analysis included 14,773 genes for the FAZ and 15,741 genes for the LAZ. The chart represents the top 10 GO listed in Tables S5, S6 in the categories of Molecular Function (blue), Biological Process (orange), and Cellular Components (green).

in the two AZs during abscission execution. Nevertheless, some differences were found between the two lists of enriched GO terms. In Group A, the GO terms were associated with phosphorylation, transport, carbohydrate metabolic processes, translation, methylation, defense response, microtubule-based processes, protein folding, and cell cycle (Figure 4). This suggests that such biological processes may be associated with leaf abscission. On the other hand, in Group B the enriched GO terms included the processes of lipid metabolism, photosynthesis, and biosynthesis (Tables S8, S9).

In the category of molecular function, the abundant transcripts in Groups A and B showed the predominant expression of genes associated with metal-ion binding, catalytic, transferase, hydrolase activities, and transferring phosphorus-containing groups. Apart of these similar gene categories, the most over-represented GO terms in Group A also included genes

associated with protein and ATP binding, while Group B also included genes associated with oxidoreductase activity (Figure 5, Tables S8, S9). Finally, within the category of cellular component, the GO terms of membrane, nucleus, cytoplasm, and integral to membrane constituted the most over-represented categories of the genes with increased transcript accumulation in both Groups A and B (Figure 6).

Differential Regulation of Genes Encoding Transcription Factors in the FAZ and the LAZ

Among the total 7896 differentially regulated genes of Groups A and B, diverse families of genes putatively encoding transcription factors (TFs) were differentially expressed (Table S10) in the pooled samples of the LAZ and the FAZ. Thus, 336 TF genes were

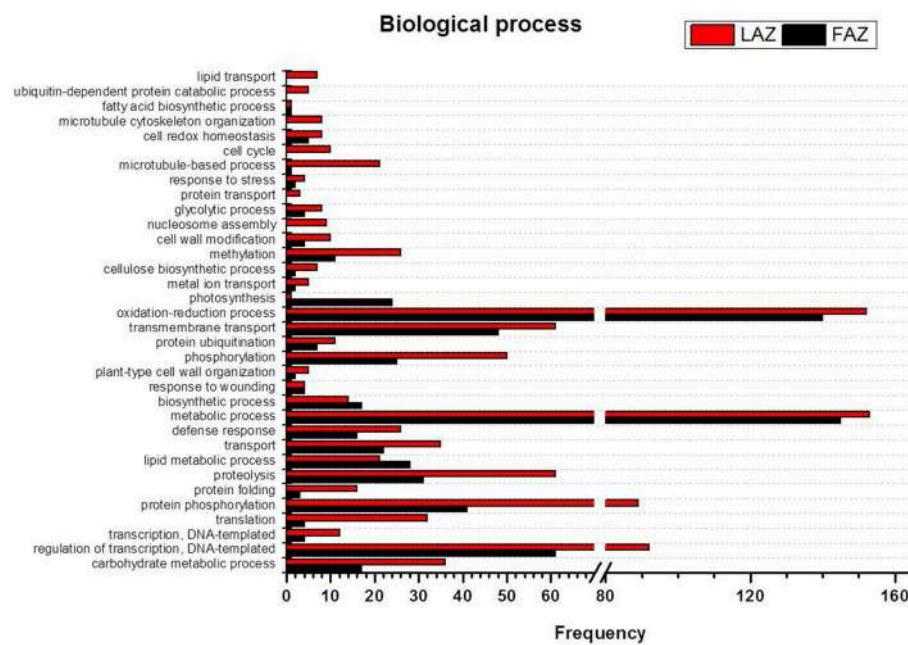


FIGURE 4 | Comparison of frequencies of the GO “biological process” term of over-expressed transcripts in the LAZ (Group A) and FAZ (Group B). The bars represent the comparison of the occurrence frequencies of the GO “biological process” term in the GO annotations of 1135 and 2066 over-expressed transcripts in the FAZ and LAZ, respectively. The frequencies are given for the most abundant biological processes.

expressed in Group A and 215 TF genes were expressed in Group B. Changes in the abundance of these 551 differentially over-expressed TF genes, belonging to the top 20 highly represented TF families, were determined in both the LAZ and the FAZ (**Figure 7A**). Additionally, diverse families of TF genes were also equally expressed in the FAZ and the LAZ (Group C). Apart from the differentially overexpressed TF genes, we also found exclusively expressed TF genes in each of the AZs. Thus, 71 and 70 TF genes, which belong to various TF families, were specifically expressed in the LAZ and the FAZ, respectively (Group A1, B1) (**Table S10, Figure 7B**).

The most abundant TF genes overexpressed in the LAZ (Group A) were: ZINC FINGER (ZF) proteins, MYB, Homeobox domain proteins, Ethylene Responsive Factor (ERF), basic leucine zipper (bZIP), basic helix-loop-helix (bHLH), and WRKY. In the FAZ (Group B) the most abundant TF genes were: MYB, ZF proteins, ERF, bHLH, NAC, Homeobox domain proteins, and MADS box (**Figure 7A**). The most abundant TF genes found in the exclusively expressed genes in the LAZ (Group A1) included: MYB, ERF, ZF proteins, bHLH, GRAS, bZIP, and B3 domain families, whereas the exclusively expressed genes in the FAZ (Group B1) was enriched with the TF genes of MYB, ZF proteins, ERF, MADS box, and B3 domain (**Figure 7B**). Although the two sub-groups (A1, B1) contained members of several TF families, in each primary Group (A, B), significant differences were found in the proportion of the TF families. Moreover, distinct TF families were differentially expressed in each Group, including AP2/ERF, Auxin Response Factor (ARF), Aux/IAA, TF E2F, and CCAAT-binding protein families in Group A, and NAC,

TCP, GATA TF—Zinc finger GATA-type, and GRAS families in Group B (**Figure 7A**). The enrichment of sequence elements in different gene groups from each cluster, in combination with the data on transcript abundance offer a tenable set of TFs which bind these elements, and that could be examined in future research.

Our results show that the most abundant TF family in Groups A and A1 or Groups B and B1 was MYB (**Figure 7**). The MYB gene (Solyc11g069030—*Bl*) was specifically expressed in the tomato FAZ compared to proximal and distal NAZ tissues (Nakano et al., 2013), and was overexpressed in the LAZ (Group A) as shown by our analysis (**Table S10**). Several MYB *blind-like* (*bli*) genes (*bli1,3,4,5,7*) (Solyc09g008250, Solyc04g077260, Solyc12g008670, Solyc08g065910, Solyc02g091980, respectively) were also expressed in all groups (**Table S10**). Our analysis show that a total number of 98 ZF TF genes were overexpressed in both AZs, being the second most highly expressed family in Groups A and B (**Figure 7A, Table S10**).

The MADS-box and GRAS TFs gene families were expressed in both AZs represented by Groups A and B (**Figure 7A, Table S10**). While the GRAS family was highly expressed in Group A1, the MADS-box family was much more highly expressed in Group B1 (**Figure 7B, Table S10**). The MADS-box and GRAS TF genes, MACROCALYX (Solyc05g012020), JOINTLESS (Solyc11g010570), SEPALLATA MADS-box Protein21 (SLMBP21), and *Ls* (Solyc07g066250), which were shown to regulate the differentiation and development of the tomato FAZ (Schumacher et al., 1999; Mao et al., 2000; Nakano et al., 2012; Liu et al., 2014), were expressed in both the FAZ

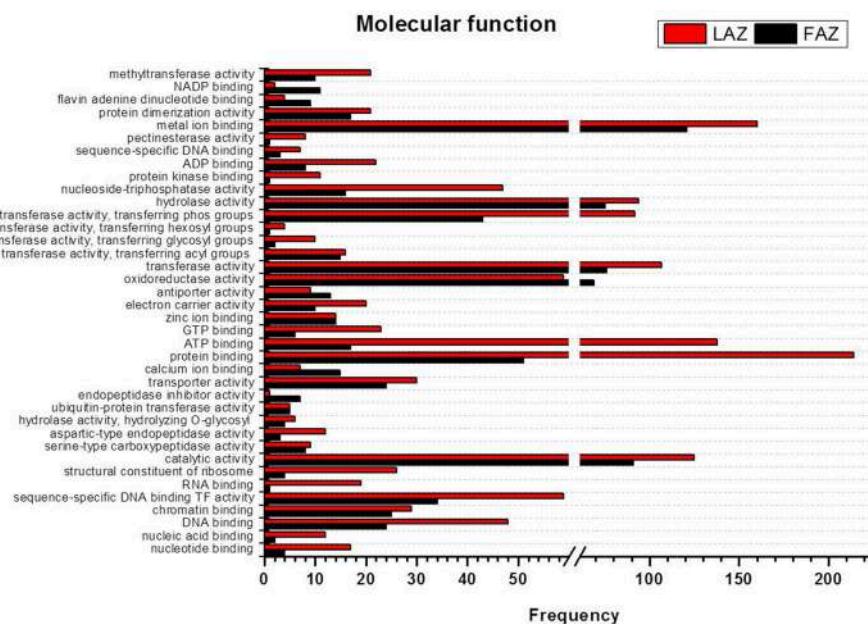


FIGURE 5 | Comparison of frequencies of the GO “molecular function” term of over-expressed transcripts in the LAZ (Group A) and FAZ (Group B). The bars represent the comparison of the occurrence frequencies of the GO “molecular function” term in the GO annotations of 1135 and 2066 over-expressed transcripts in the FAZ and LAZ, respectively. The frequencies are given for the most abundant molecular functions.

and the LAZ (Table S10). The *MACROCALYX* gene was highly expressed in the tomato FAZ (Group B), whereas the *JOINTLESS* gene was expressed at a similar level in both the FAZ and the LAZ (Group C) (Table S10). Therefore, we speculate that a similar type of organ identity specification, reported for the FAZ, might also operate in the LAZ.

The MADS-box gene *Tomato AGAMOUS-LIKE12* (*TAGL12*), which is known to be expressed during tomato seed and fruit development (Busi et al., 2003), was upregulated in the FAZ after flower removal (Meir et al., 2010). Our analysis data show that *TAGL12* was equally present in both the FAZ and the LAZ (Group C), while *TAGL2* (Solyc02g089200—*LeSEPI*) was highly over-expressed in the FAZ (Group B) compared to the LAZ (Group A) (Table S10), in accordance with our previous report (Meir et al., 2010). Several GRAS TFs, including *GRAS2* (Solyc07g063940), *GRAS5* (Solyc09g018460), *GRAS7* (Solyc07g065270), and *GRAS9* (Solyc06g036170), were exclusively over-expressed in the FAZ (Group B), whereas *GRAS4* (Solyc01g100200) was overexpressed in the LAZ (Group A) (Table S10). This suggests that different GRAS TF family members probably mediate abscission-responsive transcription in both flowers and leaves.

The WRKY TF family identified in multiple crop species, was implicated to operate in various biological processes in plants, especially in regulating defense mechanisms against biotic and abiotic stresses (Rushton et al., 2010). So far, 137, 89, and 81 WRKY genes were identified in rice, Arabidopsis, and tomato, respectively (Zhang et al., 2011; Huang et al., 2012). Our analyses revealed that 17 and 8 WRKY genes were overexpressed in the tomato LAZ and FAZ, respectively (Table S10). These results are

consistent with previous studies showing upregulation of several WRKY TF genes in fruit AZ during abscission of mature melon and olive fruit (Corbacho et al., 2013; Gil-Amado and Gomez-Jimenez, 2013). *WRKY1* and *WRKYIId-1* (AY157063) genes were upregulated in the tomato FAZ at the early and late stages of pedicel abscission (Meir et al., 2010). In the present study, we show that the *WRKYII* (Solyc01g079360) gene was expressed in both tomato AZs, but was highly expressed in the FAZ (Group B) compared to the LAZ (Table S10). This suggests that the WRKY TFs have a role in both AZs in mediating the late events of the abscission process.

Recently, 159 and 152 *bHLH* TFs genes were identified in the tomato genome (Sun et al., 2015; Wang et al., 2015). Out of the 159 identified *bHLH* genes in tomato, we could detect 137 *bHLH* TFs in both AZs, which were distributed among all the groups presented in Figure 2, indicating that this TF family is associated with the abscission process. Out of these 137 AZ-associated *bHLH* genes, 30 genes were expressed in Group A, 28 genes in Group B, 67 genes in Group C, and 7 and 5 genes were exclusively present in the LAZ (Group A1) and the FAZ (Group B1), respectively (Figure 7, Table S10). Most of the *bHLH* TFs were overexpressed in both the FAZ and the LAZ (Group A and B) (Table S10).

Most of the *bZIP* TFs genes (22) were present and overexpressed in the LAZ (Group A), compared to only five genes in the FAZ (Group B) (Figure 7A, Table S10). In the exclusively expressed categories, five *bZIP* TF genes were present in the LAZ compared to one gene in the FAZ (Figure 7B, Table S10). One of the *bZIP* TF gene (BG631669) was reported to be downregulated in the FAZ at an early stage of tomato pedicel abscission

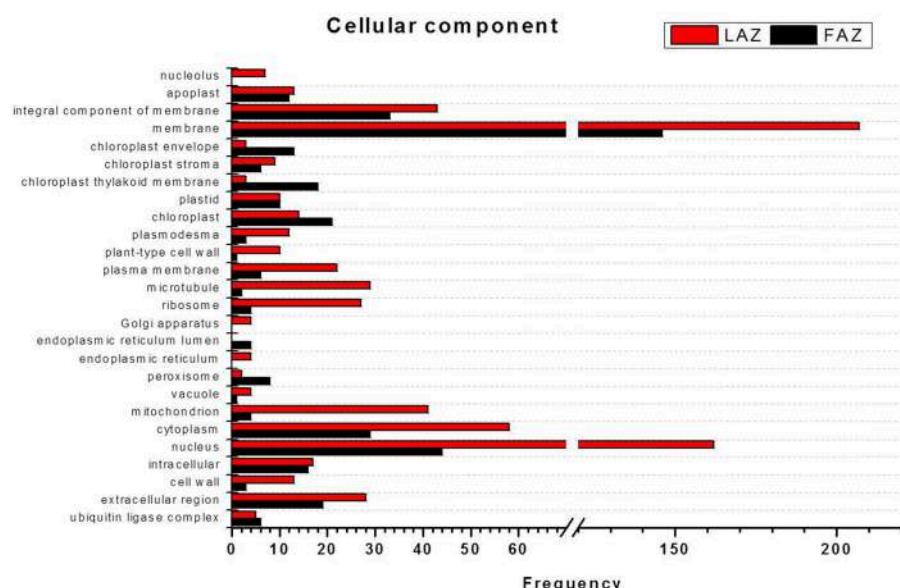


FIGURE 6 | Comparison of frequencies of the GO “cellular component” term of over-expressed transcripts in the LAZ (Group A) and FAZ (Group B).

The bars represent the comparison of the occurrence frequencies of the GO “cellular component” term in the GO annotations of 1135 and 2066 over-expressed transcripts in the FAZ and LAZ, respectively. The frequencies are given for the most abundant cellular components.

(Meir et al., 2010). Most members of the AP2/ERF, B3 domain, *bHLH*, *bZIP*, *MADS*, *MYB*, *WRKY*, *ZF*, *Homeobox*, and *Ethylene Responsive Factor* (ERF) gene families were overexpressed in the tomato LAZ samples compared to the FAZ, while most members of the NAC families were less expressed in the LAZ samples (Group A) (Figure 7).

In the present study, we identified common and distinct TFs that were not previously related to abscission. Our results also show that distinct patterns of transcriptional regulation occur in the tomato FAZ and LAZ.

Key Meristem Genes in the AZs

A long list of shoot apical meristem (SAM) genes were similarly expressed in both the LAZ and the FAZ (Group C) and in the differentially regulated groups (Group A and B) (Table S3). The key *Arabidopsis* SAM activity genes and their orthologs were preferentially expressed in tomato FAZ (Nakano et al., 2012; Wang et al., 2013). Our data show that the well-documented key meristem genes, *KNOTTED2* protein/*Tkn3/KNAT6* (Solyc05g005090), *BEL1-like homeodomain protein4* (*TBL4/BELL-like homeodomain protein3*) (*BLH*) (Solyc08g065420), along with the *LATERAL ORGAN BOUNDARIES DOMAIN PROTEIN1* (*LBD1*) (Solyc11g072470), and the tomato auxiliary meristem gene, *Bl* (Solyc11g069030), which encodes a *MYB* TF, were highly expressed in the tomato LAZ (Group A) compared to the FAZ (Table 3). On the other hand, the WUSCHEL-related homeobox-containing protein4 (*WUS*) gene (Solyc02g083950), *OVATE FAMILY PROTEIN (OFP)* gene (Solyc02g085500), another *MYB-Cpm10/MYB78* gene (Solyc05g053330), *Goblet* (Solyc07g062840), a NAC domain *TF* gene, and the *Ls* (Solyc07g066250), a GRAS family *TF* gene,

were highly expressed in the tomato FAZ compared to the LAZ (Group B) (Table 3).

Cell Wall Related Genes in the FAZ and the LAZ

Our RNA-Seq analyses show that several genes encoding cell wall and middle lamella degradation and remodeling factors, which are the main targets at the late stages of the abscission process, were expressed in both the tomato FAZ and LAZ. These include *PG*, *Cel*, *XTH*, and *EXP* genes (Table 4). These genes were previously demonstrated to be highly expressed in the AZs of a large number of abscising organs (Lashbrook et al., 1994; del Campillo and Bennett, 1996; Cho and Cosgrove, 2000; Taylor and Whitelaw, 2001; Roberts et al., 2002; Ogawa et al., 2009; Meir et al., 2010). The previously characterized tomato AZ-specific *PG* genes (*TAPGs*), *TAPG1,2,3,4,5*, were expressed in both the FAZ and the LAZ, with *TAPG4* expression being the highest and *TAPG3* expression being the lowest in both AZs (Table 4). These results are in agreement with previous works showing that *TAPG1,2,4* were specifically expressed in tomato FAZ (Lashbrook et al., 1994; Kalaitzis et al., 1997; Wang et al., 2013), and their expression was inhibited by 1-methylcyclopropene (1-MCP) in a positive correlation with its inhibitory effect on pedicel abscission (Meir et al., 2010).

The expression levels of genes encoding cellulase enzymes, such as *Cel1,2,3,4,5,6,7,8*, are detailed in Table 4. *Cel2,5,6,7,8* were highly expressed in the LAZ (Group A), with *Cel5* showing the highest expression among all the *Cel* family genes in both AZs (Table 4). Only the expression of *Cel1* was higher in the FAZ (Group B) than in the LAZ, while *Cel3* and *Cel4* had similar expression levels in both AZs (Table 4). The expression levels

of several *XTH* genes, *SIXTH1,2,6,9*, was higher in the LAZ than in the FAZ samples, while the other genes of this family were similarly expressed in both AZs (**Table 4**). It was previously reported that *XTH8,9* were highly and specifically expressed in tomato FAZ following ethylene treatment (Wang et al., 2013), and *XTH6* was specifically up-regulated in the tomato FAZ following flower removal, and this increased expression was

inhibited by 1-MCP pretreatment (Meir et al., 2010). *XTH1,2* genes were also over-expressed in the LAZ during ethylene-induced citrus leaf abscission (Agustí et al., 2008). These results further confirm the role of ethylene in inducing abscission via increased expression of *XTH* genes in both the LAZ and the FAZ.

In general, all the members of the *EXP* gene family, which were expressed in both tomato AZs, were highly upregulated in the LAZ compared to the FAZ (**Table 4**). The expression level of several *EXP* genes, *LeEXP1,5,9,11,18*, was particularly high in the LAZ.

Differential Regulation of Genes Associated with Hormonal Signal Transduction

The RNA-Seq data for both the FAZ and the LAZ samples were analyzed for the KEGG pathway database to examine the potential involvement of consensus sequences in hormonal signal transduction pathways. The pathway-based analysis helped us to understand the biological functions and their interactions (Figures S3, S4). The KEGG categories and list of transcripts in each sample with their expression values in the LAZ and the FAZ are listed in **Table 5**. The genes related to the different plant hormones, auxin, ethylene, jasmonic acid (JA), abscisic acid (ABA), brassinosteroids, cytokinins, and gibberellins (GA), and other signaling-related factors (protein kinases), were present in both AZs (**Table 5**). Most of the genes were equally expressed in both AZs (Group C), but some genes of each hormone category were differentially expressed in the LAZ or the FAZ, belonging to Groups A or B, respectively (**Figure 2**). The data suggest specific roles for this family members in each AZ.

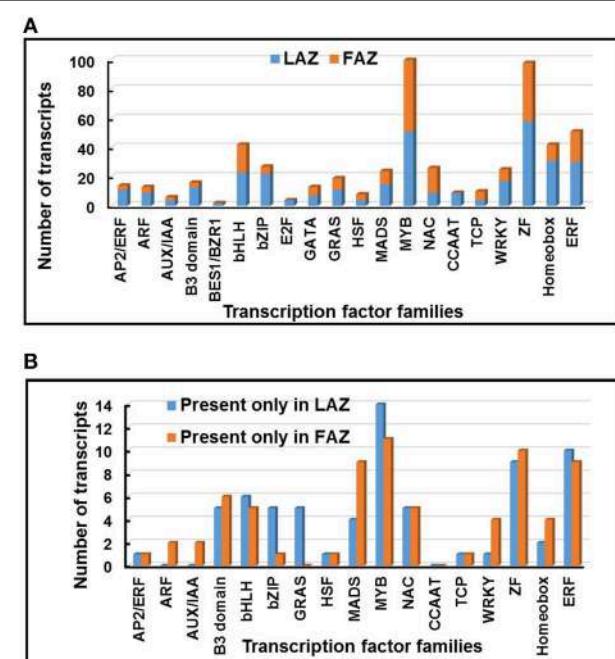


FIGURE 7 | Distribution of abscission-regulated transcription factor (TF) families over-expressed in the LAZ or in the FAZ (A), and exclusively expressed only in the LAZ or FAZ (B) during abscission. The changes in the abundance of 551 TF transcripts belonging to 20 families were determined in graph (A) for Group A (LAZ) and Group B (FAZ). The changes in the abundance of 141 TF transcripts belonging to 20 families were determined in graph (B) for Group A1 (LAZ) and Group B1 (FAZ). The Groups were classified according to the categories presented in **Figure 2**.

TABLE 3 | Differential expression patterns of shoot meristem genes in the tomato LAZ and FAZ.

Gene ID	Solyc gene description ^a	Expression level		Log ₂ (Ratio) ^b
		LAZ	FAZ	
Solyc08g065420*	<i>BEL1-like homeodomain protein 3 (BLH)/TBL4</i>	12,966	2564	2.34
Solyc07g066250*	<i>GRAS family TF (LATERAL SUPPRESSOR-Ls)</i>	45	783	-4.11
Solyc05g005090*	<i>Knotted1-like homeobox protein H1 (Knotted2 – KNAT6)/TKn3</i>	7975	2577	1.63
Solyc02g083950*	<i>WUSCHEL-related homeobox-containing protein4 (WUS)</i>	36	178	-2.32
Solyc11g069030*	<i>MYB TF (B)</i>	9703	1971	2.30
Solyc11g072470*	<i>LOB domain protein1(LBD1)</i>	27,817	6660	2.06
Solyc02g085500*	<i>Ovate protein</i>	1343	4640	-1.79
Solyc05g053330*	<i>MYB TF</i>	9779	22,747	-1.22
Solyc07g062840	<i>NAC domain protein (GOBLET)</i>	647	2715	-2.07

*Genes which were shown to be preferentially expressed in the tomato FAZ (Meir et al., 2010; Nakano et al., 2013; Wang et al., 2013).

^aAccording to the Tomato Sol Genomic Network database (<http://solgenomics.net>).

^bLog₂ of the gene expression ratio between LAZ and FAZ. Ratio = LAZ/FAZ.

TABLE 4 | Differential expression patterns of cell wall-related genes in the tomato LAZ and FAZ.

Gene ID	Gene name	Nucleotide Accession number	NCBI description	Expression level		Log ₂ (Ratio)
				LAZ	FAZ	
POLYGALACTURONASES						
Solyc02g067630	TAPG1	AF001000	Polygalacturonase 1	93,779	65,322	0.5
Solyc02g067640	TAPG2	AF001001	Polygalacturonase 2	19,182	58,301	-1.6
Solyc02g067650	TAPG3	AF000999	Polygalacturonase 3	661	399	0.7
Solyc12g096750	TAPG4	AF001002	Polygalacturonase 4	93,458	298,246	-1.7
Solyc12g096740	TAPG5	AF001003	Polygalacturonase 5	40,338	169,016	-2.1
Solyc12g019180		AF072732	Polygalacturonase 7	819	37	4.5
Solyc12g096730	TPG6	AF029230	Polygalacturonase	258	123	1.1
Solyc03g116500	XOPG1	AF138858	Polygalacturonase	8036	2546	1.7
Solyc08g060970	PGcat	AF118567	Polygalacturonase	15,150	10,785	0.5
Solyc04g015530	PS-2	EU111748	Dehiscence polygalacturonase	0	2774	0.0
Solyc10g080210	PG-2a	X04583	Polygalacturonase-2a	0	11	0.0
Solyc07g065090		L26529	Polygalacturonase inhibitor protein	49,824	52,806	-0.1
CELLULASES						
Solyc08g081620	Cel1	U13054	Endo-1,4-beta-glucanase	7882	27,048	-1.8
Solyc09g010210	Cel2	U13055	Endo-1,4-beta-glucanase	33,292	4618	2.8
Solyc01g102580	Cel3	U78526	Endo-1,4-beta-glucanase	31,216	37,492	-0.3
Solyc09g075360	Cel4	U20590	Endo-1,4-beta-glucanase	3210	3800	-0.2
Solyc08g083210	Cel5	AF077339	Endo-1,4-beta-glucanase	256,927	10,075	4.7
Solyc05g005080	Cel6	NA	Endo-1,4-beta-glucanase	87,695	30,178	1.5
Solyc11g040340	Cel7	Y11268	Endo-1,4-beta-glucanase	14,458	1561	3.2
Solyc08g082250	Cel8	AF098292	Endo-1,4-beta-glucanase	47,547	7409	2.7
XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASES						
Solyc01g099630	SIXTH1	D16456	Endo-xyloglucan transferase	299,193	15,747	4.2
Solyc07g009380	SIXTH2	AF176776	Xyloglucan endotransglycosylase lexet2	19,890	3315	2.6
Solyc03g093080	SIXTH3	AY497476	Xyloglucan endotransglucosylase-hydrolase XTH3	11,463	7902	0.5
Solyc03g093110	SIXTH3	AY497476	Xyloglucan endotransglucosylase-hydrolase XTH3	10,544	6556	0.7
Solyc03g093120	SIXTH3	AY497476	Xyloglucan endotransglucosylase-hydrolase XTH3	11,633	6461	0.8
Solyc03g093130	SIXTH3	AY497476	Xyloglucan endotransglucosylase-hydrolase XTH3	12,099	16,273	-0.4
Solyc11g065600	SIXTH4	AF186777	Xyloglucan endotransglycosylase	83	795	-3.3
Solyc01g081060	SIXTH5	AY497475	Xyloglucan endotransglucosylase-hydrolase XTH5	32,612	54,930	-0.8
Solyc11g066270	SIXTH6	AY497477	Xyloglucan endotransglucosylase-hydrolase XTH6	30,396	4588	2.7
Solyc02g091920	SIXTH7	AY497478	Xyloglucan endotransglucosylase-hydrolase XTH7	11,361	16,524	-0.5
Solyc04g008210	SIXTH8	AB036338	endoxyloglucan transferase	14,512	18,467	-0.3
Solyc12g011030	SIXTH9	AY497479	xyloglucan endotransglucosylase-hydrolase XTH9	25,489	12,237	1.1
Solyc07g056000	SIXTH10	X82684	Xyloglycan endo-transglycosylase	21,410	28,794	-0.4
Solyc12g017240	SIXTH11	X82685	Xyloglucan endo-transglycosylase	6763	3776	0.8
Solyc07g052980	SIXTH16	DQ098654	Xyloglucan endotransglucosylase-hydrolase XTH16	43,636	186,375	-2.1
EXPANSINS						
Solyc01g090810	LeEXPB1	DQ234354	Beta-expansin precursor	2151	0	
Solyc03g093390	LeEXPB2	DQ205653	Beta expansin precursor (EXPB2)	102	17	2.6
Solyc05g007830		AC154033	Alpha-expansin 1 precursor	172,124	9441	4.2
Solyc01g112000	LeEXLA1	DQ178133	Expansin-like protein precursor (EXLA1)	46,270	23,553	1.0
Solyc06g051800	LeEXP1	U82123	Fruit ripening regulated expansin	74,430	3752	4.3
Solyc06g049050	LeEXP2	AF096776	Expansin LeEXP2	35,951	10,479	1.8
Solyc03g031840	EXP A3	AF059487	Expansin precursor (EXP A3)	18,068	12,366	0.5
Solyc09g010860	EXP A4	AF059488	Expansin precursor (EXP A4)	5088	3355	0.6
Solyc02g088100	EXP A5	AF059489	Expansin precursor (EXP A5)	67,484	21,494	1.7
Solyc10g086520	EXP A6	AF059490	Expansin (EXP A6)	6244	253	4.6
Solyc03g115300	EXP A7	AF059491	Expansin	112	75	0.6
Solyc12g089380	EXP A8	AF184232	Expansin EXP A8	396	75	2.4
Solyc06g005560	EXP 9	AJ243340	Expansin 9	60,173	13,676	2.1
Solyc03g115890	EXP A10	AF184233	Expansin EXP A10	1183	349	1.8
Solyc04g081870	Exp 11	AF218775	Expansin precursor (Exp 11)	81,922	3546	4.5
Solyc06g076220	Exp 18	AJ004997	Expansin 18	99,291	2685	5.2

TABLE 5 | The KEGG categories and their expression values for plant hormone signaling-related genes in the tomato LAZ and FAZ.

Gene ID	KEGG ID	KEGG description	Expression level		Log ₂ (Ratio)
			LAZ	FAZ	
AUXIN					
Solyc10g076790.1.1	K13946	Auxin influx carrier (AUX1 LAX family) <i>LAX4</i>	601	3295	-2.5
Solyc11g013310.1.1	K13946	Auxin influx carrier (AUX1 LAX family) <i>LAX3</i>	19,674	29,200	-0.6
Solyc10g055260.1.1	K13946	Auxin influx carrier (AUX1 LAX family) <i>LAX5</i>	89	50	0.8
Solyc03g120390.1.1	K14484	Auxin-responsive protein IAA (<i>IAA8</i>)	5982	4732	0.3
Solyc03g120500.1.1	K14484	Auxin-responsive protein IAA (<i>IAA6</i>)	19,397	60,354	-1.6
Solyc04g076850.1.1	K14484	Auxin-responsive protein IAA (<i>IAA9</i>)	50,252	53,992	-0.1
Solyc06g008590.1.1	K14484	Auxin-responsive protein IAA (<i>IAA10</i>)	1653	745	1.1
Solyc06g066020.1.1	K14484	Auxin-responsive protein IAA	941	1001	-0.1
Solyc09g083280.1.1	K14484	Auxin-responsive protein IAA (<i>IAA23</i>)	9659	2545	1.9
Solyc09g083290.1.1	K14484	Auxin-responsive protein IAA (<i>IAA24</i>)	74,088	3881	4.3
Solyc09g090910.1.1	K14484	Auxin-responsive protein IAA (<i>IAA 25</i>)	1874	3577	-0.9
Solyc12g007230.1.1	K14484	Auxin-responsive protein IAA (<i>IAA26</i>)	19,121	21,111	-0.1
Solyc03g121060.1.1	K14484	Auxin-responsive protein IAA (<i>IAA14</i>)	35,233	115,921	-1.7
Solyc08g021820.1.1	K14484	Auxin-responsive protein IAA (<i>IAA21</i>)	7129	790	3.2
Solyc09g065850.1.1	K14484	Auxin-responsive protein IAA (<i>IAA3</i>)	17,164	13,737	0.3
Solyc01g096070.1.1	K14486	Auxin response factor (<i>ARF18</i>)	20,948	4373	2.3
Solyc01g103050.1.1	K14486	Auxin response factor (<i>ARF1</i>)	25,076	18,211	0.5
Solyc02g077560.1.1	K14486	Auxin response factor (<i>ARF3</i>)	12,141	8367	0.5
Solyc04g081240.1.1	K14486	Auxin response factor (<i>ARF5</i>)	2305	2776	-0.3
Solyc07g042260.1.1	K14486	Auxin response factor (<i>ARF19</i>)	2634	3400	-0.4
Solyc08g008380.1.1	K14486	Auxin response factor (<i>ARF9B</i>)	3429	2597	0.4
Solyc12g005310.1.1	K14487	Auxin responsive GH3 gene family (<i>GH3-15</i>)	2800	798	1.8
Solyc01g107390.1.1	K14487	Auxin responsive GH3 gene family (<i>GH3-2</i>)	212	2279	-3.4
Solyc10g008520.1.1	K14487	Auxin responsive GH3 gene family (<i>GH3-10</i>)	3559	10,863	-1.6
Solyc02g084010.1.1	K14488	SAUR family protein (<i>SAUR33</i>)	1843	2835	-0.6
Solyc07g014620.1.1	K14488	SAUR family protein (<i>SAUR63</i>)	2031	1128	0.8
Solyc01g091030.1.1	K14488	SAUR family protein (<i>SAUR1</i>)	13,748	7391	0.9
Solyc01g110580.1.1	K14488	SAUR family protein (<i>SAUR5</i>)	552	151	1.9
Solyc01g110770.1.1	K14488	SAUR family protein (<i>SAUR10</i>)	283	63	2.2
Solyc03g082520.1.1	K14488	SAUR family protein (<i>SAUR36</i>)	1320	13,445	-3.3
Solyc03g082530.1.1	K14488	SAUR family protein (<i>SAUR37</i>)	3442	23,779	-2.8
Solyc02g079190.1.1	K14485	Transport inhibitor response 1	28,232	34,580	-0.3
Solyc09g074520.1.1	K14485	Transport inhibitor response 1	17,058	18,054	-0.1
Solyc03g118740		Auxin efflux carrier (<i>SIPIN1</i>)	1559	4048	-1.4
Solyc04g007690		Auxin efflux carrier (<i>SIPIN3</i>)	3123	2283	0.5
Solyc05g008060		Auxin efflux carrier (<i>SIPIN4</i>)	21,059	23,775	-0.2
Solyc01g068410		Auxin efflux carrier (<i>SIPIN5</i>)	1681	163	3.4
Solyc06g059730		Auxin efflux carrier (<i>SIPIN6</i>)	290	47	2.6
Solyc10g080880		Auxin efflux carrier (<i>SIPIN7</i>)	514	182	1.5
Solyc02g087660		Auxin efflux carrier (<i>SIPIN8</i>)	190	218	-0.2
Solyc10g078370		Auxin efflux carrier (<i>SIPIN9</i>)	1284	2617	-1.0
ETHYLENE					
Solyc01g009170.1.1	K14514	Ethylene-insensitive protein 3 (<i>LeEIL2</i>)	53,217	61,035	-0.2
Solyc01g014480.1.1	K14514	Ethylene-insensitive protein 3	5896	5983	0.0
Solyc01g096810.1.1	K14514	Ethylene-insensitive protein 3 (<i>LeEIL3</i>)	73,340	97,915	-0.4
Solyc06g073720.1.1	K14514	Ethylene-insensitive protein 3 (<i>LeEIL1</i>)	46,978	73,358	-0.6
Solyc06g073730.1.1	K14514	Ethylene-insensitive protein 3 (<i>LeEIL4</i>)	47,534	50,923	-0.1
Solyc09g007870.1.1	K14513	Ethylene-insensitive protein 2 (<i>EIN2</i>)	22,094	24,356	-0.1

(Continued)

TABLE 5 | Continued

Gene ID	KEGG ID	KEGG description	Expression level		Log ₂ (Ratio)
			LAZ	FAZ	
Solyc04g014530.1.1	K14516	Ethylene-responsive transcription factor1 (<i>ERF.C2</i>)	2600	2038	0.4
Solyc05g051200.1.1	K14516	Ethylene-responsive transcription factor1 (<i>ERF.C1</i>)	25,783	70,599	-1.5
Solyc05g051180.1.1	K14516	Ethylene-responsive transcription factor1	1084	0	0.0
Solyc09g089930.1.1	K14516	Ethylene-responsive transcription factor1 (<i>ERF.E2</i>)	5735	9067	-0.7
Solyc11g011740.1.1	K14516	Ethylene-responsive transcription factor1 (<i>ERF</i>)	3712	165	4.5
Solyc11g011750.1.1	K14516	Ethylene-responsive transcription factor1	1982	0	0.0
Solyc07g008250.1.1	K14515	EIN3-binding F-box protein	180,332	61,554	1.6
Solyc12g009560.1.1	K14515	EIN3-binding F-box protein	70,361	47,713	0.6
Solyc08g060810.1.1	K14515	EIN3-binding F-box protein	43,384	91,970	-1.1
Solyc06g053710.1.1	K14509	Ethylene receptor [EC:2.7.13.-] (<i>ETR4</i>)	39,361	33,454	0.2
Solyc11g006180.1.1	K14509	Ethylene receptor [EC:2.7.13.-] (<i>ETR5</i>)	5931	4233	0.5
Solyc12g011330.1.1	K14509	Ethylene receptor [EC:2.7.13.-] (<i>ETR1</i>)	7997	9721	-0.3
Solyc09g075440.1.1	K14509	Ethylene receptor [EC:2.7.13.-] (<i>ETR3</i>)	41,038	25,627	0.7
JASMONIC ACID (JA)					
Solyc10g011660.1.1	K14506	Jasmonic acid-amino synthetase	25,179	79,073	-1.7
Solyc03g118540.1.1	K13464	Jasmonate ZIM domain-containing protein	1637	4703	-1.5
Solyc03g122190.1.1	K13464	Jasmonate ZIM domain-containing protein	6892	147,758	-4.4
Solyc12g009220.1.1	K13464	Jasmonate ZIM domain-containing protein	8060	98,488	-3.6
Solyc01g005440.1.1	K13464	Jasmonate ZIM domain-containing protein	18,902	218,372	-3.5
Solyc11g011030.1.1	K13464	Jasmonate ZIM domain-containing protein	1381	81,182	-5.9
Solyc12g049400.1.1	K13464	Jasmonate ZIM domain-containing protein	5763	189,025	-5.0
Solyc05g052620.1.1	K13463	Coronatine-insensitive protein 1 (<i>COI1</i>)	7622	8202	-0.1
ABSCISIC ACID (ABA)					
Solyc06g050500.1.1	K14496	Abscisic acid receptor PYR/PYL family	7053	9257	-0.4
Solyc08g076960.1.1	K14496	Abscisic acid receptor PYR/PYL family	6901	4195	0.7
Solyc03g095780.1.1	K14496	Abscisic acid receptor PYR/PYL family	3927	940	2.1
Solyc10g076410.1.1	K14496	Abscisic acid receptor PYR/PYL family	13,049	3674	1.8
Solyc12g095970.1.1	K14496	Abscisic acid receptor PYR/PYL family	26	306	-3.6
Solyc04g078840.1.1	K14432	ABA responsive element binding factor	19,141	13,753	0.5
Solyc10g081350.1.1	K14432	ABA responsive element binding factor	7695	3965	1.0
Solyc01g108080.1.1	K14432	ABA responsive element binding factor	11,528	18,411	-0.7
Solyc09g009490.1.1	K14432	ABA responsive element binding factor	1641	23	6.2
BRASSINOSTEROID (BA)					
Solyc01g104970.1.1	K13416	Brassinosteroid insensitive 1-associated receptor kinase 1 [EC:2.7.11.1 2.7.10.1]	14,379	17,613	-0.3
Solyc10g047140.1.1	K13416	Brassinosteroid insensitive 1-associated receptor kinase 1 [EC:2.7.11.1 2.7.10.1]	11,969	12,680	-0.1
Solyc04g039730.1.1	K13416	Brassinosteroid insensitive 1-associated receptor kinase 1 [EC:2.7.11.1 2.7.10.1]	4539	6692	-0.6
Solyc04g051510.1.1	K13415	Protein brassinosteroid insensitive 1 [EC:2.7.11.1 2.7.10.1] (<i>tBRI1/SR160</i>)	25,691	15,587	0.7
Solyc01g080880.1.1	K14500	BR-signaling kinase [EC:2.7.11.1]	43,070	28,006	0.6
Solyc10g085000.1.1	K14500	BR-signaling kinase [EC:2.7.11.1]	8438	2869	1.6
Solyc02g072300.1.1	K14502	Protein brassinosteroid insensitive 2 [EC:2.7.11.1]	67,130	52,758	0.3
Solyc07g055200.1.1	K14502	Protein brassinosteroid insensitive 2 [EC:2.7.11.1]	49,911	44,967	0.2
Solyc04g079980.1.1	K14503	Brassinosteroid resistant 1/2	33,117	15,846	1.1
Solyc02g063010.1.1	K14503	Brassinosteroid resistant 1/2	22,858	26,736	-0.2
PROTEIN KINASES					
Solyc04g012160.1.1	K14498	Serine/threonine-protein kinase SRK2 [EC:2.7.11.1]	10,971	3460	1.7
Solyc04g074500.1.1	K14498	Serine/threonine-protein kinase SRK2 [EC:2.7.11.1]	11,080	7213	0.6
Solyc09g009090.1.1	K14510	Serine/threonine-protein kinase CTR1 [EC:2.7.11.1] (<i>CTR3-S/MAPKKK68</i>)	6144	6104	0.0

(Continued)

TABLE 5 | Continued

Gene ID	KEGG ID	KEGG description	Expression level		Log ₂ (Ratio)
			LAZ	FAZ	
Solyc10g083610.1.1	K14510	Serine/threonine-protein kinase CTR1 [EC:2.7.11.1] (CTR1-S/MAPKKK77)	10,408	10,150	0.0
Solyc10g085570.1.1	K14510	Serine/threonine-protein kinase CTR1 [EC:2.7.11.1] (CTR4-S/MAPKKK78)	3629	5872	-0.7
Solyc12g019460.1.1	K14512	Mitogen-activated protein kinase 6 [EC:2.7.11.24]	23,304	21,882	0.1
Solyc08g014420.1.1	K14512	Mitogen-activated protein kinase 6 [EC:2.7.11.24]	15,807	13,380	0.2
Solyc04g008110.1.1	K14489	Arabidopsis histidine kinase 2/3/4 (cytokinin receptor) [EC:2.7.13.3]	4945	2291	1.1
Solyc05g015610.1.1	K14489	Arabidopsis histidine kinase 2/3/4 (cytokinin receptor) [EC:2.7.13.3]	11,349	20,362	-0.8
Solyc07g047770.1.1	K14489	Arabidopsis histidine kinase 2/3/4 (cytokinin receptor) [EC:2.7.13.3]	4949	4851	0.0
CYTOKININS					
Solyc06g048930.1.1	K14492	Two-component response regulator ARR-A family	2613	35,896	-3.8
Solyc01g065540.1.1	K14491	Two-component response regulator ARR-B family	2713	5276	-1.0
Solyc04g008050.1.1	K14491	Two-component response regulator ARR-B family	4064	5417	-0.4
Solyc05g014260.1.1	K14491	Two-component response regulator ARR-B family	11,991	12,016	0.0
Solyc05g054390.1.1	K14491	Two-component response regulator ARR-B family	28,980	22,296	0.4
Solyc07g005140.1.1	K14491	Two-component response regulator ARR-B family	6591	4443	0.6
Solyc12g010330.1.1	K14491	Two-component response regulator ARR-B family	6610	6061	0.1
GIBBERELLIN (GA)					
Solyc04g078390.1.1	K14495	F-box protein GID2	44,092	37,565	0.2
Solyc11g011260.1.1	K14494	DELLA protein (LeGA)	27,680	14,393	0.9
OTHERS					
Solyc01g102300.1.1	K12126	Phytochrome-interacting factor 3	9224	5635	0.7
Solyc07g043580.1.1	K16189	Phytochrome-interacting factor 4	10,518	6044	0.8
Solyc03g006960.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	1381	1769	-0.4
Solyc03g007230.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	25,140	59,268	-1.2
Solyc03g096670.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	47,447	64,439	-0.4
Solyc03g121880.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	61,668	38,683	0.7
Solyc05g052980.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	44,904	49,082	-0.1
Solyc06g051940.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	4900	1780	1.5
Solyc06g076400.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	6832	9804	-0.5
Solyc07g040990.1.1	K14497	Protein phosphatase 2C [EC:3.1.3.16]	20,065	20,418	0.0
Solyc02g092980.1.1	K14505	Cyclin D3, plant	18,872	2447	2.9
Solyc12g088650.1.1	K14505	Cyclin D3, plant	5434	1717	1.7
Solyc08g076930.1.1	K13422	Transcription factor MYC2	14,494	28,347	-1.0
Solyc07g040690.1.1	K14508	Regulatory protein NPR1	10,172	9185	0.1
Solyc10g079460.1.1	K14508	Regulatory protein NPR1	6214	7924	-0.4
Solyc02g069310.1.1	K14508	Regulatory protein NPR1	9608	10,302	-0.1
Solyc07g044980.1.1	K14508	Regulatory protein NPR1	34,016	38,526	-0.2
Solyc10g079750.1.1	K14508	Regulatory protein NPR1	4588	587	3.0
Solyc04g054320.1.1	K14431	Transcription factor TGA	9563	5811	0.7
Solyc05g009660.1.1	K14431	Transcription factor TGA	1746	1997	-0.2
Solyc10g080410.1.1	K14431	Transcription factor TGA	85	1312	-4.0
Solyc10g080780.1.1	K14431	Transcription factor TGA	4146	6741	-0.7
Solyc04g072460.1.1	K14431	Transcription factor TGA	7045	14,224	-1.0

such as *Like Auxin (LAX)* influx carriers and *Pin-formed (PIN)* efflux carrier genes. Aux/IAA proteins are negative repressors which bind to the Auxin-Responsive Elements (AREs) of the target gene promoters, leading to activation or repression of the target genes, and their degradation is promoted by auxin (Worley et al., 2000; Overvoorde et al., 2005). The members of

the tomato *Aux/IAA* gene family were differentially regulated in the tomato FAZ and LAZ (Table 5). The *SlIAA6,9,25,26,14* genes were overexpressed in the FAZ, with *SlIAA14* showing the highest expression among this family members. On the other hand, *SlIAA8,10,23,24,21,3* were overexpressed in the LAZ, with *SlIAA24* showing the highest expression level among

this family members. The *Aux/IAA1,3,4,7,8,9,10* genes were downregulated in tomato FAZ and served as good markers for auxin depletion after flower removal (Meir et al., 2010). Similarly, *CitAux/IAA3,4,18,19* genes were downregulated in citrus fruit AZ during fruitlet abscission (Xie et al., 2015). Genetic mutation and expression analysis demonstrated that *ARF* genes could regulate plant organ abscission (Ellis et al., 2005; Guan et al., 2014). The results presented in **Table 5** show that *SlARF18,1,3,9B* genes were over-expressed in the LAZ, whereas *SlARF5,19* genes were over-expressed in the FAZ. The expression of *SlARF1* was the highest in both AZs compared to the other family members of this gene.

The tomato homolog of *METHYLESTERASE1* (*MES1*) (AK328818, Solyc03g070380) was highly expressed in the LAZ (Group B) compared to the FAZ (Group A), whereas the *DWARF IN LIGHT1 (DFL1)/auxin-inducible GH3.9* gene (AK319847, Solyc07g063850) was expressed equally at very high levels in both AZs (Group C) (Table S3). *MES1* converts the storage form of IAA to the active free form, whereas *DWARF IN LIGHT1* does the opposite, i.e., it converts the active free IAA to the inactive conjugated form (Staswick et al., 2005; Woodward and Bartel, 2005; Yang et al., 2008; Ludwig-Müller, 2011). In addition, conjugation of IAA to amino acids provides a negative feedback loop for controlling auxin homeostasis, and the *SlGH3-2,10,15* genes, which control IAA conjugation, were found to respond quickly to exogenous auxin application (Kumar et al., 2012; Meir et al., 2015). Our KEGG analysis revealed that *SlGH3-2,10* genes were overexpressed in the FAZ, whereas *SlGH3-15* was over-expressed in the LAZ (**Table 5**).

The auxin influx and efflux transporters PIN and AUX/LAX proteins mediate the auxin polar transport (Vanneste and Friml, 2009), resulting in directional auxin flow and creation of auxin gradients (Bainbridge et al., 2008; Petrásek and Friml, 2009). The auxin influx carrier genes, *SlLAX3,4*, were overexpressed in the tomato FAZ compared to the LAZ, whereas *SlLAX5* was expressed at low levels in both AZs, but was comparatively expressed higher in the LAZ (**Table 5**). The auxin efflux carrier genes, *SlPIN1,4,9* were over-expressed in the FAZ compared to the LAZ, whereas *SlPIN3,5,6,7* were over-expressed (by ~2- to 10-fold) in the LAZ compared to the FAZ (**Table 5**). Reduced auxin levels were attributed to increased activity of auxin efflux transporters in Arabidopsis and other systems (Sorefan et al., 2009; Meir et al., 2015).

Ethylene

Many genes related to different steps of the ethylene signaling transduction pathway were expressed in both the tomato AZs following flower removal and leaf deblading (**Table 5**). Out of six genes encoding for ethylene receptors in tomato (Klee, 2002, 2004), four genes were presented in both AZs. Among them, *ETR3,4,5* were expressed at very high levels in the LAZ, whereas *ETR1* was expressed higher in the FAZ (**Table 5**). These data confirm previous results demonstrating the expression of *ETR1,4* genes in tomato FAZ during pedicel abscission (Payton et al., 1996; Meir et al., 2010). The *Constitutive Triple Response1 (CTR1)* gene product acts downstream of the ethylene receptors, and belongs to the Arabidopsis *RAF Mitogen-Activated Protein Kinase Kinase (MAPKKK)* subfamily, which negatively regulates ethylene signal transduction (Kieber et al., 1993). Recent studies of genome-wide analysis of the *Mitogen-Activated Protein Kinase (MAPKK)* and *MAPKKK* gene families in tomato revealed five and 89 genes, respectively (Wu et al., 2014). The predicted transmitter domain of *ETR1* and the regulatory domain of *CTR1* were found to interact directly (Clark et al., 1998; Huang et al., 2003; Binder, 2008). Our analysis data show that three *CTR* genes, *CTR1-SlMAPKK77*, *CTR3-SlMAPKK68*, and *CTR4-SlMAPKK78*, were expressed in both AZs, with *CTR1* showing the highest expression (**Table 5**). All the *CTR* genes identified in this study belong to the *RAF MAPKKK* sub-family according to the classification detailed previously (Wu et al., 2014).

Downstream ethylene signaling events are mediated by ERFs, which are plant-specific TFs which belong to the large AP2/ERF super-family (Riechmann et al., 2000), containing a *cis*-acting ethylene-responsive element named the GCC-box in their promoter regions (Fujimoto et al., 2000). The GCC-box interacts with *trans*-acting factors termed ethylene-responsive element-binding proteins, which are required for ethylene regulation in many plant species (Ohme-Takagi and Shinshi, 1995). Our data show that *SlERF.C1* and *SlERF.E2* genes were highly expressed in the FAZ, while *SlERF.C2* and *SlERF* (Solyc11g011740) genes were highly expressed in the LAZ (**Table 5**). Interestingly, two other *ERF* genes (Solyc05g051180, Solyc11g011750) were present only in the LAZ (Group A1) (**Table 5**). The *ERF1c* gene (AY044236, *SlERF.C1*) was proposed to be involved in the late stages of flower pedicel abscission (Meir et al., 2010). Several other tomato *ERF* genes, *SlERF52* (Solyc03g117130), *SlERF56* (Solyc09g066360), *SlERF68* (Solyc08g078180), *ERF1* (AF502085.1, *SlERF.E2*), and *ERF2* (AI776626, Solyc09g089910), were overexpressed in both the FAZ and the LAZ (Table S3).

Ethylene Insensitive2 (EIN2) was reported to act downstream of *CTR1* as a positive regulator of the ethylene signaling pathway (Alonso et al., 1999). *LeEIN2* was expressed in both the FAZ and the LAZ at similar levels (**Table 5**). *EIN3*, which acts downstream of *EIN2*, belongs to a multi-gene family designated as *Ethylene Insensitive-Like (EIL)* in tomato. *EIN3* encodes a downstream component of the ethylene signal transduction pathway, which ultimately activates ethylene-responsive genes (Ecker, 1995; Roman et al., 1995). *LeEILs* are functionally redundant and positive regulators of multiple ethylene responses throughout plant development (Tieman et al., 2001). Our data show that all the *EIL* genes, *LeEIL1,2,3,4*, were expressed at high levels in both AZs, with the highest levels in the FAZ (**Table 5**).

Other Hormones

Several genes involved in pathways related to other phytohormones were expressed in both the FAZ and the LAZ (**Table 5**). Thus, a gene involved in JA perception and signaling *COI1* was highly expressed in the FAZ (**Table 5**). This gene encodes an F-box protein, which is required for JA signaling in Arabidopsis (Xie et al., 1998). A gene involved in brassinosteroid biosynthesis and signaling encoding for the *BRI1* protein—*tBRI1/SR160* (Koka et al., 2000; Montoya et al., 2002), showed a high expression in both the FAZ and the LAZ, with the highest level in the LAZ (**Table 5**). A gene involved in GA

signaling, *LeGAI* (Martí et al., 2007; Bassel et al., 2008), also showed a higher expression in the LAZ compared to the FAZ (Table 5).

Designing and Preparation of an AZ-Specific Microarray for Transcriptomic Abscission Research in Tomato

The above comparisons between gene expression in the FAZ and the LAZ were based on samples pooled from individual samples taken at the different time points during pedicel and petiole abscission following abscission induction (Figure 1). The pooled samples limit the identification of specific gene expression profiles at the timing of the spatial events of the abscission process. To perform an analysis that enables to reveal the sequences of the molecular events involved in petiole and pedicel abscission following induction by artificial auxin depletion (IAA source removal), it is necessary to develop a low-cost method than the RNA-Seq, but with comparable robustness, to perform transcriptomic analyses. For this purpose, we developed an AZ-specific microarray based on the RNA-Seq results.

The RNA-Seq analysis of the tomato FAZ and LAZ samples revealed a total number of 40,959 transcripts, including 31,298 transcripts analyzed for sequence similarity to the known database of sequences, 8823 novel tomato transcripts (novel ORF predictions), and 838 transcripts annotated with *A. thaliana* and *N. tabacum* (Table 6). The NGS annotated and the novel transcripts from the tomato FAZ and LAZ, together with sequences of known abscission-related genes originated from various sources detailed in Table 6, were used to design the AZ-specific microarray chip. We included all these available transcripts to enrich this customized microarray chip with previously reported AZ-related genes. As a result, the eArray was finalized with 100,276 probes for the 41,315 transcripts (Table 6).

A 4x180K gene expression array was designed with the probes having 60-mer oligonucleotides from annotated and novel transcriptome sequenced data and gene sequence related to *Nicotiana tabacum* and *A. thaliana*. The 4x180K array comprised of 180,880 features including 176,026 probes and 4854 Agilent controls. All the oligonucleotides were designed and synthesized *in situ* according to the standard algorithms and methodologies used by Agilent Technologies for 60 mer *in situ* oligonucleotide DNA microarray (Table 6). The probes were designed in both sense and antisense orientations and with multiple probes for each transcripts (Table 6, Figure S5). Blast analyses were performed against the complete set of sequence databases to check the specificity of the probes. Finally, 96,152 probes were designed, and 79,874 specific probes were duplicated to fill the remaining spots (Table 6). The detailed list of transcripts, probes, and cross hybrid probe details is presented in Table S11. The designed AZ-specific microarray chip (AMADID: 043310; Genotypic Technology Private Limited, India) is now available from the company for abscission research upon our approval.

Validation of Differentially Regulated Genes in the LAZ and the FAZ

To verify the results of the RNA-Seq analyses, we characterized the expression patterns of nine arbitrarily selected genes, which were differentially regulated in the LAZ and the FAZ, using quantitative reverse transcription-PCR (qRT-PCR). We randomly selected the genes from the four differentially expressed groups, Group A, Group A1, Group B, and Group B1 (Figure 2). In the tomato AZs, the expression levels of the auxin-related genes, *SIIA24*, *SIARF18*, *GH3-15*, and *NPR1-like protein* (*Solyc10g079750*) mRNAs, highly increased in the LAZ (Group A) (Figures 8A–D). In Group A1, the *LOB domain proteins* (*Solyc02g086480*), *Peroxidase1* (*Solyc10g076210*), *1-aminocyclopropane-1-carboxylate oxidase* (*Solyc06g060070*),

TABLE 6 | Probe design summary for the AZ-specific microarray chip.

Transcript category	No. of transcripts	No. of probes per transcript	Total No. of probes
Annotated transcripts (RNA-Seq)	31,298	2 (1 sense and 1 antisense)	62,596
Novel transcripts (RNA-Seq)	8823	4 (2 sense and 2 antisense)	35,292
Annotated with <i>Arabidopsis thaliana</i> & <i>Nicotiana tabacum</i>	838	2 (1 sense and 1 antisense)	1676
Total No of transcripts resulted from RNA-Seq study	40,959		
Previously reported transcripts related to the abscission process	356	2 (1 sense and 1 antisense)	712
Total No. of transcripts for Tomato Array	41,315		100,276
No. of probes collected after redundant/ probe selection criteria			93,674
Additional transcripts for technical quality control	50	4 (2 sense and 2 antisense)	200
No. of Agilent probes added in the Array		2 (1 sense and 1 antisense)	2278
Total No. of probes designed for Tomato Array		(93,674 + 200 + 2278) = 96,152	
Total no of probes replicated to fill the remaining spots#		79,874	
Total no of probes in the final array		176,026	

The transcripts were obtained from various sources: RNA-Seq annotated and novel transcripts of the FAZ and LAZ prepared as detailed in Tables 1, 2; previously reported transcripts related to abscission (Meir et al., 2010; Wu et al., 2011), and additional transcripts obtained from Mark L. Tucker (USDA-ARS, USA). The probe was designed specifically for both sense and antisense orientations for the specific transcripts. The microarrays were designed for the transcripts listed in the table, using the Agilent custom gene expression microarray, 4X180K. The microarray was finalized with 176,026 probes including the technical quality and Agilent control grid probes.

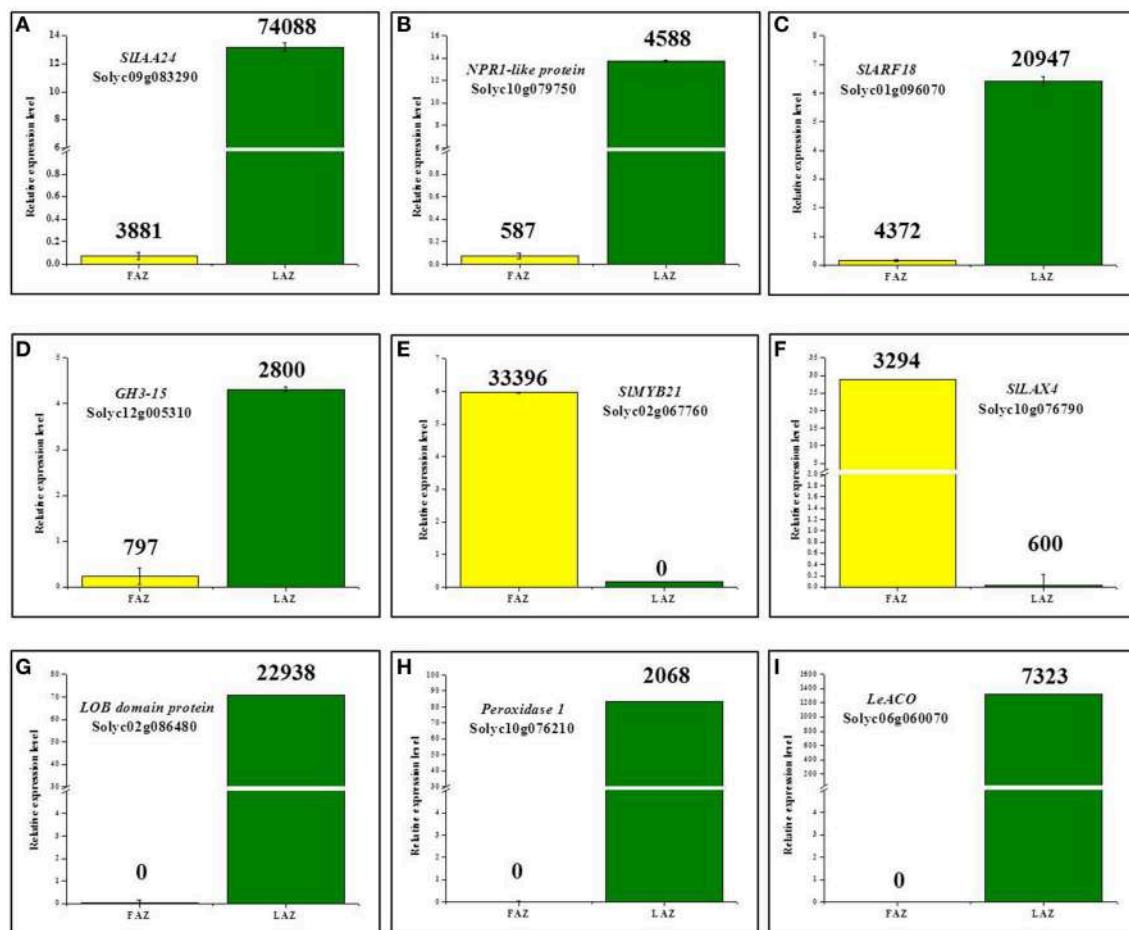


FIGURE 8 | Validation by qPCR of differential expression patterns of selected genes in the FAZ and LAZ pooled samples following abscission induction. Expression levels were measured for tomato Aux/IAA24 (*SIIAA24*) (A), *NPRI*-like protein (B), Auxin response factor18 (*SIARF18*) (C), *Gretchen Hagen-3-15* (*GH3-15*) (D), *SIMYB21* (E), *Like Aux4* (*SILAX4*) (F), *LOB domain protein* (G), *Peroxidase 1* (H), and 1-Aminocyclopropane-1-Carboxylate-Oxidase (*LeACO*) (I). The relative quantification of the gene expression level in the qPCR assay was determined by the comparative C_T method $2^{-\Delta\Delta C_T}$, using *ACTIN* as a reference gene. The results are means of three biological replicates \pm SD. The values presented on top of each bar indicate the expression levels derived from the RNA-Seq data. Transcript identities are indicated in the graphs by their gene ID. The qPCR and RNA-Seq analyses were performed with different samples taken from independent biological replicates of two separate experiments.

were exclusively expressed in the LAZ (Figures 8G–I). The auxin influx carrier gene *SILAX4* (Solyc10g076790) was highly expressed in the FAZ (Group B) (Figure 8F). In the group of genes exclusively expressed in the FAZ (Group B1), a MYB TF, *SIMYB21* (Solyc02g067760) was highly expressed, but it also exhibited a low expression in the LAZ (Figure 8E), which was not spotted by the RNA-Seq analysis. The qPCR performed in this study for the selected genes confirms of the RNA-Seq data. The slight variations obtained between the qRT-PCR and RNA-Seq results are due to the newly pooled samples used for the qRT-PCR, similar to the method used for the RNA-Seq analysis. It seems, therefore, that in general the qRT-PCR technique validated the RNA-Seq data of expression patterns of all the selected transcripts in pooled samples of the LAZ and the FAZ during the abscission process.

DISCUSSION

The present study was based on examination of the effects of auxin depletion, obtained by organ removal, on the molecular changes occurring during the abscission process in tomato FAZ and LAZ (Figure 1). Quantitative measurements of the changes in endogenous auxin content following flower removal were already performed in tomato FAZ, and indeed showed a significant reduction in auxin content with time (Guan et al., 2014). Basically, our study included two parts: (1) Analysis of the RNA-Seq transcriptome with the pooled samples strategy and the preparation of the customized AZ-specific microarray based on it; (2) Use of the transcriptome data for comparative analysis of global gene expression in the tomato FAZ and LAZ. The customized AZ-specific chip was further used for specific analyses at each time point with the appropriate replicates. Part

of the microarray results was recently published elsewhere (Kim et al., 2015; Meir et al., 2015), and part are still being analyzed. Hence, the present study does not include the microarray data, as its main aim was to expose the occurrence of global changes.

Transcriptome Assembly and Design of an AZ-Specific Array

Transcriptome studies were previously performed in the tomato FAZ and flower NAZ using microarrays (Meir et al., 2010, 2011; Nakano et al., 2012, 2013; Wang et al., 2013; Ma et al., 2015). However, a comparative transcriptome profiling of the FAZ and the LAZ was not performed, and no comprehensive cell separation stages in the FAZ (0–14 h after flower removal) and the LAZ (0–96 h after leaf deblading) were addressed by RNA-Seq. The aim of the present research was to study the transcriptome of tomato FAZ and LAZ pooled samples of the various time points, using Illumina PE sequencing and *de novo* assembly to permit assessment of expression across the entire genome. In addition, the data generated were used to create an AZ-specific microarray chip that enables to study the specific abscission stages. We have utilized both the *de novo* as well as the reference-based assemblies of tomato transcriptome to generate a more comprehensive and unbiased representation of the FAZ and the LAZ pooled samples during tomato flower and leaf abscission process. Stringent categories were applied for both the generated *de novo* assembly (BLAST with 50% identity and 70% query coverage) (Tables S5, S6) and the reference-based assembly (BLASTN with *E*-value of e^{-5}). It should be noted, that after the genome alignment with the current tomato reference genome, the analysis revealed only 76 and 90 unannotated transcripts (FASTA file) for the FAZ and the LAZ, respectively. These results suggest that the sequences generated are of tomato genes rather than sequences of non-coding RNAs, UTRs, unannotated introns, or contaminated sequences from other organisms, such as fungi, bacteria, and viruses. The assembly statistics revealed that the merged assembly had better characteristics, such as higher N50 value, average, and longest contig length, total number of contigs, and etc., which usually serve as hallmarks to assess the assembly quality (Table S2). The coverage of the transcriptome was comprehensive enough to discover major genes of several metabolic and regulatory pathways. Particularly, genes associated with plant hormonal signaling components could be mapped to their relevant pathways.

We used the strategy of transcriptome sequencing (RNA-Seq) of pooled samples (RNA pooled equally from all time points), since there are specific transcripts of various transcriptional networks and genes of various functions which are induced at specific time points in the AZ (Meir et al., 2010). The pooling strategy ensures that a given transcript will be present in abundance during the RNA-Seq analysis, rather than analyzing it at a specific time point in which it might not be highly expressed. In the present study, we compared the global transcriptomes generated from the FAZ and the LAZ rather than performing a direct quantitation of the expressed genes in each abscission stage. The differential expression levels described in this study are related to the fold changes of the transcripts in the FAZ and the LAZ pooled samples. However, by pooling samples from different

time points during the abscission process we lost the resolution to detect dynamic and specific changes in gene expression at specific time points. Hence, for the differential expression analysis, we utilized the transcriptome data to generate an AZ-specific microarray chip, which allowed a cost effective way to detect all the dynamic changes in gene expression at all stages of the abscission process. We had to design this AZ-specific chip, since the commercial Affymetrix Tomato Gene Chip used in our previous studies (Meir et al., 2010) contained only about 10,000 genes, and many AZ-specific genes were not included in this array. Transcriptome sequencing data were previously utilized to create customized microarrays suitable for various research needs in Syrian golden hamsters (Ying et al., 2015) and *Camellia sinensis* (Wang et al., 2014a). Customized microarrays are used for global transcriptome analysis in various organisms/systems (Lipovich et al., 2014; Yasuike et al., 2015), and strand-specific customized arrays were designed for studying NATs in the Human genome (Yelin et al., 2003) and mouse (Kiyosawa et al., 2005).

The additional novelty of our AZ-specific microarray is that the probes were designed in both sense and antisense orientations, and multiple probes were used for each transcripts (Table 6, Figure S5). Thus, this developed tomato AZ-specific chip contains much more transcripts than any other commercial microarray chip. Therefore, it can serve as an excellent means to further study the tomato AZ transcriptome. We have utilized the AZ-specific microarray chip (GSE45355; GSE45356; GSE64221) for the transcriptome analysis of several genes in the tomato FAZ and LAZ samples. Part of these analyses at specific time points following abscission induction, which represent various abscission stages, was already published. These publications showed changes in expression of specific groups of tomato genes, such as auxin-related genes in the FAZ and LAZ (Meir et al., 2015), and genes associated with cell wall, boundary layer, pathogen-related, and lipid transport in the FAZ and NAZ (Kim et al., 2015; Meir et al., 2015). Regarding the clear advantages of this AZ-specific microarray, these recent studies enabled the exploration of genes responsible for abscission induction, execution and synthesis of the defense layer processes in the tomato LAZ and FAZ.

The rapid technological progress in NGS, which led to the generation of the Strand-specific RNA-Seq, can provide a bp resolution. Thus, several cheap library construction protocols are already available (Zhong et al., 2011), and can be utilized for similar research needs. Our choice of a customized microarray platform was driven by cost advantages relative to RNA-Seq, for robustness of its own kind with well-standardized methods, and by the known inadequacies of commercial microarray platforms, which under-represents genomic complexity.

Annotation Analysis Characterizes Abscission as a Dynamic Process

The GO terms of “protein binding,” “oxidation-reduction process,” and “membrane” were the most represented ones among the categories of molecular function, biological process, and cellular component, respectively, in both tomato AZs (Figure 3). The data show that both the FAZ and the LAZ share a similar type of gene enrichments in all the three

categories of biological process, molecular function, and cellular component, with only few differences, when samples of all-time points following abscission induction were pooled together. These annotations of the over-expressed categories provide a useful basis for studying gene functions, cellular structures, and processes in the two examined AZs (Tables S8, S9). Our data are consistent with the data of the *Arabidopsis* stamen AZ (Cai and Lashbrook, 2008) and olive fruit AZ transcriptomes (Gil-Amado and Gomez-Jimenez, 2013) in the category of cellular component, where the GO term “membrane” is the most represented. In the olive fruit AZ transcriptome, the most represented term in the category of biological process was metabolic process; whereas in the tomato FAZ and LAZ our data show that the terms of “oxidation-reduction process” and “metabolic process” were represented at similar levels (**Figure 3**). In the laminar AZ of citrus leaves, the most important GO terms represented cell organization, biogenesis/metabolic process, metabolism of fatty acids, carbohydrate metabolism, response to biotic and abiotic stimuli, and transport (Agustí et al., 2012). Our data in tomato (Tables S8, S9, **Figure 4**) confirm that these terms were highlighted in the over-expressed groups in the LAZ (Group A). This is in agreement with earlier studies of the abscission process in *Arabidopsis* and olive (Cai and Lashbrook, 2008; Gil-Amado and Gomez-Jimenez, 2013), suggesting that most genes involved in AZ functions are being shared at all stages of the abscission process of various organs, although there might be significant changes in the transcriptional activities at each stage of the abscission process.

Comparative Gene Expression in the Tomato FAZ and LAZ

Transcription Factors Gene Families

TFs play key roles in plant development and act as major switches of various transcriptional regulatory networks by temporarily and spatially regulating the transcription of their target genes. Recent reports highlighted the involvement of various TFs in organ abscission and dehiscence processes, FAZ development, and formation of protective layers (Meir et al., 2010, 2011; Nakano et al., 2012; Corbacho et al., 2013; Gil-Amado and Gomez-Jimenez, 2013; Parra et al., 2013; Liu et al., 2014). Since many genes are involved in the abscission process, it is not feasible to manipulate such a complex and dynamic process by modifying a single gene expression, and therefore efforts have been focused on specific TFs that control the entire pathways (Nath et al., 2007; Van Nocker, 2009; Ma et al., 2015). Many TFs families associated with FAZ development during tomato flower abscission process was already characterized, and it was interesting to study if these TFs families are also involved in tomato leaf abscission.

MYB is one of the largest plant TF families (Riechmann et al., 2000), which is involved in various regulatory pathways (Jin and Martin, 1999). Our results show that several MYB genes were upregulated and exclusively present in the FAZ and LAZ (Table S10). In tomato, it was shown that the *Blind* (*Bl*) gene, which encodes the R2R3 class MYB TF, was upregulated in the AZ-forming lines (Nakano et al., 2012), and it interacted with *jointless* in controlling the meristem cell fate (Schmitz et al.,

2002; Quinet et al., 2011). In rice, a single non-synonymous substitution (G to T) in the shattering gene *sh4*, encoding a MYB3 DNA-binding domain, resulted in AZ dysfunction and incomplete development, leading to reduced seed shattering (Li et al., 2006). Most members of the MYB families were shown to be abundantly present in melon fruit AZ (Corbacho et al., 2013) and were upregulated in olive fruit AZ (Gil-Amado and Gomez-Jimenez, 2013) during fruit abscission. Additionally, our data show that *SlMYB43—THM16* (Solyc11g011050), which is a homolog to *AtMYB43* and *PtrMYB152*, was present in both tomato AZs and overexpressed in group A (Table S10). This indicates that this gene regulates secondary cell wall biosynthesis similarly to *Arabidopsis* MYBs (Wang et al., 2014b). Other MYB genes, such as *SlMYB3* (Solyc06g065100)—*At1g22640* (salicylic acid- and ABA-inducible TF), *SlMYB108* (Solyc12g099130)—*AT3G06490* (ethylene- and JA-inducible TF), were expressed in both tomato AZs (Table S10). This supports the idea that these MYB proteins act as critical components of multiple hormone-mediated transcriptional cascades and cell wall biogenesis, which regulate tomato flower and leaf abscission.

ZF proteins regulate many developmental and stress responses (Takatsujii, 1998, 1999). Our results show that 98 ZF TF genes were overexpressed in both AZs, being the second most highly expressed family in Groups A and B (**Figure 7A**, Table S10). In *Arabidopsis*, *ZINC FINGER PROTEIN2* was upregulated in the stamen AZ, and its overexpression delayed the abscission process and contributed to the AZ development (Cai and Lashbrook, 2008). The C2C2 type ZF genes were upregulated in the tomato FAZ upon ethylene-induced abscission (Wang et al., 2013).

Most of the MADS-box and GRAS TFs gene families were expressed in both tomato AZ tissues represented by Groups A and B (**Figure 7A**, Table S10). Since our pooled LAZ and FAZ samples were taken when the AZs were already well defined, the higher expression of MADS-box and GRAS TFs gene families in these samples suggest that these TF genes also regulate the late stages of the abscission process, when the AZ is already differentiated. It was previously proposed that MADS box TFs might substantially contribute to the specificity of the identity of the pedicel regions (Nakano et al., 2012), and might form region-specific protein complexes similar to the floral quartets model of flower organ identification (Theissen and Saedler, 2001). Our data show that the MADS-box gene *MACROCALYX* (Solyc05g012020) is highly expressed in the tomato FAZ (Group B), whereas the *JOINTLESS* (Solyc11g010570) gene was expressed at a similar level in both the FAZ and the LAZ (Group C) (Table S10). Therefore, we speculate that a similar type of organ identity specification, reported for the FAZ, might also operate in the LAZ.

Our results show that 137 *bHLH* TFs genes, out of 159 identified *bHLH* genes in tomato, were overexpressed in both the FAZ and the LAZ (Table S10). These genes were distributed among all the groups presented in **Figure 2**, indicating that the *bHLH* TF family is associated with the abscission process. It should be emphasized that our expression studies were performed with pooled samples, and therefore they do not represent the pattern of abscission progress. Most members of the *bHLH* TF gene family were downregulated during

abscission of mature olive fruit (Gil-Amado and Gomez-Jimenez, 2013). Similarly, the *bHLH* TF gene (AW648468) was sharply downregulated in the tomato FAZ after flower removal (Meir et al., 2010). Mutation in the *myc/bHLH* gene *ALCATRAZ* in Arabidopsis delayed fruit dehiscence by blocking the separation of the valve cells from the replum (Rajani and Sundaresan, 2001). MYB and bHLH proteins (MYC type bHLH) interact to form multi protein complexes to regulate gene transcription (Wolberger, 1999; Zimmermann et al., 2004). It seems therefore, that *bHLH* and *MYB* TFs manifest a potential interaction necessary for the regulation of genes operating in downstream events in the FAZ and the LAZ during flower and leaf abscission.

The majority of the bZIP TFs were present and overexpressed in the tomato LAZ (Group A, A1) (**Figure 7**, Table S10). Among the bZIP TFs, the *HY5* (Solyc08g061130) gene was present and equally expressed in both AZs (Group C). Most members of the bZIP family genes and *HY5* were specifically induced and abundantly present in melon fruit AZ (Corbacho et al., 2013), and were upregulated in olive fruit AZ during abscission (Gil-Amado and Gomez-Jimenez, 2013; Parra et al., 2013). The *bZIP* gene (BG631669) was downregulated at an early stage of tomato pedicel abscission (Meir et al., 2010). The TGA type *bZIP* genes were found to be involved in plant development (Izawa et al., 1993), auxin-induced stress responses (Pascuzzi et al., 1998), and regulation of abscission-specific *Cel* gene expression (Tucker et al., 2002). This suggests that these TF genes might act as positive regulators of abscission signaling. Our results suggest that different bZIP TFs probably mediate the abscission-responsive transcription processes in flowers and mainly in leaves. Taken together, our data corroborate that in the tomato FAZ and LAZ, TFs belonging to these families may potentially act to trigger the transcriptional cascade during abscission and formation of the defense layer. Additional research is needed to reveal the molecular basis of the regulation of expression of these genes.

Key Meristem Genes

Reports demonstrating the expression of key meristem genes and their functional association with the AZ are emerging, implying that the undifferentiated AZ cells have the capability to differentiate in response to various stimulators. Most of the key meristem genes were expressed in both tomato AZs (**Table 3**). The *Tkn3/KNAT6*, *TBL4*, *LBD1*, *Bl* were highly expressed in the tomato LAZ (Group A) compared to the FAZ (**Table 3**). The KNOX family genes regulate the size and proliferation of the AZ cells during floral organ abscission (Shi et al., 2011). Moreover, *TKN3* and *BL4*, encoding KNOX and BELL family TFs, form a heterodimer required for SAM functioning (Rutjens et al., 2009). On the other hand, the *WUS*, *OFP*, *MYB-Cpm10/MYB78*, *Goblet*, *Ls* were highly expressed in the tomato FAZ compared to the LAZ (Group B) (**Table 3**). *WUSCHEL-related homeobox-containing protein* (*WUS*) and KNOX gene families are key genes in the regulation of the maintenance of undifferentiated cells (Long et al., 1996; Mayer et al., 1998; Lenhard et al., 2002). It was previously reported that the homologs of *WUS* and its potential functional partner *OFP* were downregulated during flower abscission (Meir et al., 2010; Wang et al., 2013). More

interestingly, the family members of *Arabidopsis KNAT6*, *BELL-like homeodomain protein 1*, and *OFP*, can potentially form ternary complexes, which are critical for meristem activities (Hake et al., 2004; Hackbusch et al., 2005; Hamant and Pautot, 2010; Li et al., 2011). Similarly, homologs of *LBD1* (Wang et al., 2013) and the SAM gene, NAC-domain TF *GLOBLET* (Berger et al., 2009), were also highly expressed in the tomato FAZ (Hu et al., 2014). *WUS*, *Bl*, and *Ls* genes displayed differential expressions between tomato wild type and the *mc* mutant, and hence showed a *jointless* phenotype (Nakano et al., 2012). On the other hand, *Bl* and *AGL12* were continuously and specifically induced in the tomato FAZ during the pedicel abscission process (Meir et al., 2010; Wang et al., 2013). The *REVOLUTA* gene is involved in apical meristem development for limiting cell divisions in *Arabidopsis* (Talbert et al., 1995). Overexpression of a microRNA166-resistant version of the tomato *REVOLUTA* (*SIREV*) (*35S::REV^{Ris}*) caused dramatic reproductive alterations, including continuous production of flowers at the FAZ (Hu et al., 2014). Our data show that *SIREV* (Solyc11g069470) was expressed equally (Group C) in both the FAZ and the LAZ samples (Table S3), indicating its importance for the development of the apical meristem in both AZs.

Our findings showing that most of the SAM and auxiliary meristem genes are preferentially expressed at differential levels in both the tomato FAZ and LAZ and interact with each other, support the idea that meristem activity genes play important roles in maintaining the undifferentiated status of cells in both AZs. The transcriptome data show that there are substantial differences between the tomato FAZ and LAZ. In particular, genes involved in key meristem functions show distinct expression patterns. These differences between the FAZ and the LAZ might explain the significant differences observed in the rate of pedicel and petiole abscission, respectively, when petiole abscission took a much longer time and had to be enhanced by ethylene (**Figure 1**). Apart of the key meristem genes, many other floral meristem genes were preferentially expressed in both the tomato FAZ and LAZ (Table S3).

Cell Wall Related Genes

Our transcriptome analyses showed that the majority of cell wall degrading and remodeling factors, including *PG*, *Cel*, *XTH*, and *EXP* genes (**Table 4**) were highly expressed in both tomato AZs. These genes were previously demonstrated to be highly expressed also in the AZs of a large number of abscission systems including tomato AZ (Lashbrook et al., 1994; del Campillo and Bennett, 1996; Cho and Cosgrove, 2000; Taylor and Whitelaw, 2001; Roberts et al., 2002; Agustí et al., 2008; Ogawa et al., 2009; Meir et al., 2010). *TAPG1* was highly expressed in the LAZ following exposure of the debladed leaf explants to ethylene (**Table 4**), thereby confirming previous reports showing that *TAPG1* transcript in the LAZ was induced by ethylene (Jiang et al., 2008) and inhibited by auxin (Hong et al., 2000). In addition, *TAPG4* showed the highest expression level in the FAZ, and therefore its promoter was used for specific silencing of genes in the tomato FAZ (Ma et al., 2015). Previous microarray experiments performed with the tomato pedicel abscission system showed that *Cel1* and *Cel5* were strongly and specifically

upregulated in the FAZ, but the expression levels of *Cel2,3,7,8* were very low in the FAZ and were not affected by flower removal (Meir et al., 2010). However, our results demonstrate that the *Cel2,5,6,7,8* genes were present and expressed at high levels in the LAZ pooled samples (**Table 4**). The functions of *Cel1* and *Cel2* were already demonstrated by antisense suppression in tomato flower and leaf abscission (Lashbrook et al., 1998; Brummell et al., 1999). *Cel1,6,9* genes were upregulated in the LAZ of soybean (*Glycine max*) explants after ethylene treatment (Tucker et al., 2007). Expansins are involved in cell wall enlargement and pectin remodifications (Lee et al., 2001; Cosgrove et al., 2002; Zenoni et al., 2011), and were reported to regulate pedicel abscission in Arabidopsis and soybean and leaflet abscission in elderberry (*Sambucus nigra*) (Cho and Cosgrove, 2000; Belfield et al., 2005; Tucker et al., 2007). We observed that the expression levels of several *EXP* genes, *LeEXP1,5,9,11,18*, were particularly high in the LAZ (**Table 4**). It was previously reported that the expression levels of *EXP3,4,5,9* genes in tomato, which were specifically and highly expressed in the FAZ, decreased during the process of pedicel abscission, and were not affected by the 1-MCP pretreatment (Meir et al., 2010). *LeEXP1,11* genes were also specifically upregulated in tomato FAZ (Wang et al., 2013).

Taken together, our data regarding the changes in the expression of genes encoding cell wall modifying enzymes, confirmed previous reports on various abscission systems. The results also indicate that both the FAZ and the LAZ have similar types of cell wall-related genes, but with different expression levels, which were generally higher in the LAZ than in the FAZ. This may be ascribed to the ethylene treatment applied to the debladed leaf explants in order to enhance the rate of leaf petiole abscission (**Figure 1B**). Ethylene treatment might also induce an increased expression of cell wall modifying genes in the LAZ.

Hormonal Signal Transduction Genes

The interplay of plant hormones during the abscission process has been widely reported. In tomato, the pedicel abscission process is inhibited by a continuous auxin flow from the flowers, and is triggered by ethylene following auxin depletion in the FAZ (Roberts et al., 1984; Meir et al., 2010). Ethylene also inhibits auxin transport, thereby enhancing auxin depletion in the AZ (Meir et al., 2015). Therefore, it was expected that genes of auxin and ethylene signal transduction cascades would be expressed predominantly in both the FAZ and the LAZ as compared to the other plant hormones. A continuous auxin flow to the AZ is required for preventing the acquisition of ethylene sensitivity by the AZ cells, which leads in turn to organ abscission (Taylor and Whitelaw, 2001). The molecular basis of auxin transport in tomato was elucidated in sympodial growth, compound leaves, fleshy fruit, and whole plants (Giovannoni, 2004; Kimura and Sinha, 2008; Nishio et al., 2010; Pattison and Catalá, 2012). This auxin flow is presumably regulated by auxin-responsive genes, belonging to three major groups, *Aux/IAA*, *SAUR*, and *GH3*. These auxin-responsive genes, which are regulated by ARFs (Guilfoyle and Hagen, 2007), are well characterized in tomato (Kumar et al., 2011, 2012; Wu et al., 2012). Our data show that various auxin-related genes, such as *Aux/IAA*, *SAUR*, *GH3*, *ARFs*,

LAX, and *PINs*, are expressed in both the tomato FAZ and LAZ at various levels (**Table 5**).

The expression level of *Aux/IAA* genes was used as a marker for auxin activity associated with inhibition of floret abscission by auxin application in *C. elegans* (Abebie et al., 2008), as well as a marker of auxin depletion in tomato FAZ following flower removal (Meir et al., 2010) and in citrus AZ during fruitlet abscission (Xie et al., 2015). We previously showed that the auxin responsive genes *IAA1,3,4,7,8,9,10* were downregulated following abscission induction in tomato flowers (Meir et al., 2010), and our present data showed that *IAA3,8,9,10* were presented and expressed in both the tomato FAZ and LAZ pooled samples at various levels (**Table 5**). The remaining genes which were already shown to be present in the FAZ, *IAA1,4,7* (Solyc06g053840, Solyc04g076850, Solyc06g053830, respectively), were shown to have equal expression in the FAZ and the LAZ (**Table S3**). These genes are not KEGG-annotated in the auxin hormonal pathway, which might indicate that that they are not significant for the auxin signaling cascade.

ARFs are TFs that bind to AREs in promoters of early auxin responsive genes and play central roles in many auxin-mediated processes, leading to activation or suppression of the selected genes. The expression pattern and the possible role of the *ARF* gene family in the tomato FAZ, as well as auxin- and ethylene-induced changes during flower abscission were comprehensively studied (Guan et al., 2014). We showed that multiple *ARF* genes were expressed in both tomato AZs (**Table 5**, **Table S3**). Upregulation of the *SlARF1,3,5,19* delayed the abscission process in the FAZ (Guan et al., 2014), indicating that these ARFs have a similar function in both AZs.

Several reports suggested that *GH3* genes are involved in flower or fruitlet abscission (Kuang et al., 2012; Wang et al., 2013; Meir et al., 2015). *MES1* converts the storage form of IAA into its active free form, whereas *Dwarf in Light1* does the opposite, i.e., converts active free IAA to an inactive conjugated form (Staswick et al., 2005; Woodward and Bartel, 2005; Yang et al., 2008; Ludwig-Müller, 2011). Our results demonstrate that *MES1* and *DFL1* (**Table S3**) as well as other *GH3* genes (**Table 5**) were expressed in both tomato AZs. Recently, we reported (Meir et al., 2015) that *GH3* genes in the FAZ were upregulated in response to auxin depletion, confirming that increased IAA conjugation is involved in the process of auxin depletion, while their expression decreased in the LAZ after leaf deblading, further confirming that *GH3* is an auxin-induced gene. In addition, conjugation of IAA to amino acids provides a negative feedback loop to control auxin homoeostasis, and the *SlGH3-2,10,15* genes, which control IAA conjugation, were found to respond quickly to exogenous auxin application (Kumar et al., 2012; Meir et al., 2015).

Reduced auxin levels in Arabidopsis were attributed to increased activity of auxin efflux transporters (Sorefan et al., 2009). Recently, it was demonstrated that the *KD1* gene played a role in modulating auxin levels in the tomato FAZ, by altering the expression profiles of the auxin efflux transporters, *PIN9* (HQ127075; *SlPIN9*) and *PIN-like3* (SL_TC197872) (Ma et al., 2015). Our data show that the auxin influx carrier genes, *SlLAX3,4,5* and the auxin efflux carrier genes *SlPIN1,3,4,5,6,7,9*, were present in both the tomato FAZ and LAZ (**Table 5**). In

addition, *SIPIN3,5,6,7* genes were overexpressed (by ~2- to 10-fold) in the LAZ compared to the FAZ (**Table 5**). Most of the auxin-related gene families are expressed in both AZs, but some members of different gene families are expressed specifically in the FAZ or the LAZ (Meir et al., 2015). The only study so far on the effect of leaf deblading on expression of auxin-related genes in the LAZ was performed in *Mirabilis jalapa* (Meir et al., 2006). The differential expression of the auxin influx and efflux carrier genes between the two AZs suggests that different genes of these families play important roles in different AZs. This might further provide us with means for selective manipulation of leaf and flower abscission by specifically manipulating auxin transport in these two AZs. Such a manipulation might be important for agricultural purposes, for example to reduce olive fruit strength before harvest without affecting leaf abscission.

Ethylene response factors (ERFs) are plant transcriptional regulators that specifically bind the GCC motif of the promoter region of ethylene-regulated genes, thereby mediating ethylene-dependent gene expressions (Ohme-Takagi and Shinshi, 1995; Solano et al., 1998). These *SIERF* genes were reported to be specifically overexpressed in the tomato FAZ compared to the proximal (basal) and distal (apical) NAZ regions (Nakano et al., 2013; Wang et al., 2013). It was recently reported that in *SIERF52*-suppressed tomato plants, flower pedicel abscission was significantly delayed compared to the wild type, and accordingly, a reduced expression of the genes encoding for the abscission-associated enzymes, cellulase and PGs, was detected (Nakano et al., 2014). Our data show that several *SIERF* genes, and particularly *SIERF52*, which were reported to be essential for flower pedicel abscission, operate also in the tomato LAZ. The downstream components (positive) of ethylene signaling, such as *EIN2* and *EILS*, were expressed in both the tomato AZs (**Table 5**). Antisense lines of *LeEIL* plants exhibited delayed flower abscission (Tieman et al., 2001), which suggests that the *LeEILS* has the same function in the tomato LAZ.

Many genes related to different steps of the ethylene signaling transduction pathway were expressed in both the tomato AZs following flower removal and leaf deblading (**Table 5**). The tomato ethylene receptor genes *ETR1,3,4,5* were expressed in both AZs, confirming previous reports that they were expressed in the FAZ (Payton et al., 1996; Meir et al., 2010). The *Constitutive Triple Response1* (*CTR1*) gene product acts downstream of the ethylene receptors, and negatively regulates ethylene signal transduction (Kieber et al., 1993). It was previously reported that *CTR1* was upregulated in early stages of the tomato pedicel abscission process, and was specifically expressed in the FAZ in the late stages of the abscission process (Meir et al., 2010). Our analysis data show that *CTR1,3,4* genes were expressed in both tomato AZs, with *CTR1* showing the highest expression (**Table 5**). This indicates that a similar cascade of events operates in both AZs.

CONCLUSIONS

This study presents a *de novo* assembly of AZ-associated transcripts and provides valuable information about their functional annotation by using an integrated approach to enrich

the transcriptome of *S. lycopersicum* FAZ and LAZ. To the best of our knowledge, this is the first comprehensive report on RNA-Seq of tomato FAZ and LAZ, which we believe would contribute to the understanding of the expression differences in these AZs. The present research identified genes that may be involved in the abscission process of tomato flowers and leaves, including genes encoding for TFs, hormone transduction components, cell wall-related enzymes, and key meristem factors. Similar gene family members were expressed in both the FAZ and the LAZ following flower or leaf removal, respectively, suggesting a similar regulation of the abscission process of these organs with few exceptions. This provides a significant improvement in our understanding of the abscission process.

We have utilized the RNA-Seq data for the development of an AZ-specific microarray chip. The AZ microarray is more comprehensive than other commercially available arrays, having more transcripts with multiple probes and probes designed in antisense direction, which might be further used to explore the roles of NATs. The AZ-specific microarray can be used for further examination of detailed gene expression in various abscission stages, and is applicable to other transcriptomic studies in tomato. The AZ-specific microarray chip provides a cost-effective approach for analysis of multiple samples in a rapid succession. Hence, this study can serve as a foundation for characterization of candidate genes, which would not only provide novel insights into understanding of the AZ development, early and late abscission events, but also will provide resources for improved tomato breeding for preventing abscission and for specific manipulation of flower or leaf abscission for horticultural uses.

SEQUENCE DEPOSITION

NGS data is submitted to the NCBI sequence read archive (NCBI-SRA) under the study ID: PRJNA192557. Individual SRS ID = SRS399193 entitled with FAZ transcriptome analysis of VF-36, and SRS401162 entitled with LAZ transcriptome analysis of VF-36. The AZ-specific microarray (AMADID: 043310) was validated and used to analyze the FAZ, flower NAZ and LAZ at various time points and deposited in the NCBI Gene Expression Omnibus (GEO) databases (<http://www.ncbi.nlm.nih.gov/geo/>) under the following locators: GSE45355; GSE45356; GSE64221. These data will be released for public access upon acceptance of this publication.

AUTHOR CONTRIBUTIONS

SS, SM and SP were responsible for the conception, design of the experiments and interpretation of data for work. SS, RM and NK were responsible for acquisition of the data and the bioinformatics analysis. SS, BK and ShS performed the laboratory experiments and analyses. SS, RJ, and MT were responsible for the design of the microarray chip. SS, SP, JR and SM were responsible for final approval of the version to be published.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01258>

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Distribution of XTH, expansin, and secondary-wall-related CesA in floral and fruit abscission zones during fruit development in tomato (*Solanum lycopersicum*)

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After fruit development is triggered by pollination, the abscission zone (AZ) in the fruit pedicel strengthens its adhesion to keep the fruit attached. We previously reported that xyloglucan and arabinan accumulation in the AZ accompanies the shedding of unpollinated flowers. After the fruit has developed and is fully ripened, shedding occurs easily in the AZ due to lignin accumulation. Regulation of cell wall metabolism may play an important role in these processes, but it is not well understood. In the present report, we used immunohistochemistry to visualize changes in the distributions of xyloglucan and arabinan metabolism-related enzymes in the AZs of pollinated and unpollinated flowers, and in ripened fruits. During floral abscission, we observed a gradual increase in polyclonal antibody labeling of expansin in the AZ. The intensities of LM6 and LM15 labeling of arabinan and xyloglucan, respectively, also increased. However, during floral abscission, we observed a large 1 day post anthesis (DPA) peak in the polyclonal antibody labeling of XTH in the AZ, which then decreased. These results suggest that expansin and XTH play important, but different roles in the floral abscission process. During fruit abscission, unlike during floral abscission, no AZ-specific expansin and XTH were observed. Although lignification was seen in the AZ of over-ripe fruit pedicels, secondary cell wall-specific cellulose synthase signals were not observed. This suggests that cellulose metabolism-related enzymes do not play important roles in the AZ prior to fruit abscission.

Keywords: abscission, XTH, expansin, fruit, flower, tomato

Introduction

Abscission is a process by which plants shed unnecessary organs such as leaves, flowers, and fruits to save metabolic energy, to protect themselves from biotic and abiotic stresses by releasing organs attacked by pathogens, and to allow seed dispersal by releasing fruits. Organ abscission occurs at a specialized region in the leaf stem and pedicel, known as the AZ. The AZ consists of several layers of microscopic cells that are distinct from surrounding cells, and which form well before organ separation (Roberts et al., 2000, 2002). Earlier studies have identified several MADS-box genes that are related to the formation of AZs, including *JOINTLESS*, a gene essential for AZ formation in tomato pedicels (Mao et al., 2000). Furthermore, one of the key enzymes in cell wall degradation,

polygalacturonase, was first found in the tomato fruit pedicel AZ. Thus, the tomato plant has become an important resource in organ abscission research (Taylor et al., 1991; Kalaitzis et al., 1995, 1997). We previously reported that during floral abscission, a large increase was observed in LM15-labeling of xyloglucan specifically at the AZ in the abscised pedicel. During fruit abscission, unlike in floral abscission, we did not observe any AZ-specific cell wall polysaccharide deposition. However, high autofluorescence was seen in the AZ of overripe fruit pedicels, suggesting secondary cell wall synthesis and lignification of the AZ prior to fruit abscission (Iwai et al., 2013). In the previous report, we did not analyze enzymatic activities of cell wall modeling enzymes, such as xyloglucan endotransglucosylase/hydrolase (XTH), expansin, and certain cell wall synthesis-related enzymes (secondary cell wall enzymes: CesA4, 7, and 8). Biochemical experiments could not be performed due to the limited number of AZ samples, making enzymatic analyses quite difficult. Therefore, in this report, we visualized XTH, expansin, and cellulose synthase using immunohistochemistry.

Xyloglucan is one of the major hemicelluloses of primary cell walls, in dicot plants, and may account for up to 10–20% of cell wall components (Fry, 1989; Hayashi, 1989). They tether cellulose microfibrils by cross-linking them through non-covalent linkages, thus providing strength to the walls during growth. Modification in the length of xyloglucans during cell expansion is primarily mediated by the enzyme XTH through endotransglycosylation, thus enabling the cell wall to expand without weakening (Smith and Fry, 1991; Fry et al., 1992; Nishitani and Tominaga, 1992). XTHs belong to a multigene family (Xu et al., 1996; Campbell and Braam, 1999; Rose et al., 2002; Yokoyama et al., 2004), which plays important roles in several different processes during cell wall modification. These include root hair initiation (Vissenberg et al., 2000, 2001), hypocotyl elongation (Potter and Fry, 1994; Catalá et al., 1997, 2001), hydrolysis of seed storage carbohydrates (de Silva et al., 1993), leaf growth and expansion (Schunmann et al., 1997), aerenchyma formation (Saab and Sachs, 1996), fruit softening (Schroder et al., 1998; Ishimaru and Kobayashi, 2002; Saladié et al., 2006), tension wood formation (Nishikubo et al., 2007, 2011), and petal abscission (Singh et al., 2011). Expansins were the first proteins characterized that directly induce the extension of the plant cell wall (McQueen-Mason et al., 1992), and are believed to be important regulators of wall extension during plant cell growth (reviewed in Lee et al., 2001; Cosgrove et al., 2002; Li et al., 2003). Expansins appear to operate by disrupting hydrogen bonds between cellulose microfibrils and xyloglucans that tether them to one another in plant cell walls (McQueen-Mason and Cosgrove, 1994, 1995; Whitney et al., 2000). In addition to this role during cell growth, expansins play an important role in fruit softening (Rose et al., 1997; Brummell et al., 1999; Anjanasree and Bansal, 2003). Similarly, expansins are expressed at the point of radicle emergence in germinating tomato seeds (Chen and Bradford, 2000) and in the micropylar endosperm of *Datura ferox* seeds in response to red light (Mella et al., 2004), suggesting that they may play a general role in promoting cell wall dissolution. Abscission and fruit softening both involve cell wall breakdown, and many of the same types of enzymes are involved in the two processes (Rose and Bennett, 1999; Rose et al., 2003). Although

there is some circumstantial evidence of an association between XTH and expansins and abscission (Cho and Cosgrove, 2000), no reports have been published showing a correlation between the activity of these proteins and organ shedding, especially during floral and fruit abscission.

In the present study, we present the first report that abscission is associated with elevated XTH and expansin, suggesting that these proteins contribute to the process of organ shedding. We also discuss the abscission systems that occur during floral and fruit abscission in tomato plants.

Materials and Methods

Plant Material and Growth Conditions

Tomato (*Solanum lycopersicum* cv Micro Tom) plants were grown inside a cultivation chamber (TOMY CL-301) under a 16 h light and 8 h dark regime, at temperatures of 26 and 22°C, respectively, and a light intensity of approximately 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Pollination

Tomato flowers were pollinated by hand. 1 day prior to flowering, the closed buds were opened using a pair of tweezers and the anthers were extracted, leaving only the pistil inside. The opened buds were pollinated the next day by rubbing a dehisced anther onto the stigma. Glassine paper bags were placed over the treated flowers at the time the anthers were extracted to avoid unwanted pollination and to protect against physical stress.

Technovit Resin Sections

Samples were fixed in 2.5% paraformaldehyde in 0.025 mM phosphate-buffered saline (PBS) and evacuated using a vacuum pump for 12 h. Fixed samples were dehydrated through the following series of EtOH concentrations: 30, 50, 70, 80, and 90% for 20 min each, and then 95 and 100% twice for 30 min. EtOH in dehydrated samples was exchanged for Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) through the following series of Technovit 7100:EtOH: 1:4, 2:3, 3:2, and 4:1 each for 30 min, and then 100% Technovit for 30 min and 12 h. Samples were then solidified in Technovit 7100 resin following the manufacturer's protocol. Embedded samples were cut into 5 μm sections using a microtome and a glass knife.

Paraffin Sections

Samples were fixed in 4% paraformaldehyde at 4°C overnight for paraffin embedding. The fixed samples were dehydrated in a graded series of ethanol (70 and 85%) followed by a 1-butanol/ethanol series (80% ethanol/1-butanol 13:7, 90% ethanol/1-butanol 9:11, 100% ethanol/1-butanol 1:3, and 100% 1-butanol). 1-butanol was replaced gradually with paraffin (Paraplast Plus; McCormick Scientific, St. Louis, MO, USA) at 60°C over two nights inside an open jar to evaporate traces of n-butanol, and was then embedded in paraffin. Sections 12 μm thick were cut using a rotary microtome (Leica RM2145), and the ribbons were placed and stretched out on albumin-glycerin-treated glass slides with distilled water (DW). The slides were dried at 45°C on a warming plate for 2 days, and stored at room temperature. For

use, the slides were deparaffinized in xylene for 10 min (twice), and then hydrated in a graded series of ethanol (100, 90, 80, 70, and 0% in DW).

Lignin Staining

The sections were soaked in DW prior to staining. Phloroglucinol staining of lignin was performed according to Tadeo and Primo-Millo (1990). A saturated solution of phloroglucinol (Sigma-Aldrich) was prepared in 20% HCl and applied to sections.

Immunohistochemical Analysis

Monoclonal rat IgG antibodies of LM6 and LM15 were purchased from PlantProbes (Leeds, UK¹). Anti XTH, CesA4, CesA7, and CesA8 rabbit antibodies were obtained from Agrisera (Vännäs, Sweden^{2,3,4,5}). Anti expansin antibody was obtained from Biocompare (South San Francisco, San Mateo, CA, USA⁶). A TSA kit with HRP-conjugated secondary antibody and Alexa Fluor 488 tyramide were purchased from Invitrogen (Carlsbad, CA, USA; cat. #T20912, #20922, respectively). We used the set of polyclonal antibodies to perform immunohistochemistry according to the manufacturer's instructions. Briefly, the sections were incubated in PBS prior to labeling, and 100 µL of the following reagents were added to the sections in consecutive order: quenching buffer (to quench endogenous peroxidase activity), 1% blocking reagent, and primary antibody diluted in 1% blocking reagent (1:30). After each addition, the sections were incubated at room temperature for 1 h. The sections were then washed three times with PBS, incubated in 100 µL horseradish peroxidase (HRP)-conjugated second antibody diluted in 1% blocking reagent (1:100) for 1 h, again washed three times with PBS, incubated in 100 µL Alexa Fluor 488 tyramide (excitation 495 nm, emission 519 nm) working solution (tyramide stock solution diluted in amplification buffer/0.0015% H₂O₂; 1:100) for 10 min at room temperature, and finally washed three times with PBS and twice with DW. Immunofluorescence was visualized using a Leica DMRB fluorescence microscope, and all of the micrographs were captured with a DFC500 Leica digital camera using the IM50 Leica software (exposure time, 1.0 s).

Results

Immunolocalization of Xyloglucan and Arabinan Epitopes During –1 DPA Flower Pedicels to Yellowing Pedicels

To investigate the distribution of cell-wall pectic arabinan and xyloglucan, we used their specific monoclonal antibodies to stain –1 DPA, 1 DPA, 2 DPA, 3 DPA pedicels and yellowing pedicels

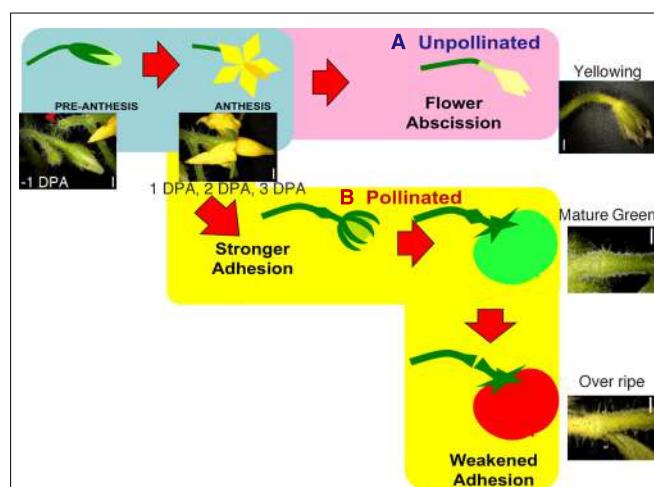


FIGURE 1 | Schematic diagram of the regulation of flower and fruit adhesion in tomato AZs. Once the flowers are successfully pollinated and fruits start to develop, the AZs enlarge and adhesion becomes stronger until the fruits fully ripen (**B**). Unsuccessful pollination leads to flower detachment from the AZ (**A**). Red arrowheads indicate pedicels separated at AZ. Bars = 1 mm. Adapted from Iwai et al. (2013).

(Figure 1). Pedicels yellowed just before abscission. Immunodot assays and competitive inhibition ELISA performed by Willats et al. (1998) and Orfila and Knox (2000) indicated that the LM6 antibody is specific to (1 → 5)-α-L-linked arabinosyl residues with extended polymer, and reacts with rhamnogalacturonan (RG)-I but not RG-II. ELISA and glycan microarray performed by Marcus et al. (2008) indicated that LM15 binds specifically to xyloglucan, and competitive inhibition ELISAs determined that the structural feature for LM15 binding is the XXXG structural motif. Therefore, we used these probes to explore the occurrence of specific cell wall polysaccharides and differences in their distribution during early fruit development.

In the pollinated flower pedicel, the intensity of LM15 xyloglucan epitopes was low in –1 DPA to 3 DPA. The intensity of LM6 arabinan and LM15 xyloglucan epitopes was low throughout the tissue (Figure 2). Some weak signals were detected in vascular bundles by LM15; however, the labeling was not specific to the AZ.

In unpollinated flower pedicels, relative to –1 DPA pedicels, the intensity of LM6 pectic arabinan labeling increased in 3 DPA, and the highest signal was detected in the yellowing stage (Figure 3). The LM6 labeling was most intense at the AZ (Figure 3). Remarkably, LM15 labeling for xyloglucan epitopes increased dramatically, preferentially at the AZ (Figure 3). These results indicate that pectic arabinan and xyloglucan increase prior to abscission. As the time of abscission approached, accumulation of xyloglucan and arabinan increased (Figure 3).

Immunolocalization of Expansin and XTH Epitopes During –1 DPA Flower Pedicels to Yellowing Pedicels

To investigate the potential role of XTH and expansin during the time course of floral abscission, we visualized changes in the distributions of XTH and expansin epitopes in –1 DPA,

¹www.plantprobes.net

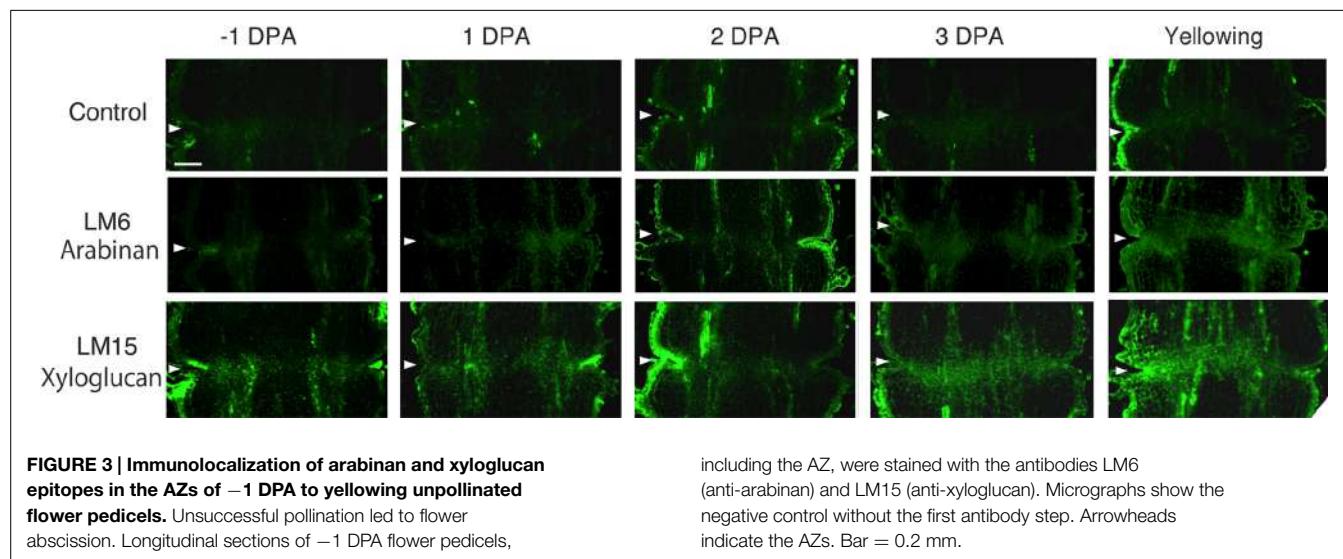
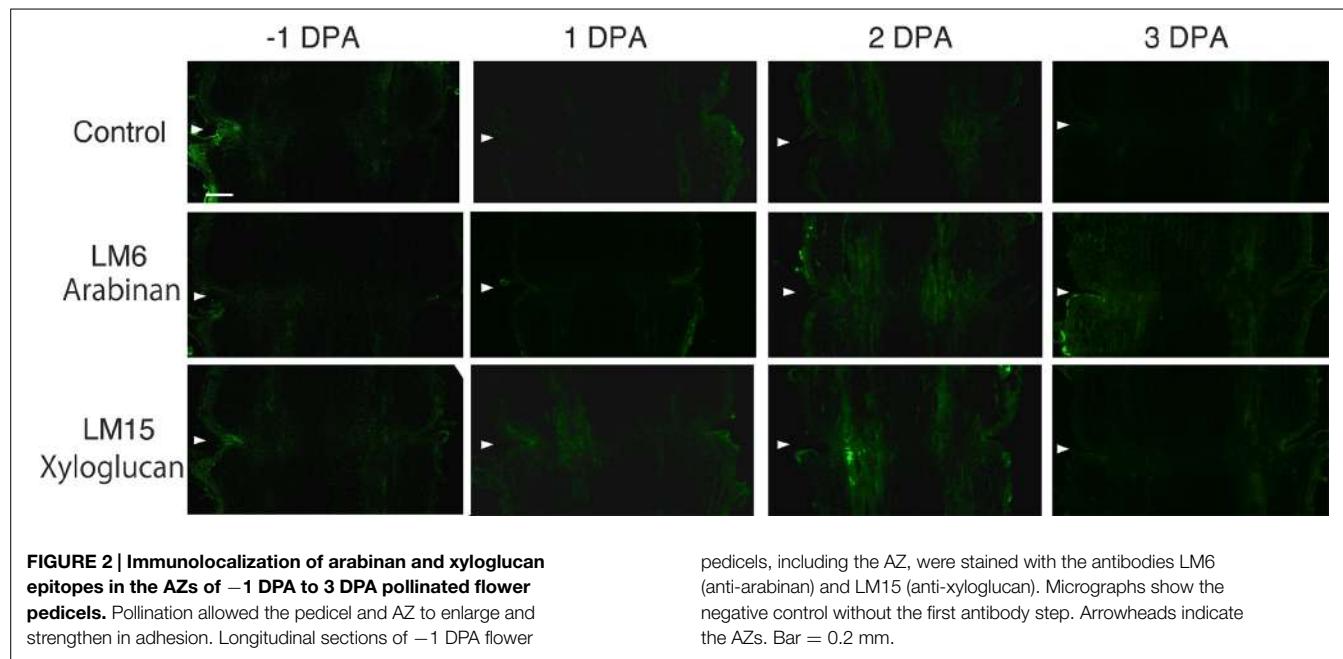
²www.agrisera.com/en/index.html

³<http://www.agrisera.com/en/artiklar/cesa4-cellulose-synthase-a-catalytic-subunit-4-udp-forming.html>

⁴<http://www.agrisera.com/en/artiklar/cesa7-cellulose-synthase-a-catalytic-subunit-7-udp-forming.html>

⁵<http://www.agrisera.com/en/artiklar/cesa8-cellulose-synthase-a-catalytic-subunit-8-udp-forming.html>

⁶<http://www.biocompare.com/9776-Antibodies/2820012-anti-EXPB-2-antibody>



1 DPA, 2 DPA, 3 DPA pedicels and yellowing pedicels. XTH was determined by immunolocalization using polyclonal antibodies (Liu et al., 2013). In tomatoes, *SIXTH1-25* was reported as the gene that encodes XTH (Muñoz-Bertomeu et al., 2013) and the antibody detected epitopes in all the XTH proteins examined (Takizawa et al., 2014). Expansin is a peripheral membrane protein that may cause loosening and extension of plant cell walls by disrupting non-covalent bonding between cellulose microfibrils and matrix glucans. Tomato expansins have also been reported (Rose et al., 2000), and we used commercial polyclonal antibodies that are reactive to β -expansin as shown in web page of antibody-online.com⁷. The epitope(s) recognized by

⁷<http://www.antibodies-online.com/antibody/726770/anti-beta+-Expansin+EXPB2/>

the anti- β -expansin is thought to occur in all tomato expansins. In the pollinated flower pedicel, the intensities of XTH and expansin epitopes were low in -1 DPA to 3 DPA (Figure 4). In the unpollinated flower pedicel, LM6 and LM15 labeling of arabinan and xyloglucan increased (Figure 3). However, during floral abscission, we observed a large 1 DPA peak in the polyclonal antibody labeling of XTH in the AZ, which then decreased (Figure 5).

Remarkably, expansin epitopes increased gradually but dramatically, specifically at the AZ, during floral abscission, whereas LM6 and LM15 labeling of arabinan and xyloglucan, respectively, also increased (Figure 3). The highest signals of expansin were detected during the yellowing stage just prior to abscission (Figure 5).

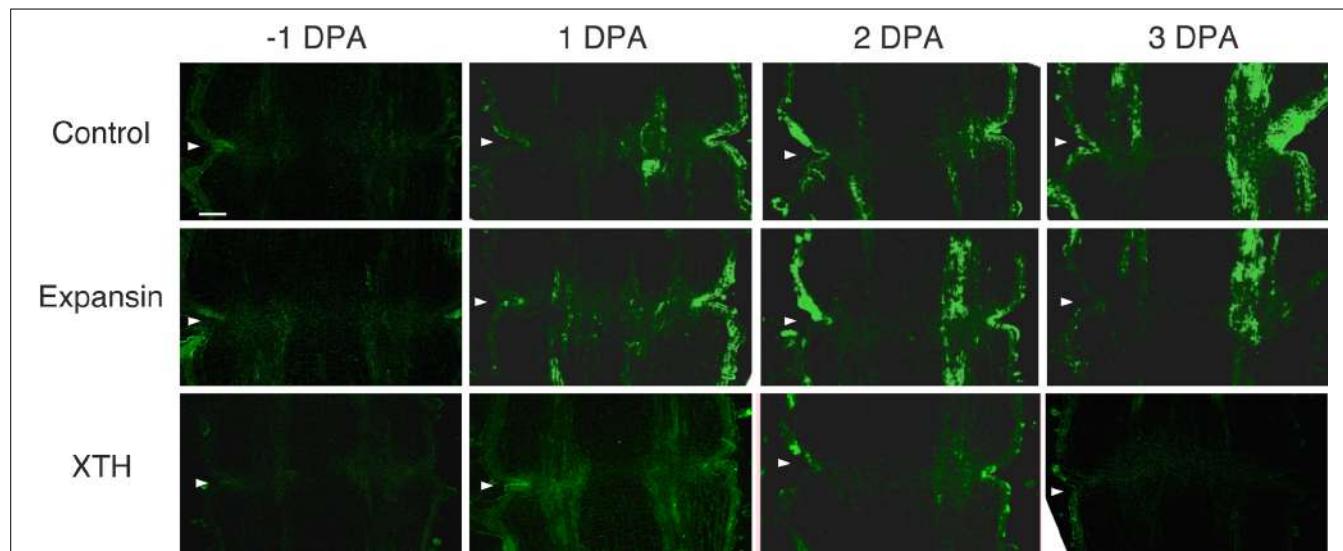


FIGURE 4 | Immunolocalization of expansin and XTH epitopes in the AZs of -1 DPA to 3 DPA pollinated flower pedicels. Pollination allowed the pedicel and AZ to enlarge and strengthen in adhesion. Longitudinal sections of

-1 DPA flower pedicels, including the AZ, were stained with the anti-expansin and anti-xyloglucan antibodies. Micrographs show the negative control without the first antibody step. Arrowheads indicate the AZs. Bar = 0.2 mm.

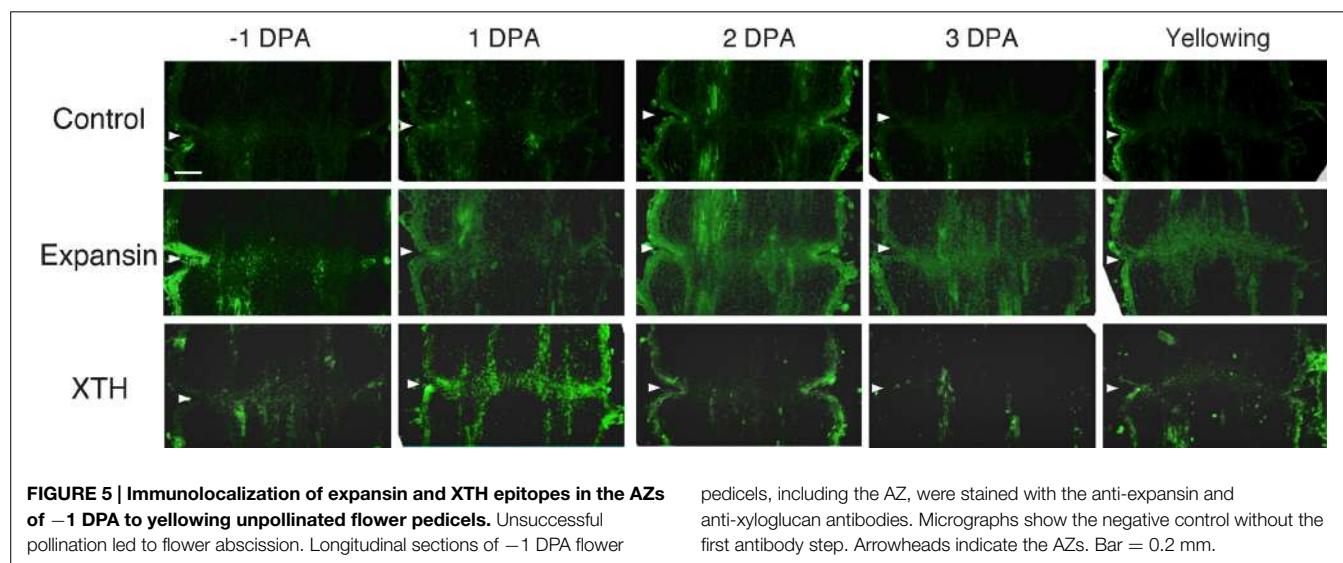


FIGURE 5 | Immunolocalization of expansin and XTH epitopes in the AZs of -1 DPA to yellowing unpollinated flower pedicels. Unsuccessful pollination led to flower abscission. Longitudinal sections of -1 DPA flower

pedicels, including the AZ, were stained with the anti-expansin and anti-xyloglucan antibodies. Micrographs show the negative control without the first antibody step. Arrowheads indicate the AZs. Bar = 0.2 mm.

Immunolocalization of XTH and Expansin Epitopes in MG Fruit Pedicels and OR Fruit Pedicels

To determine whether flower and fruit abscission from the pedicel AZ occur by similar mechanisms, the distribution of XTH and expansin epitopes were investigated by applying the same set of polyclonal antibodies to mature green (MG) and over ripe (OR) fruit pedicel sections, each representing a stage in which adhesion at the AZ was strengthened or loosened, respectively. In MG fruit pedicels, the intensities of XTH and expansin epitope labeling were relatively low (Figure 6). We detected vascular bundles of cell wall loosening protein epitopes, but they were not as intense (Figure 6). We did not observe AZ-specific labeling of any epitopes.

The immunolabeling pattern of OR fruit pedicels did not significantly differ from that of MG fruit pedicels (Figure 6); there were high labeling intensities of both XTH and expansin in the vascular bundles (Figure 7). The dramatic increase in expansin, specifically seen at the AZ, of the abscising flower pedicel was not observed in the loosened AZ of the OR fruit pedicel, nor was any AZ-specific labeling detected by immunostaining.

Immunolocalization of Secondary Cell wall Synthase and Lignin Staining of MG and OR Fruit Pedicels

To determine the function of secondary CESA proteins (CESA4, CESA7, and CESA8) during fruit abscission, we used specific polyclonal antibodies to examine MG and OR fruit pedicel

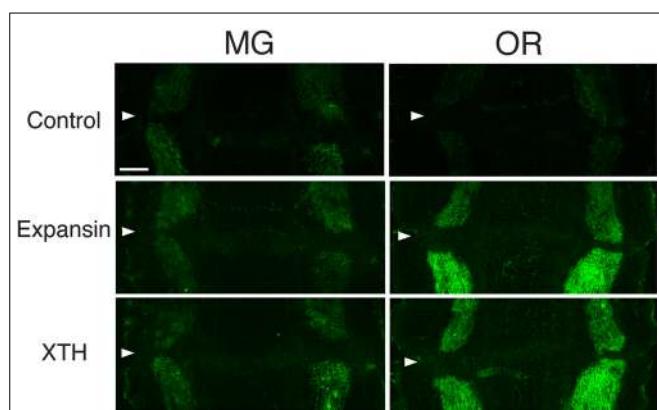


FIGURE 6 | Immunolocalization of expansin and XTH epitopes in the AZs of mature green (MG) and over-ripe (OR) fruit pedicels.

Longitudinal sections of MG fruit pedicels, including the AZ, were stained with the anti-expansin and anti-xyloglucan antibodies. Micrographs show the negative control without the first antibody step. Arrowheads indicate the AZs. Bar = 0.5 mm.

level of cellulose synthase A catalytic subunit 4, 7, and 8 are quite high compared with tomato CESA like genes (87, 91 and 88% identical, respectively), and there is one ortholog for the each CESAs in the tomato genome. Therefore, these commercial polyclonal antibodies of CESA4, 7, and 8 recognize CESA4, 7, and 8 of tomato with high probability. The results of immunostaining indicated no significant increase, decrease, or distributional change in cell wall epitopes in the AZs of loosening OR fruit pedicels. However, phloroglucinol staining was specifically observed at the AZs of the OR fruit pedicels (Figure 7). The lignin staining by phloroglucinol was not seen in the AZs of MG fruit pedicels (Figure 7), suggesting that the lignification was associated with an abscission-specific event.

Discussion

We observed natural abscission at two different stages of fruit development in the tomato pedicel: in unsuccessfully pollinated flower pedicels during the time of fruit set, and at the end of fruit development in pedicels of fully ripened fruits. However, the actual shedding event seemed to occur differently in the two scenarios (Figure 1). In OR fruit, adhesion at the AZ weakened and became vulnerable to physical stress, but was kept intact until harvested by hand, whereas in unpollinated flowers, shedding occurred naturally. We previously reported that during floral abscission, a large increase was observed in LM15 labeling of xyloglucan, specifically at the AZ in the abscised pedicel. During fruit abscission, unlike in floral abscission, no AZ-specific deposition of cell wall polysaccharides was observed. However, high autofluorescence was seen in the AZ of overripe fruit pedicels, suggesting secondary cell wall synthesis and lignification of the AZ prior to fruit abscission (Iwai et al., 2013). Because of the small size and limited amount of AZ samples that can be examined, enzymatic analyses of cell wall modifying activities are quite difficult, therefore we visualized the presence of XTH, expansin, and cellulose synthase using immunohistochemistry.

To analyze the changes occurring within the AZ, cell-wall polysaccharides were visualized by immunolocalization. Previous research has identified a number of cell wall-degrading enzymes, including endoglucanases (Tucker and Milligan, 1991; Tucker et al., 1991; del Campillo and Bennett, 1996; Mishra et al., 2008), polygalacturonases (Kalaitzis et al., 1995, 1997; Gonzalez-Carranza et al., 2002, 2007), and XTHs (Campbell and Braam, 1999) that are involved in the abscission of tomato fruits. The levels of these enzymes increase during the abscission process; therefore, we focus on the cell wall and its remodeling, which may be the target or product of enzyme activity during abscission of the reproductive organ.

At the beginning of the study, we speculated that cell wall loosening through the abscission of the xyloglucan moieties by xyloglucan endohydrolase (XEH) activity might be important. However, during floral abscission, we observed a large 1 DPA peak in the polyclonal antibody labeling of XTH in the AZ, which then decreased (Figure 5). In addition, the intensity of LM15 labeling for xyloglucan epitopes increased dramatically, preferentially at the AZ (Figure 3). These results indicate that gradual xyloglucan accumulation is important for the abscission event. XTHs were

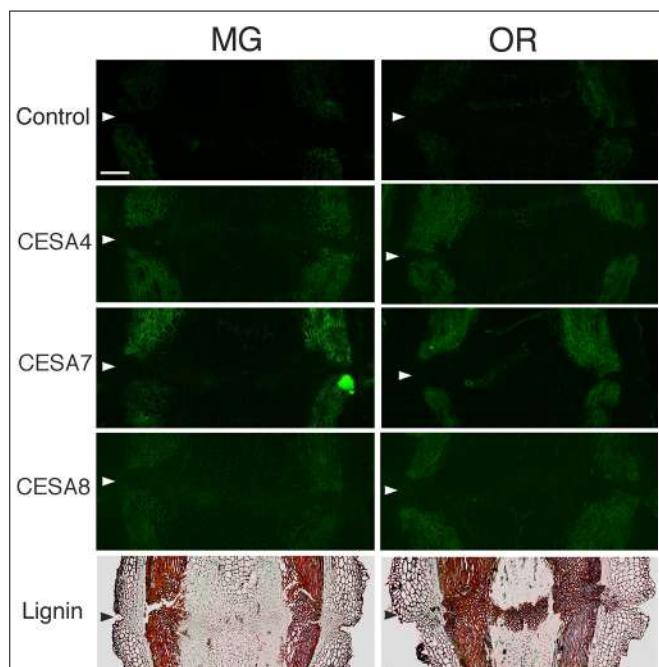


FIGURE 7 | Immunolocalization of CesA4, CesA7 and CesA8 epitopes in the AZs of mature green (MG) and over-ripe (OR) fruit pedicels.

Longitudinal sections of MG fruit pedicels, including the AZ, were stained with the anti-CesA4, 7, and 8 antibodies. Micrographs show the negative control without the first antibody step. Phloroglucinol staining for lignin of AZs in MG and OR fruit pedicels. Arrowheads indicate the AZs. Bar = 0.5 mm.

sections. CESA4, CESA7, and CESA8 are the most major marker enzymes for secondary cell wall synthesis (Carroll et al., 2012). The immunogens of the polyclonal antibodies of CESA4, CESA7, and CESA8 were produced from a recombinant peptide of cellulose synthase A catalytic subunit 4 (Q84JA6), 7 (Q9SWW6) and 8 (Q8LPK5), respectively⁸. Sequence similarity at the amino acid

⁸www.agrisera.com product No. AS12 2582, AS12 2581, and AS12 2580.

not detected in last stage of abscission (yellowing). This result indicates that XTH protein is degraded in the relatively short period. Similarly the rapid turnover of XTH protein was reported during the expansion of tomato fruit (Catalá et al., 2000). LeEXT1 levels were high in young expanding fruit, exhibiting a peak at the fruit expansion stage, but decreased dramatically at the expanded fruit stage. Almost all XTHs studied to date have shown endo-transglucosylase activity (Vissenberg et al., 2000; Baumann et al., 2007). The increase in xyloglucan endotransglucosylase (XET) action indicates active cell wall remodellings and reconstructions of the xyloglucan related cell walls in the AZ during the 1 DPA stage. It is likely that changes mediated by XET action may allow easier accessibility of the cell wall to other cell wall hydrolytic enzymes, thus accelerating abscission. Conversely, abscission is associated with an increase in cell size in *Arabidopsis* and citrus (Bleecker and Patterson, 1997; Agustí et al., 2009). XET action could be required for rearrangement of the cross-linking cell wall hemicelluloses during cell function enlargement. This could lead to a decrease in cell wall mechanical strength, and hence abscission.

Expansin associated with the process of wall extension during cell growth (Li et al., 2003). It has, however, become clear that expansins also make a significant contribution to the process of fruit softening, which involves wall breakdown, rather than expansion. It has been observed that fruit softening and abscission share a number of features in common (Rose et al., 2003). *Arabidopsis* expansin, AtExp10, resulted in the expression of β -glucuronidase specifically at the base of leaf petioles and siliques where they join the inflorescence stem. The authors proposed that this indicated a role of AtExp10 in abscission (Cho and Cosgrove, 2000). Expansins have functions that may increase disorder in cellulose crystals, making glucan chains more susceptible to hydrolysis (Cosgrove, 1999). In our data, AZ localize expansin epitopes increased dramatically and the highest levels of expansin were detected during the yellowing stage just prior to abscission (Figure 5). These results suggest that expansins may increase disorder in cellulose crystals, making the glucan chains more susceptible to hydrolysis. This suggests that expansins play a role in abscission by promoting the degradation of the linkage between cellulose and other components in the cell wall.

A stable level of xyloglucan may be important during rapid cell expansion, which is accompanied by rapid cell wall synthesis, and suggests functions related to XTH and expansin. Cell wall remodeling through rearrangement of the wall xyloglucans by the XET action of one to several XTHs might be essential determinants in the process of abscission. And expansin increase may be a trigger function of floral abscission.

In the case of leaflet abscission in *Sambucus nigra*, ethylene-promoted expansin was important for undergoing cell separation

(Belfield et al., 2005). Likewise ethylene-responsive XTH was also important for petal abscission in rose (Singh et al., 2011). Therefore, ethylene might be important for floral abscission in tomato.

The immunostaining results from both MG and OR fruit pedicel AZs were similar, showing a relatively high intensity of XTH and expansin labeling of HG at the epidermis in and around the vascular bundles. During fruit development and formation, the pedicel itself, as well as the vascular bundles, thickens and its diameter increases, as can be seen by comparing flower and fruit pedicel micrographs (Figures 2–5). The strong XTH and expansin labeling in the MG fruit pedicels may be due to changes in the cell walls; possibly a new cell-wall network is created by bonding xyloglucan and cellulose. The labeling intensity of cell wall modifying proteins may reflect newly created cellulose-pectin binding that occurs via xyloglucan-based side chains that may lead to the development of stronger vascular tissue for delivering large amounts of water to the developing fruit.

Although strong labeling was observed in the vascular bundles and epidermis, no AZ-specific XTH and expansin epitope labeling was observed in either the MG or OR fruit pedicel AZs (Figure 6 and 7). The dramatic deposition of expansin in the flower AZ prior to abscission was not apparent in the OR fruit AZ (Figure 6 and 7). In our previous report, no AZ-specific LM6 and LM15 epitope labeling was observed in the MG or OR fruit pedicel AZs. It showed that the intensity of arabinan and xyloglucan labeling was low in both the MG and OR pedicels (Iwai et al., 2013). However, AZ-specific lignification was observed in the AZ of the OR fruit pedicel. These results suggest that lignification was due to secondary cell wall components, including lignin and other phenolics (Figure 7). However, AZ-specific secondary CESA proteins (CESA4, CESA7, and CESA8) were not detected during fruit abscission. These results suggest AZ-specific lignification might not be associated with normal secondary cell wall synthesis. Floral abscission following unsuccessful pollination is fundamentally different from that following fruit ripening. Floral abscission occurs by a remodeling involving the deposition of cell wall polysaccharides, whereas fruit abscission occurs through lignin deposition.

The results of our study suggest that floral abscission, determined by the successfulness of pollination, and fruit abscission that occurs post-ripening, are regulated by different mechanisms—floral abscission through the remodeling of cell wall polysaccharides, and fruit shedding through lignification.

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Ethephon induced abscission in mango: physiological fruitlet responses

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Fruitlet abscission of mango is typically very severe, causing considerable production losses worldwide. Consequently, a detailed physiological and molecular characterization of fruitlet abscission in mango is required to describe the onset and time-dependent course of this process. To identify the underlying key mechanisms of abscission, ethephon, an ethylene releasing substance, was applied at two concentrations (600 and 7200 ppm) during the midseason drop stage of mango. The abscission process is triggered by ethylene diffusing to the abscission zone where it binds to specific receptors and thereby activating several key physiological responses at the cellular level. The treatments reduced significantly the capacity of polar auxin transport through the pedicel at 1 day after treatment and thereafter when compared to untreated pedicels. The transcript levels of the ethylene receptor genes *MiETR1* and *MiERS1* were significantly upregulated in the pedicel and pericarp at 1, 2, and 3 days after the ethephon application with 7200 ppm, except for *MiETR1* in the pedicel, when compared to untreated fruitlet. In contrast, ethephon applications with 600 ppm did not affect expression levels of *MiETR1* in the pedicel and of *MiERS1* in the pericarp; however, *MiETR1* in the pericarp at day 2 and *MiERS1* in the pedicel at days 2 and 3 were significantly upregulated over the controls. Moreover, two novel short versions of the *MiERS1* were identified and detected more often in the pedicel of treated than untreated fruitlets at all sampling times. Sucrose concentration in the fruitlet pericarp was significantly reduced to the control at 2 days after both ethephon treatments. In conclusion, it is postulated that the ethephon-induced abscission process commences with a reduction of the polar auxin transport capacity in the pedicel, followed by an upregulation of ethylene receptors and finally a decrease of the sucrose concentration in the fruitlets.

Keywords: ethylene receptors, fruitlet abscission zone, fruitlet pedicel, fruitlet pericarp, gene expression, polar auxin transport capacity, soluble carbohydrates

Abbreviations: ACT, β -ACTIN; AZ, abscission zone; At, *Arabidopsis thaliana*; cDNA, complementary desoxyribonucleic acid; DAT, days after treatment; ERS1, ETHYLENE RESPONSE SENSOR 1; ET1, ETHYLENE RESISTANT 1; ET, ethephon treatment; ET600, ethephon treatment 600 ppm; ET7200, ethephon treatment 7200 ppm; FDF, fruitlet detachment force; IAA, indole-3-acetic acid; Mi, *Mangifera indica*; MES, 2-(N-morpholino) ethanesulfonic acid; PAT, polar auxin transport; qPCR, quantitative real-time PCR; PCR, polymerase chain reaction; rcf, relative centrifugal force; RNA, ribonucleic acid; RTE1, REVERSION-TO-ETHYLENE SENSITIVITY1; TUB, α -TUBULIN; UBI, UBIQUITIN.

Introduction

Plant organ shedding or abscission is a highly coordinated process governed by the interplay of several plant metabolites, in particular phytohormones, carbohydrates, and polyamines (Sexton and Roberts, 1982; Malik and Singh, 2003; Xie et al., 2013). Abscission can be initiated in response to disease pressure, pest injury, or climate extremes, leading to interorgan competition for assimilates (Patterson and Bleeker, 2004; Botton et al., 2011). Understanding the regulation of genes encoding for proteins involved in synthesis, perception, and transport of these abscission relevant metabolites is of paramount importance for increasing the productivity of horticultural crops. This fundamental knowledge can be specifically utilized for devising practical solutions, ranging from marker-assisted genotype selection to crop management strategies using for example effective and growth stage dependent irrigation strategies and applications of plant growth regulators (Estornell et al., 2013).

Fruit drop is a yield-limiting factor for the production of several specialty crops, for example sweet cherry (Blanusa et al., 2005), litchi (Kuang et al., 2012), or mango (Singh et al., 2005). Of particular concern in many mango production systems worldwide is the extensive fruitlet drop. This major production constraint has been extensively studied at the orchard level (Singh et al., 2005) and was also a key research objective by Hagemann et al. (2014) who investigated the potential use of plant growth regulators, irrigation techniques, and cropping systems for improving fruit retention in mango. Both, biotic and abiotic factors have been frequently suggested as the key triggers for inducing fruitlet drop in mango (Singh et al., 2005). Biotic factors are mainly the lack of pollination or fertilization of flowers and pest or disease pressure that subsequently lead to seed degeneration (Singh and Arora, 1965). Abiotic factors associated with fruitlet drop are extensive drought periods, extreme ambient air temperatures or dry and strong winds (Burondkar et al., 2000; Singh et al., 2005; Hagemann et al., 2014, 2015). In plants these factors generally reduce the auxin efflux from as well as the carbohydrate influx to the fruitlet, thus the demand of the growing fruitlet is not sufficiently matched by its supply (Wünsche and Ferguson, 2005; Estornell et al., 2013). This was shown for example in litchi, where branch girdling and defoliation, clearly limiting the carbohydrate supply to the fruitlets, resulted in a decrease of fruitlet auxin concentration which in turn led to abscission (Kuang et al., 2012). This result supports the theory for mango that a reduced basipetal transport of seed-derived auxin through the pedicel (Chacko et al., 1970; Prakash and Ram, 1984; Roemer et al., 2011) and the subsequently increased sensitivity for ethylene in the pedicel abscission zone (AZ) induces fruitlet abscission (Estornell et al., 2013).

Ethylene is perceived by binding to two sub-families of specific ethylene receptors, which control a downstream signal cascade (see reviews of Binder, 2008; Stepanova and Alonso, 2009). Five ethylene receptors have been identified in the model plant *Arabidopsis* (*Arabidopsis thaliana* (L.) HEYNH.; Binder, 2008) and homologous genes were subsequently described for several crop plants, e.g., six receptors in tomato (Alexander and

Grierson, 2002), nine in apple (Ireland et al., 2012), and at present two in mango (Martínez et al., 2001; Ish-Shalom et al., 2011). Based on assessing the triple-response to varying degrees of ethylene perception of *Arabidopsis* mutants, it was found that a malfunction of one or more receptors can mostly be compensated by the other receptors, however, double mutants of the receptors *ETHYLENE RESISTANT 1* (*AtETR1*) and *ETHYLENE RESPONSE SENSOR 1* (*AtERS1*) exhibit the most severe deficiencies (Binder, 2008). The plant response to ethylene is regulated by receptor specific elements, as for example the *REVERSION-TO-ETHYLENE SENSITIVITY 1* (*AtRTE1*) that exclusively modulates the function of the *AtETR1* (Shakeel et al., 2013) or by receptor-receptor interaction through building homo- and heterodimers or clusters of higher complexity (Gao et al., 2008). These experiments on receptor functionality led to the development of a hierarchical model resulting in *AtETR1* and *AtERS1* being the predominant receptors. Specifically, Patterson and Bleeker (2004) showed in ethylene-insensitive *etr1-1* *Arabidopsis* mutants that *ETR1* delays abscission by reducing the enlargement of the proximal cells within the separation layer. In this context, it is important to note, that O’Malley et al. (2005) showed a positive and linear correlation between ¹⁴C-ethylene binding activity and the transcript level of ethylene receptors in *Arabidopsis* and suggested a similar correlative relationship between the transcript and protein level of ethylene receptors. Given the numerous regulatory mechanisms of the ethylene response, it is remarkable that fruitlet and mature fruit abscission seem always associated with a strong upregulation of *ERS1* but not of *ETR1* in pedicels of mango (Ish-Shalom et al., 2011), orange (John-Karuppiah and Burns, 2010), peach (Rasori et al., 2002), and apple (Dal Cin et al., 2008).

Ethephon (2-Chloroethylphosphonic acid) is an ethylene releasing chemical and commonly used to induce thinning of fruitlets or to facilitate the fruit harvesting process (Dennis, 2000; John-Karuppiah and Burns, 2010; Ish-Shalom et al., 2011). In the presence of ethylene, the cells within the fruit pedicel AZ produce cell wall degrading enzymes, thereby inducing the disintegration of the separation layer in the AZ and ultimately leading to the detachment of the fruit (Leslie et al., 2007). Ethephon has previously been used to study the regulation of the mango ethylene receptors *MiERS1* and *MiETR1* during the fruitlet abscission process in laboratory-based experiments (Ish-Shalom et al., 2011). Consequently, the aim of the present study was to investigate the physiological and molecular mechanisms of ethephon-induced fruitlet abscission in mango under field conditions. In particular, emphasis was given on analyzing carbohydrate concentration, polar auxin transport (PAT) capacity and the transcription of ethylene receptors of individual fruitlets and pedicels before and after ethephon spray applications. Moreover, new ethylene receptor versions were identified and their expression patterns interpreted.

Materials and Methods

Plant Material and Experimental Site

Experiments were conducted over two consecutive fruit growth cycles in 2011 and 2012 in the Tú Nang commune (20°37'0 N,

106°4'60 E) near the township Yên Châú, Province Sơn La, North Vietnam. The mango (*Mangifera indica* L.) trees of the local cultivar "Hôî" were between 10 and 15 years of age. For details on orchard management and phenology see Hagemann et al. (2014).

Treatments and Experimental Design

To investigate the physiological and molecular mechanism of fruitlet (pea size; ~4 weeks after full bloom) abscission in mango, fruitlet drop was induced by ethephon spray applications during the critical midseason drop stage. Consequently, there was a greater probability that all fruitlets investigated at each sampling time were at a similar abscission stage. In 2011 and 2012, 12 trees were randomly selected for each of the following treatments: water control and two ethephon (Flordimex 420, Spiess Urania, Germany) treatments at a concentration of 7200 ppm (ET7200) and 600 ppm (ET600). The latter treatment was applied in 2012 to compare the results to those of Ish-Shalom et al. (2011). All treatments were sprayed to run-off with 5 ppm surfactant (Ethalfix® Pro, Syngenta, Switzerland) using a low-pressure handhold sprayer (Gloria, Typ 133, Witten, Germany). For each experimental tree, healthy appearing panicles were randomly tagged at 1 week after full bloom ($\geq 90\%$ of all panicles are at least to 80% flowering). For each treatment, six trees with 10 panicles each were used for assessing fruit drop, whereas six trees with 40 panicles each were used for taking fruit samples.

Fruitlet Drop Assessment and Sampling

Fruit retention was recorded every 2 days for the first 4 counting dates and weekly thereafter and expressed as the average fruit number of all initially tagged panicles. Sampling for gene expression and carbohydrate analysis commenced about 2 days (2 ± 1) prior to treatment and continued 1, 2 (only in 2012), and 3 days after treatment (DAT). At each sampling day, 12 fruitlets (averagely two fruitlets from one panicle per tree) were collected for each treatment at noontime. Fruitlet detachment force (FDF) was determined with a gauge (PCE-FM50, Germany) by fixing an individual fruitlet in a customized bracket, holding the entire panicle in position while concomitantly pulling the fruitlet until detachment. In addition, the location of the detachment at the AZ or along the pedicel was recorded and the diameter, length, and weight of each sampled fruitlet as well as the pedicel diameter at mid-position were measured. Each fruitlet was then cut in half and the seed was scored either healthy or degenerated when symptoms of degradation, discoloration, or shrivel were noticed. From each fruitlet, the following parts were sampled for analysis (Figure 1): (1) a 4 mm long pedicel fragment, including the AZ, for gene expression analysis, and fruitlet pericarp for (2) gene expression and (3) carbohydrate analyses. All samples were immediately snap frozen in liquid nitrogen and stored until further processing at -80°C for gene expression analysis and at -30°C for carbohydrate analysis.

Sampling for polar auxin transport (PAT) assay were taken at 2 days (2 ± 1) prior to treatment and at 2 DAT in 2011, whereas at 1 and 3 DAT in 2012. At each sampling day, six panicles (one panicle per tree) were collected per treatment at noontime. The cut end of each panicle was placed in a falcon tube filled with water and transported in sealed styrofoam boxes

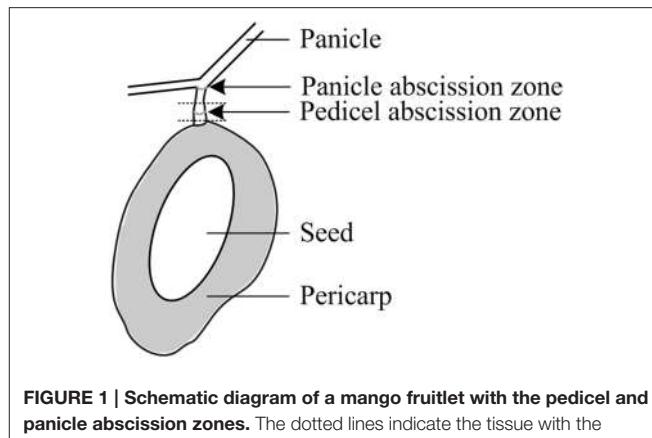


FIGURE 1 | Schematic diagram of a mango fruitlet with the pedicel and panicle abscission zones. The dotted lines indicate the tissue with the pedicel abscission zone used for sampling and various analyses.

to the laboratory within 2 h of sampling. Two fruitlets per panicle served for taking records of diameter, length, and weight as well as pedicel diameter at mid-position. The AZ was sampled by cutting 4 mm to either side of the AZ with two parallel mounted razor blades and processed as described in Section Polar Auxin Transport Assay.

Gene Analysis

RNA Extraction and cDNA Synthesis

Frozen fruitlet pedicels and pericarp were ground in liquid nitrogen to fine powder. Total ribonucleic acid (RNA) was extracted from 100 mg subsamples with the MasterPure Plant RNA Purification Kit (Epicentre, USA), following the manufacturer's recommendations. In addition, to reduce the phenolic compounds from the fruitlet pericarp, polyvinylpolypyrrolidone was added in a first step of the extraction process. Genomic desoxyribonucleic acid (DNA) was eliminated with DNaseI and this was subsequently tested by polymerase chain reaction (PCR). RNA samples were stored at -80°C until complementary DNA (cDNA) was synthesized using the TaqMan Reverse Transcription Kit (Applied Biosystems, USA) and following the protocol of the manufacturer. For cDNA synthesis 500 ng of total RNA was used for each reaction. cDNA quality was tested by quantitative real-time PCR (qPCR), using a Rotor-Gene 6000 cycler (Corbett, Australia) with the following conditions: initial denaturation (3 min; 95°C); 40 cycles of denaturation (20 s, 95°C), annealing (20 s, 58°C), and extension (20 s, 72°C); followed by a melt curve from 60°C to 99°C in 0.5 K steps.

Gene Identification

Specific primers for *MiETR1* were designed (Genbank ID: AF227742.1; Table 1). Conserved regions of *ERS*-like sequences from woody plants and *Arabidopsis* were identified by alignments to design degenerate primers. Nested PCRs were performed to verify sequence specificity before cloning. The PCR products were then ligated into the pGEM-T vector (Promega, VIC, Australia) following the manufacturer's recommendations. After blue-white selection, a colony PCR with gene specific primers (Table 1) was performed to verify positive clones for

TABLE 1 | Primers specific for mango genes used for quantitative real-time PCR analysis.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
<i>MiACT</i>	CCCTGAAGAGCACCCA	AGTTGTACGACCACCTGGC	156
<i>MiUBI</i>	AAGATCCAGGACAAGGAGG	GGACCAGGTGGAGCG	125
<i>MiTUB</i>	ATCAACTACCAGGCCACC	CCTTCCTCCATAACCTCAC	184
<i>MiETR1</i>	CCAAGGAGAATTGCATGAG	GGCAGCTTGCTCCTC	141
<i>MiERS1</i>	TGGCGACAAGAACGACTG	GCCAGTCTTGAAAGACTC	116
<i>MiERS1m</i>	GCGCTGTAATGAACCATGA	TCTTGGTATCGTGTGTC	151
<i>MiERS1s</i>	TCTAGTGTATGCTAACTGC	GTGCTACCTTGTCAGC	115

β -ACTIN (*MiACT*), UBIQUITIN (*MiUBI*), α -TUBULIN (*MiTUB*), ETHYLENE RESISTANT 1 (*MiETR1*), ETHYLENE RESPONSE SENSOR 1 (*MiERS1*), and the two *MiERS1* versions *MiERS1m* and *MiERS1s*. (*Mangifera indica* abbreviated as *Mi*).

subsequent plasmid extraction (QIAprep Miniprep, Qiagen, Germany) and sequencing (GATC, Germany). Using degenerate primers to identify the homolog to the *Arabidopsis AtERS1*, three different versions of mango *ERS1* were detected and confirmed by sequencing: a version with the full length sequence (*MiERS1*) that is comparable to the *AtERS1*, a medium sized *MiERS1m* with a length of 1203 nucleotides, and a short *MiERS1s* with a length of 561 nucleotides. The sequences were confirmed to be *MiERS1*-like by BLAST search using the NCBI online tool (<http://blast.ncbi.nlm.nih.gov>) and following the recommendations of Samach (2012).

Gene Expression Studies

The transcription levels of *MiETR1* and the three versions of *MiERS1* were analyzed by qPCR. The efficiency of each primer pair was determined with DART tool (Peirson et al., 2003). Primer specificity was confirmed by melt curve analyses for each individual run and by sequencing of the resulting amplicons. Relative expression of the target genes was analyzed with the efficiency corrected $\Delta\Delta Ct$ -method using the DART tool (Peirson et al., 2003). A pool-sample, composed of 1 μ l cDNA, was used in each run as a reference for the relative gene expression and as a standard for the different runs. Three potential reference genes, β -ACTIN (*MiACT*), α -TUBULIN (*MiTUB*), and UBIQUITIN (*MiUBI*), were evaluated for their expression stability in the pericarp and pedicel from control and ET7200. *MiACT* was selected as reference gene because it revealed the highest expression stability based on the analysis with the BestKeeper tool (Pfaffl et al., 2004).

Analysis of Soluble Carbohydrates

The concentration of fruit soluble carbohydrates was analyzed for all fruitlets that were used for gene expression studies in 2012. Individual fruitlets were ground to a homogenous powder under liquid nitrogen with an impact ball mill (CryoMill, Retsch, Germany). A subsample of 50 mg was taken and re-suspended in 950 μ l bi-distilled water, diluted 1:4 and vortexed thoroughly for 1 min. The debris was removed by centrifugation (5 min, 18.000 rcf, 20°C) and 750 μ l were collected from the supernatant. Because of the high content of organic acids in the sample, which are disturbance variables in the analytical process, acids were removed from the sample fraction with a strong anion exchange column (Strata-X-A 33u, Phenomenex,

CA, USA). Therefore, the columns were pre-conditioned with 8 ml of 0.1 M sodium hydroxide followed by 2 ml of water. The sample was then transferred to the column, eluted with 3 ml water and concentrated to a dry pellet with a rotary evaporator set-up (RC1022, RVT4104, VLP120; Thermo Fisher Scientific Inc., MA, USA). The pellet was re-suspended in 600 μ l of water, filtered through a nylon filter with a pore size of 0.45 μ m (Wicom, Germany) and injected into the high performance liquid chromatography (HPLC) sampler (Bischoff, Germany). The HPLC setup consisted of a guard column, Hamilton PRP-X400, and a main column, Hamilton HC-75 Ca²⁺ (Hamilton, NV, USA), connected to a refractometric detector (Model 8120; Bischoff, Germany). The carbohydrate separation was done isocratically with bi-distilled water as mobile phase facilitated by two HPLC-pumps (HPLC-Compact-Pump 2250, Bischoff, Germany). The analysis conditions were 80°C at a flow rate of 1.2 ml min⁻¹. The amounts of glucose, fructose, and sucrose were quantified using respective standards (Sigma-Aldrich, MO, USA).

Polar Auxin Transport Assay

To assess the basipetal (polar) auxin transport, the basal end of the fruitlet pedicel was placed onto 96 well-microplates (Greiner bio-one, Germany). Each well-contained 300 μ l solidified buffer with 0.05 M 2-(N-morpholino) ethanesulfonic acid (MES), adjusted to pH 5.2, and 1.2% Agar-Agar. A donor block with a volume of 50 μ l, shaped as concave disc and consisting of MES buffered 1.5% Agar-Agar, was immediately placed onto the apical side of the pedicel. The acropetal auxin transport was also determined by using 12 additional pedicels in reverse orientation in order to measure the non-polar auxin transport. A droplet of 10 μ l [³H]-IAA (indole-3-acetic acid labeled with tritium at the 5' carbon atom of the indol ring with a specific activity of 962 GBq mmol⁻¹; Amersham plc, UK) was applied into the cavity of the donor block. Each plate was placed in a dark box with 100% relative humidity and incubated for 8 h at 25°C. After the incubation, the donor block, the pedicel, and the agar of the receiving well (receiver block) were placed into different plastic scintillator vials and stored at -20°C until extraction. For extraction 2 ml of scintillation liquid (Quickzint 212, Zinsser Analytic, Germany) was added to each vial and the samples were incubated at room temperature for 10 days on a rotary shaker at 200 rpm. Thereafter, the [³H]-IAA activity as disintegration per

minute (dpm) was measured with a liquid scintillation counter (Tri-Carb 3110 TR, PerkinElmer, USA) for 5 min.

Statistical Analysis

The effects of the ethephon treatments on the expression level of ethylene receptors and the concentration of soluble fruit carbohydrates were evaluated by pairwise comparison of the means at a probability level of $p \leq 0.05$ and the Fisher's least significant difference (LSD) (SAS 9.3; SAS Institute Inc., NC, USA). Model assumptions (normality and variance homogeneity) for the analysis of variance (ANOVA) were checked by examining the residual plots. For analysis of the ethylene receptor expressions, a transformation with the common logarithm was used to stabilize the variance at high expression levels (Rocke and Durbin, 2001), however, the untransformed means are presented in the figures. The results of the PAT experiment and of the FDF measurements did not meet the assumption of variance homogeneity, thus an ANOVA based on ranks (Dunn's post-test) was used to identify differences between treatment groups. In all models various covariates were tested for significant influences on treatment effects.

Results

Eethephon Induced Fruitlet Abscission

Eethephon was used to induce abscission of fruitlets at pea size stage in midseason, allowing the analysis of specific molecular and physiological parameters throughout the process of abscission. Both ethephon concentrations induced an immediate and a much stronger fruitlet abscission than the control treatment (Figure 2A). However, 95% of all fruitlets abscised within 8 days after ET7200 application, whereas it required 6 additional days for ET600 treated fruitlets to reach this level. It is important to note that while ET7200 defruited completely all panicles within 1 month, the ET600 resulted in 2% fruitlets per panicle (Figure 2A). The FDF was significantly reduced by approximately 85% in the ET7200 at 1 DAT and in the ET600 at 2 DAT, respectively, when compared to the control (Figure 2B, Supplementary Figure 1). The FDF in the ET7200 remained extremely low at 2 DAT and was zero at 3 DAT, whereas in the ET600 at 3 DAT it was similar to that of controls (Figure 2B). While all ET7200 treated fruitlets detached at the AZ, a close to 100% abscission at the AZ occurred only at 2 DAT for ET600 fruitlets (Figure 2C). This corresponds in all cases with extremely low FDF values (Figures 2B,C). However, the ET600 application detached only about 50% fruitlet at the AZ at 1 and 3 DAT, which corresponds with relatively high FDF values due to higher detachment forces needed to pull-off the remaining 50% fruitlet somewhere along the pedicel. In contrast, approximately 30% of the controls detached at the AZ, thereby about 70% broke at different locations of the pedicel (Figure 2C). These results are in good agreement with the findings in the previous year (2011), specifically, an ET7200 induced continuous decrease of FDF to zero concomitantly with an increase in fruitlet detachment at the AZ to 100% at 3 DAT (Supplementary Figure 1). Overall, about one third of all fruitlets evaluated showed visible symptoms of

seed degeneration; however, this did not seem to be related to the ethephon treatments or FDF (Figure 2D).

Expression of Ethylene Receptors in the Pedicel

Both ethephon treatments led to a specific receptor transcription pattern in the pedicel, with little response of *MiETR1* and a strong upregulation of *MiERS1* (Figures 3A,B, Supplementary Figure 2). *MiETR1* was not significantly regulated by ET7200, except at 1 DAT in 2012 (Figure 3A, Supplementary Figure 2). In contrast, the expression of *MiERS1* shows a strong response to both ethephon concentrations. ET7200 led to a six and three times higher expression level at 1 DAT in 2011 and 2012, respectively, compared to the control (Figure 3B, Supplementary Figure 2B). The ET7200 induced *MiERS1* upregulation remained higher than the control at the following sampling days, although this was not significant at 3 DAT in 2011 (Figure 3B, Supplementary Figure 2B). The ET600 led to an increasingly stronger *MiERS1* transcription, with a significant *MiERS1* upregulation at 2 and 3 DAT but not at 1 DAT unlike in the case of the ET7200 (Figure 3B).

Three homologs of the *Arabidopsis* ethylene receptor *AtERS1* have been identified. According to a BLAST analysis all three *MiERS1* versions are highly similar (identity values of 98–99%) to the two full length *MiERS1* GenBank accessions (JN851132.1, JF323582.1). These two accessions derived from the cultivar "Kent," thus the 1–2% sequence differences are likely a result of a few nucleotide polymorphisms between the cultivars "Höi" and "Kent." The "Höi" *MiERS1* full length has a coding sequence of 1890 nucleotides while the other versions, *MiERS1m* and *MiERS1s* are shorter with 1203 nucleotides and 561 nucleotides, respectively. In contrast to the *MiERS1*, which was detected in all samples (100%), transcripts of *MiERS1m* and *MiERS1s* could only be detected in a much reduced number of samples, although *MiERS1m* was more frequently detected than *MiERS1s* (Figures 3C,D). Nevertheless, transcripts of both shorter receptor versions were detected more often in pedicels of treated fruitlets than in controls (Figures 3C,D). The regulation of *MiERS1m* and *MiERS1s* in the pedicel appears to be erratic, therefore a statistical analysis was not possible (Supplementary Figure 3).

Expression of Ethylene Receptors in the Fruitlet Pericarp

The two receptors *MiETR1* and *MiERS1* were expressed in the fruitlet pericarp with a similar timely pattern in both experimental years (Figures 3E,F, Supplementary Figure 4). Both receptors were significantly upregulated at all DAT following the ET7200 application compared to the control. In contrast, the ET600 led to a significant upregulation of the *MiETR1* only at 2 DAT (Figure 3E, Supplementary Figure 4A), while the transcription level of *MiERS1* was similar to that of the controls at all sampling dates (Figure 3F, Supplementary Figure 4B). Both short versions of *MiERS1* were rarely detected in the fruitlet pericarp (data not shown) and consequently analysis of these receptor versions was not further pursued.

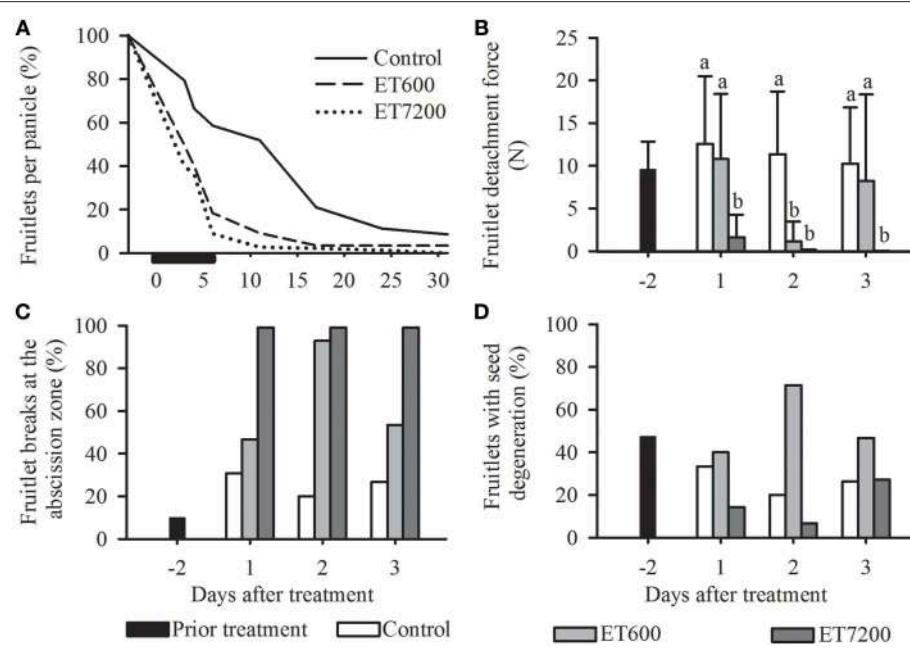


FIGURE 2 | The effect of the ethephon treatment 600 ppm (ET600) or 7200 ppm (ET7200) spray applications on average (A) fruitlet retention, (B) fruitlet detachment force of fruitlets detaching at the abscission zone or along the pedicel, (C) percentage of fruitlet detachment at the abscission zone (the remainder to 100% are fruitlets detaching along the pedicel) and (D) seed degeneration in comparison to the control at 1, 2, and 3 days after treatment. (A) Horizontal black bar indicates time until 95% of the fruits have abscised in response to ET7200. (B) Homogeneous subgroups with no significant difference ($p \leq 0.05$) are indicated by same letters. Error bars show standard deviation.

Polar Auxin Transport Capacity

The non-polar, acropetal transport capacity of 40 ± 20 dpm was always significantly lower than the PAT capacity of pedicels from control fruitlets (Figure 4). Both ethephon concentrations effectively decreased the PAT capacity of the pedicel at each sampling time (Figure 4, Supplementary Figure 5); however, ET7200 reduced the PAT capacity to a greater extent than the ET600.

Analysis of Soluble Carbohydrates

Among all the analyzed carbohydrates, a clear response to both ethephon treatments was only found for sucrose, indicated by significantly lower concentrations in treated fruitlets than those in controls at 2 DAT (Figure 5, Supplementary Figure 6). While the sucrose concentration in ET7200 treated fruitlets remained low at 3 DAT, it was not different between ET600 treated fruitlets and controls. Ethephon did not affect the concentration of fructose in the fruitlets (Supplementary Figure 6A). Fruitlet concentration of glucose was significantly increased only at 3 DAT by ET600 compared to the control, whereas was not affected by ET7200 (Supplementary Figure 6B).

Discussion

The current study supports earlier findings (Malik et al., 2003) that ethephon induced fruitlet abscission in mango is a concentration dependent response: the ET7200 led to

a complete loss of fruitlets while approximately 2% of fruitlets were retained in the ET600 at 1 month after spray application (Figure 2A). This clearly indicates that the fruitlet abscission response to ET600 is less pronounced and hence proportionally fewer about-to-abscise fruitlets with a greater FDF value were sampled at 3 DAT when compared to the ET7200.

Irrespective of the treatment applied, low FDF values were symptomatic for fruitlets breaking at the abscission zone (Hagemann et al., 2015, in press) and are indicative of an advanced abscission process. Developmental disorders or nutritional stress during embryogenesis, leading to seed degeneration, was previously suggested as another symptomatic cause of fruitlet abscission (Singh, 1961; Botton et al., 2011). However, despite 30% of the fruitlets containing degenerated seeds, it appeared to be related neither with the point of detachment (data not shown) nor with the ethephon treatments (Figure 2D). Nevertheless, the ET7200 must have induced specific morphological changes at the cellular level within 24 h that led to low FDF values and fruitlets detaching at the AZ, the weakest point along the pedicel (Figures 2B,C). In contrast, this response was only seen 48 h after the ET600. Indeed, microscopy studies of Barnell as early as in 1939 showed for mango that cellular changes within the AZ located between the fruitlet base (flower receptacle) and the pedicel, e.g., meristematic activity and swelling of cell walls, allow a fruitlet to separate with a clean break (Barnell, 1939). Moreover, the action of cell wall degrading

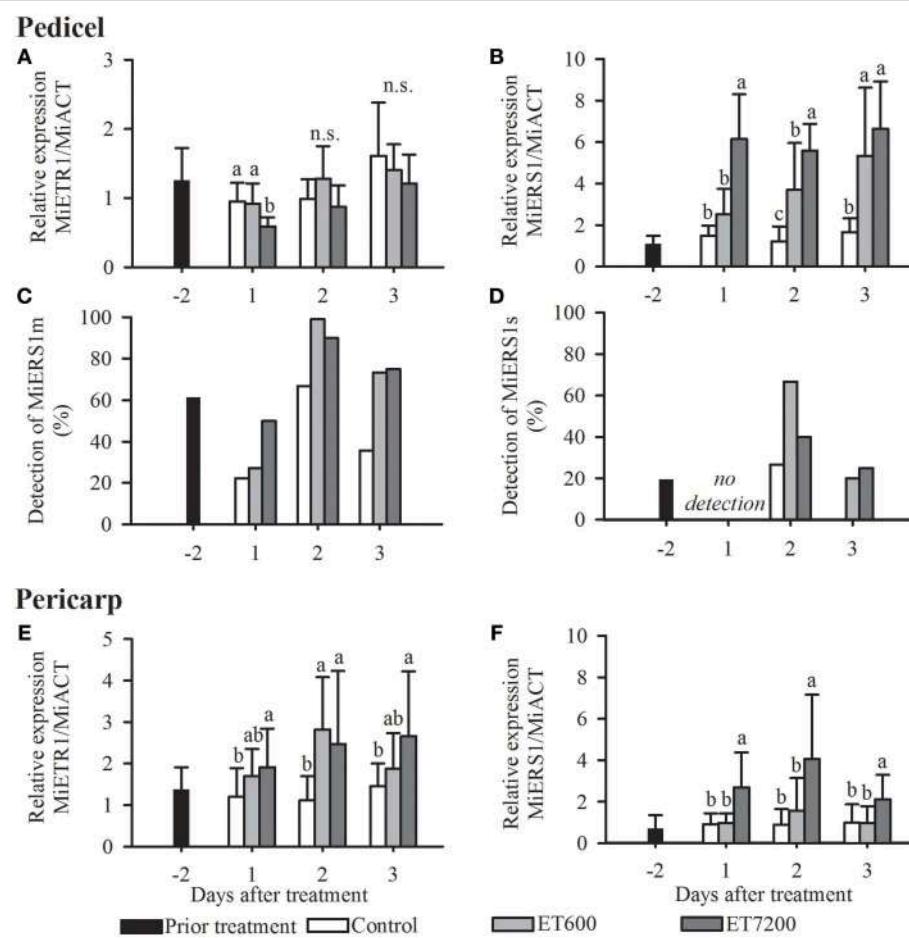


FIGURE 3 | Expression of the ethylene receptors in the pedicel (A–D) and pericarp (E,F) of pea sized mango fruitlets in response to the ethephon treatment 600 ppm (ET600) and 7200 ppm (ET7200) in comparison to the control at 1, 2, and 3 days after treatment. (A) Expression of *MiETR1* and **(B)** *MiERS1* in the pedicel, **(C)** detection of transcription of *MiERS1m* and **(D)** *MiERS1s* in the pedicel, and **(E)** expression of *MiETR1* and **(F)** *MiERS1* in the pericarp. Homogeneous subgroups with no significant difference ($p \leq 0.05$) are indicated by same letters. Error bars show standard deviation.

enzymes and an increase of turgor pressure are necessary for fruitlet detachment and prior to that, specific genes must have been differentially expressed to induce the AZ (Roberts et al., 2002).

Ethylene receptors were examined as the target genes since the ethylene signaling pathway has been linked to the induction of the AZ and fruitlet abscission (Xie et al., 2013). Of the two ethylene receptors so far described for mango, the *MiETR1* has been reported to be upregulated in the pericarp but not in the pedicel of fruitlets induced for abscission (Martínez et al., 2001; Ish-Shalom et al., 2011). In contrast, *MiERS1* has been reported to be upregulated in the pedicel but not in the pericarp of abscission-induced fruitlets (Ish-Shalom et al., 2011). The current results confirm the findings of Ish-Shalom et al. (2011) that ethephon does not upregulate *MiETR1* but *MiERS1* in the pedicel by using the more sensitive qPCR method instead of the Northern blot (Dean et al., 2002). The about five-times higher concentration than the one used by Ish-Shalom et al. (2011), 1400 vs. 7200 ppm,

led to at least 48 h longer upregulation of the *MiERS1* (Figure 3B, Supplementary Figure 2). In general, the *ERS1* responds with an upregulation in the fruitlet pedicels and leaf petioles of different tree crops, including mango, within 24 h of an abscission inducing treatment (Rasori et al., 2002; John-Karuppiah and Burns, 2010; Ish-Shalom et al., 2011). These results corroborate the hypothesis that the role of the *ERS1* in organ abscission is highly conserved in plants. The newly identified short *MiERS1* versions *MiERS1m* and the *MiERS1s* may also be associated with fruitlet abscission because their probability of detection and their expression level were higher in pedicels of ethephon treated and thus abscising fruitlets than in untreated controls (Figures 3C,D, Supplementary Figure 3).

The *MiETR1* upregulation in the pericarp of ethephon treated fruitlets was more pronounced following the ET7200 compared to the ET600 (Figure 3E). The ET600 induced significant upregulation of the *MiETR1* but not of the *MiERS1* in the pericarp, corresponds to the findings of Ish-Shalom et al. (2011).

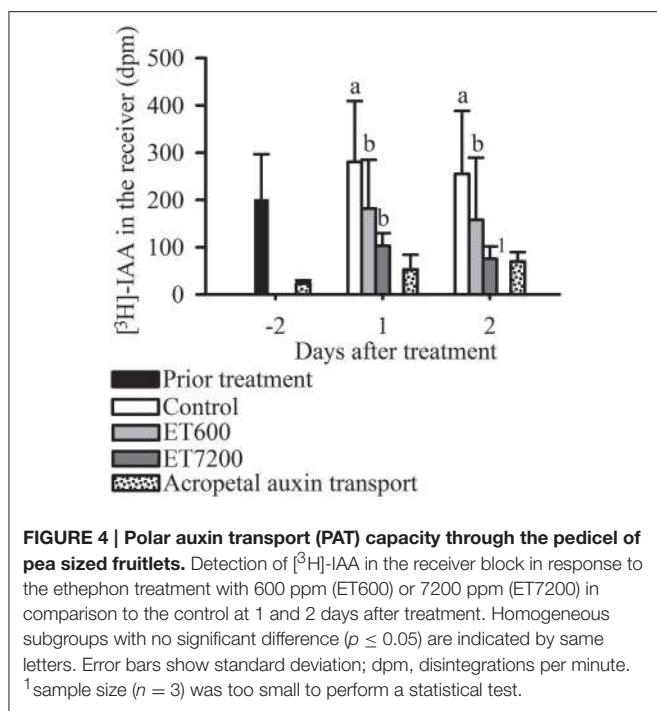


FIGURE 4 | Polar auxin transport (PAT) capacity through the pedicel of pea sized fruitlets. Detection of [^3H]-IAA in the receiver block in response to the ethephon treatment with 600 ppm (ET600) or 7200 ppm (ET7200) in comparison to the control at 1 and 2 days after treatment. Homogeneous subgroups with no significant difference ($p \leq 0.05$) are indicated by same letters. Error bars show standard deviation; dpm, disintegrations per minute. ¹sample size ($n = 3$) was too small to perform a statistical test.

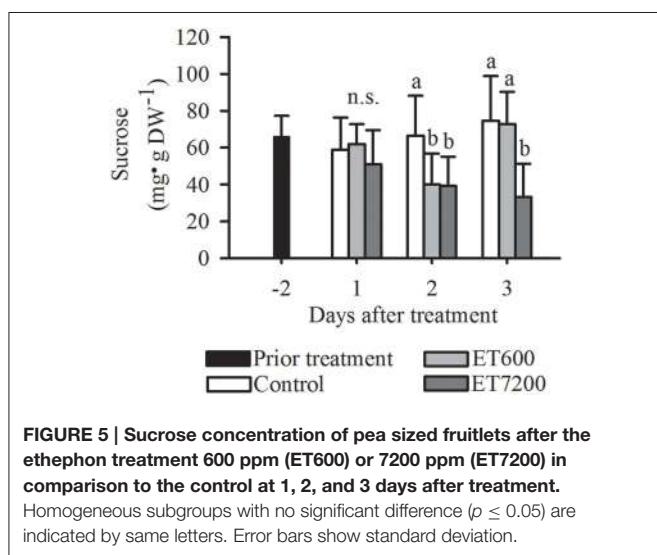


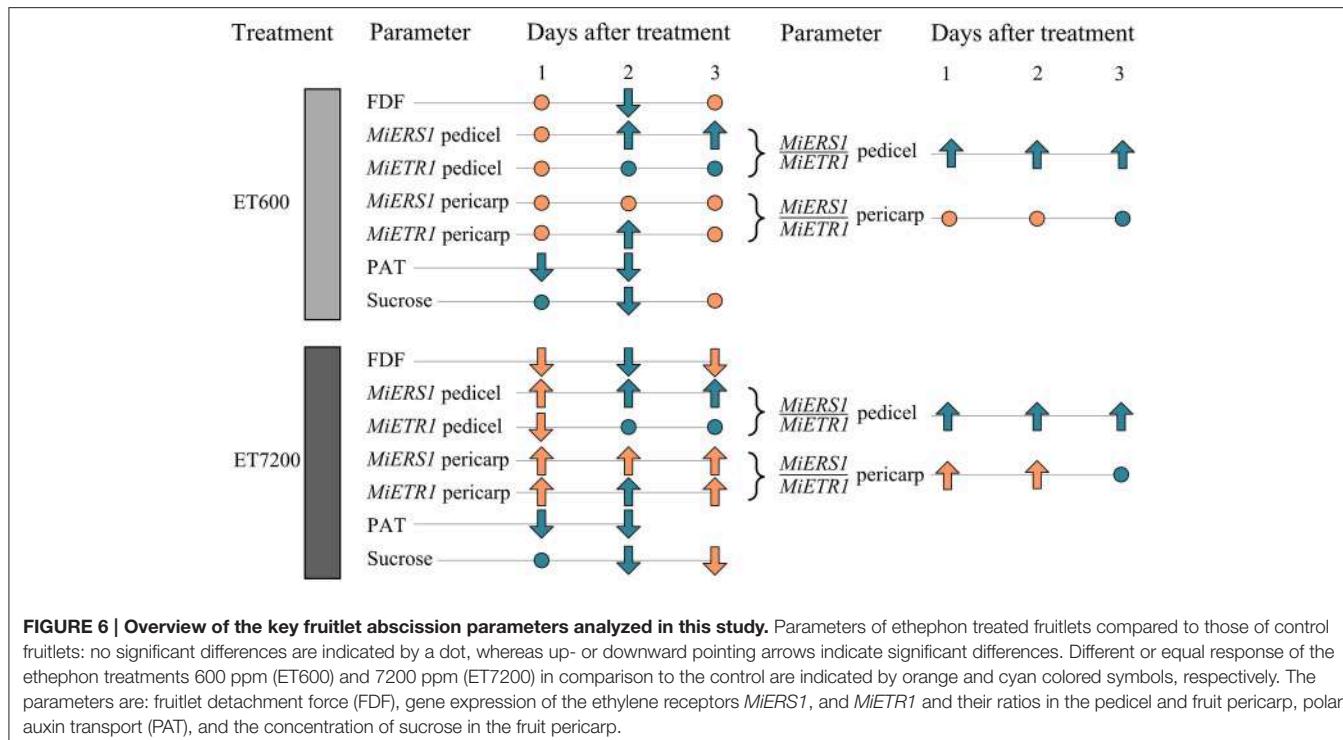
FIGURE 5 | Sucrose concentration of pea sized fruitlets after the ethephon treatment 600 ppm (ET600) or 7200 ppm (ET7200) in comparison to the control at 1, 2, and 3 days after treatment. Homogeneous subgroups with no significant difference ($p \leq 0.05$) are indicated by same letters. Error bars show standard deviation.

It is important to note that Ish-Shalom et al. (2011) applied a higher concentration of ethephon at a lower temperature (1400 ppm at 20°C) compared to the current study (600 ppm at 29°C), hence both studies are comparable due to the temperature-depending effect of ethephon (Yuan and Burns, 2004). In contrast, the ET7200 led to a significant upregulation of both ethylene receptors in the pericarp of fruitlets from 1 DAT onwards (Figures 3E,F). Thus, the expression pattern of both receptors clearly indicates an ethephon (ethylene) concentration dependent response. It is likely that ET7200 induced a greater endogenous autocatalytic ethylene synthesis, which largely contributes to a longer lasting and significantly greater ethylene receptor response. It may also be that the

ethylene sensitivity threshold of the AZ is in part maintained through the ET7200 application despite a 50% ethephon degradation within 1 DAT (Domir and Foy, 1978). In the natural abscission process, it is suggested that fruitlet-derived ethylene is synthesized in the pericarp and diffuses to the AZ (Nunez-Elisea and Davenport, 1986; Malik et al., 2003) where it induces the upregulation of ethylene receptors (Stepanova and Alonso, 2009) prior to the induction of the abscission process. These findings lead to the hypothesis that during natural abscission, ethylene receptors are first upregulated in the fruitlet and then in the pedicel (Hagemann et al., in press). Chemical induction of the abscission by ethephon would result in a simultaneous upregulation of ethylene receptors in fruitlets and pedicels (Figure 3).

Another key element of the abscission process is the auxin signaling (Xie et al., 2013), which was expressed as PAT capacity in the present study. Untreated mango fruitlets transported only 5% of the radioactively labeled auxin through an 8 mm long pedicel within 8 h, while it was 38% through 4 mm sweet cherry pedicels within 3 h (Else et al., 2004) and 5–13% through 15 mm lupine hypocotyls within 8 h (Sánchez-Bravo et al., 1992). In mango the vascular system is in close association with resin canals and exudates rich in carbohydrates and phenolic compounds cause a rapid sealing of the cut surface (Joel, 1981; Lima Filho, 2004), thus likely reducing the PAT capacity. However, a sealing of the cut surface was prevented by immediately placing a physiologically-buffered agar block on the cut surfaces. Both ethephon treatments reduced the PAT capacity of mango fruitlet pedicels within 24 h (Figure 4) which supports earlier findings that the transcript of an auxin efflux carrier responsible for the basipetal auxin transport (Friml, 2003) was reduced within 24 h of ethylene treatment (Dal Cin et al., 2009). Experiments with *Arabidopsis* seedlings showed that ethylene biosynthesis pathway enzymes respond to varying auxin concentrations (Abel et al., 1995), suggesting that a reduced PAT through the pedicel can also induce endogenous ethylene evolution in pedicels and in turn trigger abscission in the AZ.

Carbohydrate deficiency is another plausible cause of fruitlet abscission (Xie et al., 2013), however, few data of carbohydrate concentrations in mango fruitlets during the main fruitlet drop stage at pea to marble size are available. Defoliation experiments with citrus have clearly shown that low sucrose concentration in fruitlets cause fruitlet abscission (Mehouachi et al., 1995) and in agreement with this finding, also low concentration of sucrose in mango fruitlet seem to be related to the abscission inducing treatment (Figure 5, Supplementary Figure 6). Sucrose concentration in the pulp of mature mango fruit ranged from 46 to 114 mg g⁻¹ dry weight, depending on cultivar, ripening stage and method used for analysis (Thanaraj et al., 2009). Moreover, it was shown earlier for mango that sucrose is the main translocation carbohydrate in support of fruit growth (Chauhan and Pandey, 1984). It is suggested that the ethephon-induced reduction of sucrose concentration in fruitlets at 2 DAT is triggered by reduced auxin signaling that subsequently reduces the sink strength for carbohydrate import into the fruitlet commencing at 1 DAT (Figure 5).



In conclusion, the data suggest that the ethephon-induced fruitlet abscission follows a different sequence of events (**Figure 6**) compared to the natural abscission process. In the latter case, resource deficiency, e.g., carbohydrate supply limitations for fruitlet growth, or seed degeneration with auxin signaling disruption are primary physiological causes (Xie et al., 2013; Hagemann et al., in press). In contrast, the ethephon-induced fruitlet abscission process responds initially with a reduction of the PAT capacity in the pedicel, followed by an upregulation of ethylene receptors and then a decline in sucrose concentration; physiological markers that were not linked to seed degeneration. Ethephon spray applications at the high concentration caused a faster abscission of mango fruitlets at the AZ than the low ethephon concentration. This might be due to a more rapid saturation of ethylene receptor binding sites in the pedicel by the high ethephon concentration, which presumably also causes a greater autocatalytic ethylene production in the pericarp and the pedicel.

An alternative explanation is provided by Dal Cin et al. (2005) who first suggested that a greater *ERS1/ETR1* ratio in both the pedicel AZ and the fruit cortex (pericarp) is a decisive trigger for fruitlet abscission during the midseason drop stage in apple. This notion was also suggested for mango (Ish-Shalom et al., 2011); however, specific evidence is provided in the present study with higher *MiERS1/MiETR1* ratios in the pericarp and the pedicel of ET7200-treated fruitlets than those of control fruitlets (**Figure 6**; Supplementary Table 1). In contrast, ET600 induced an increased *MiERS1/MiETR1* ratio in the pedicel but not in the pericarp, suggesting that the receptor regulation in the pericarp is not the primary determining

factor in both ethephon-inducing fruitlet abscission treatments (**Figure 6**; Supplementary Table 1). However, the 1-day earlier reduction of FDF in the ET7200 than the ET600 might be associated with the higher *MiERS1/MiETR1* ratios in both pedicel and pericarp. Following the *MiERS1/MiETR1* ratio concept, the ethephon-induced fruitlet abscission process commences with a reduction of the PAT capacity and an upregulation of ethylene receptors.

The findings of this study contribute to further our understanding of the regulation of ethylene signaling in horticultural crops. The *ERS1* versions might also be able to interact with one another to build receptor clusters as previously described by Gao et al. (2008) for *Arabidopsis*. It was shown that some receptors and in particular *ERS2* and the *ETR1* form preferentially heterodimers which in turn can form receptor clusters through the GAF domains (Gao et al., 2008), known as common elements of ethylene receptors necessary for the receptor-receptor interaction (Binder, 2008; Gao et al., 2008). Moreover, it was concluded that a given receptor within the cluster can laterally transmit the signal of detecting an ethylene molecule to neighboring receptors, thereby amplifying the signal and subsequently inducing the ethylene response at even low ethylene concentrations. Liu and Wen (2012) discussed different scenarios of receptor cluster function. Following the model for family 1 receptor clusters with a strong signal output, the identified *MiERS1* versions could lead to reduced ethylene sensitivity when expressed and translated. These cluster functions could partly explain the numerous ethylene-induced plant responses, e.g., the different abscission response to ethylene of leaves and fruit as shown for citrus (John-Karuppiah and Burns,

2010). The findings may also provide new breeding targets for mango as for example selecting genotypes for mutated ERS1 receptors that are less sensitive to ethylene and thereby less prone to fruitlet abscission.

Author Contributions

The authors have drafted and contributed to the concept and design of the work together. Further the authors have critically revised the work for its scientific significance and approved this version of this manuscript for publication. Additionally the specific contributions of the authors were as followed:

MHH conducted the sample and data collection in Vietnam, the sample processing, as well as technical and statistical data analysis. He adapted and further developed the HPLC-sample extraction and the qPCR data processing. Further he drafted the publication and adapted the layout.

PW developed the concept of the molecular biological part of the work. He established the RNA extraction and analysis including the cloning and sequence evaluation. Further he identified the genes and established the corresponding qPCR analysis.

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MH supported the establishment of the field trials and orchard management in Vietnam. Further he developed the concept of the polar auxin transport assay including sample extraction, analysis and data interpretation.

JW drafted the main concept of the experimental design. He supervised the study and critically revised the outcomes for their overall scientific impact. Together with the corresponding authors he developed the data analysis, data interpretation and the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00706>

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Genome-wide digital transcript analysis of putative fruitlet abscission related genes regulated by ethephon in litchi

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The high level of physiological fruitlet abscission in litchi (*Litchi chinensis* Sonn.) causes severe yield loss. Cell separation occurs at the fruit abscission zone (FAZ) and can be triggered by ethylene. However, a deep knowledge of the molecular events occurring in the FAZ is still unknown. Here, genome-wide digital transcript abundance (DTA) analysis of putative fruit abscission related genes regulated by ethephon in litchi were studied. More than 81 million high quality reads from seven ethephon treated and untreated control libraries were obtained by high-throughput sequencing. Through DTA profile analysis in combination with Gene Ontology and KEGG pathway enrichment analyses, a total of 2730 statistically significant candidate genes were involved in the ethephon-promoted litchi fruitlet abscission. Of these, there were 1867 early-responsive genes whose expressions were up- or down-regulated from 0 to 1 d after treatment. The most affected genes included those related to ethylene biosynthesis and signaling, auxin transport and signaling, transcription factors (TFs), protein ubiquitination, ROS response, calcium signal transduction, and cell wall modification. These genes could be clustered into four groups and 13 subgroups according to their similar expression patterns. qRT-PCR displayed the expression pattern of 41 selected candidate genes, which proved the accuracy of our DTA data. Ethephon treatment significantly increased fruit abscission and ethylene production of fruitlet. The possible molecular events to control the ethephon-promoted litchi fruitlet abscission were prompted out. The increased ethylene evolution in fruitlet would suppress the synthesis and polar transport of auxin and trigger abscission signaling. To the best of our knowledge, it is the first time to monitor the gene expression profile occurring in the FAZ-enriched pedicel during litchi fruit abscission induced by ethephon on the genome-wide level. This study will contribute to a better understanding for the molecular regulatory mechanism of fruit abscission in litchi.

Keywords: *Litchi chinensis* Sonn., fruitlet abscission, digital transcript abundance, ethephon, gene

Introduction

Fruit abscission, occurring during fruit development, is characterized through a high coordination of biochemical events that take place in a group of specialized cells located between the pedicel and fruitlet, known as abscission zones (AZs, Bonghi et al., 2000; Sun et al., 2009). In agricultural production, shedding of fruit is a major limiting factor of yield. Over the last few decades, it is widely believed that abscission involves in multiple changes in cell structure, metabolism and gene expression, and divides into four major steps (Patterson, 2001; Estornell et al., 2013): (i) the ontogeny of AZ, (ii) the acquisition of competences to respond to abscission signals, (iii) the onset of the cell separation, (iv) the differentiation of a protective layer. It is well achieved that plant hormones are deeply involved in abscission, and ethylene operates as an efficient accelerator for organ abscission. Although there is no clear and sufficient evidence for a direct link between the ethylene perception and the onset of abscission, it is well supported that the development of this process is concomitant with an increase in the production of ethylene (Zhu et al., 2008). In fact, application of ethephon, an ethylene-releasing compound, effectively hastens the abscission of fruit in apple (Yuan, 2007; Kolarić et al., 2011), sweet orange (John-Karuppiah and Burns, 2010), sweet cherry (Smith and Whiting, 2010), and olive (Zahra, 2014). However, aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, blocked the fruit abscission promoted by auxins in apple (Zhu et al., 2008), while 1-Methylcyclopropene (1-MCP), an inhibitor of ethylene perception, did not affect the abscission-promoted effect of ethephon in orange (John-Karuppiah and Burns, 2010). In this regard, understanding the regulatory effects of ethylene on abscission is important for the fruit industry.

Up to date, gene expression and enzymatic studies on organ abscission have shown that ethylene either facilitates the efficacy of ethylene signaling pathways (Li and Yuan, 2008; John-Karuppiah and Burns, 2010), or activates the synthesis and secretion of several cell wall and middle lamella hydrolytic enzymes associated with the separation of cells at the AZ, such as cellulase (Abeles and Leather, 1971; MacDonald et al., 2011) and polygalacturonase (Taylor et al., 1993). A transcriptome analysis could be one of the most powerful tools to understand complicated transcriptional regulation during plant organ shedding. In order to provide a new insight into the molecular basis of ethylene-mediated abscission, there are few cases of transcriptome analyses performed using ethylene-treated pedicels as materials. In the study of ethylene-promoted citrus leaf abscission, Agustí et al. (2008, 2009) discovered the preferential accumulation gene families in laminar AZ after ethylene treatment, such as cell wall modification, lipid transport, protein biosynthesis and degradation, transcription factors (TFs), stress and pathogen-related genes and some special genes involved in signaling events. In tomato, Wang et al. (2013) compared the transcriptome difference between the AZ and neighboring portion (the basal and apical) of pedicel in a time course after ethylene treatment, proposing a possible regulatory scheme involving in tomato flower abscission. However, the

comparative analysis of the transcriptome profiles involved in ethylene-promoted fruit abscission using AZ as materials is lacking, although it has long been observed that application of exogenous ethylene accelerates fruit abscission.

Litchi (*Litchi chinensis* Sonn.), an important economic fruit crop in subtropical area, has been challenged by massive fruit drop, one of the major factors causing a low yield (Yuan and Huang, 1988; Mitra et al., 2003). For example, a medium size tree may produce about 60,000 female flowers but, typically, less than 5% of flowers develop into mature fruits (Stern et al., 1995). Yuan and Huang (1988) reported that there were three to four waves of physiological fruit drop throughout fruit development in 70~90 days depending on cultivars. Wave I, wave II, and wave III of abscission occurred around 1 week, 3 weeks, and 6~7 weeks after full bloom, respectively, but wave IV was specific to cultivars with aborted seeds and occurred 2~3 weeks before harvest. Previously, few studies focus on the molecular regulation mechanism of litchi fruit abscission. Through the application of ethephon, ethylene has been proved to have an unequivocal promotive effect on litchi fruitlet abscission and increase the expression of *LcPG1* encoding a pectin-degrading enzyme (Peng et al., 2013). On the other hand, there was circumstantial evidence that there had a higher fruit abscission rate and *ACO* (1-aminocyclopropane-1-carboxylic acid oxidase) gene expression level in fruits treated by NAA (naphthalene acetic) spraying, suggesting a potential role in fruit abscission (Wu et al., 2013). Nevertheless, the comprehensive transcriptome-wide expression profiling analysis under ethylene-induced abscission has not yet been documented in litchi.

In this experiment, we performed a genome-wide digital transcript analysis on fruit abscission zone (FAZ) enriched pedicel at 0, 1, 2, 3 d time points of ethephon treatment. Our results showed that a total of 6167 ethylene-regulated genes were preferentially expressed in ethephon-treated FAZ-enriched tissues. Among them, 2730 candidate genes were considered to be involved in ethylene-promoted fruit abscission process by further Gene Ontology (GO) and KEGG pathway enrichment analyses. It was demonstrated that a range of functional categories such as plant hormone synthesis and signaling, carbohydrate metabolism, TFs and cell wall modification, were highly regulated by ethylene. These results will provide a new insight of the ethylene regulatory fruit abscission molecular mechanism in litchi.

Materials and Methods

Plant Materials and Treatment

Nine 9-year-old litchi trees (*L. chinensis* Sonn. cv. Feizixiao) were randomly selected in an orchard located at South China Agricultural University in 2012 (Guangzhou, China), and blocked into three biological replicates of three trees each. At 25 d after anthesis, 20 fruit-bearing shoots (about 5~8 mm in diameter) located in different directions from each tree were tagged. Ten of them were dipped in 250 mg L⁻¹ ethephon solution (containing 0.05% Tween-80 surfactant) for 1 min, while the remaining 10 shoots dipped in water were used as control. Three out of ten treated shoots were used to monitor fruit

abscission dynamic and the others were used for sampling. Samples were conducted at 0, 1, 2, and 3 d after treatment. Fruitlet and FAZ-enriched pedicels were collected immediately after the samples were taken back to laboratory on ice. FAZ-enriched pedicels were excised by cutting around 2 mm at each side of the abscission fracture plane (**Supplementary Figure 1**). After separation, all tissues were quickly frozen in liquid nitrogen and stored at -80°C for future analysis.

Determination of Fruit Abscission and Ethylene Production Rate of Fruit

Cumulative fruit abscission rate (CFAR) was calculated according to our previous method (Kuang et al., 2012). Ethylene production was measured according to the method described by Yan et al. (2011) with some modifications. Two fruit from each treatment on each tree were collected and enclosed in a 30 mL airtight syringe equipped with a rubber piston for 2 h at 25°C . Air within the syringe was forced into an airtight container filled with saturated salt water with a needle inserted to allow replacement. After all the samples were collected, 1 mL air sample was then withdrawn from the headspace of the container with a syringe and injected into a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) fitted with a flame ionization detector and an activated alumina column (200 cm \times 0.3 cm). The injector temperature was 120°C ; the column temperature was kept at 60°C and the detector temperature at 60°C . Helium was used as carrier gas at a flow rate of 30 mL min $^{-1}$. The ethylene production rate was expressed as microliters of $\text{C}_2\text{H}_4 \text{ kg}^{-1} \text{ h}^{-1}$.

Digital Transcript Abundance Library Preparation and Illumina Sequencing

Total RNA from FAZ-enriched pedicel was isolated using Column Plant RNAout 2.0 kit (TIANDZ, Inc, China). The quantity and quality of RNA samples were evaluated using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Equal amounts of total RNA from three biological replicates were pooled to construct seven libraries named CK0, CK1, CK2, CK3, ETH1, ETH2, and ETH3. For example, CK1 and ETH1 were the libraries from pedicels harvested at 1 d after water and ETH treatment, respectively. After RNA extraction, mRNA purification by Oligo (dT), fragmentation, cDNA synthesis by random hexamer primers, size selection and PCR amplification were performed by BGI-Shenzhen as described previously (Li et al., 2013).

Data Analysis for Digital Transcript Abundance Profiles

High-quality reads used for further downstream processing were filtered through the standard Illumina pipeline to remove the low-quality reads and those containing adaptor/primer contaminations. All clean reads were mapped back to the litchi genome (<http://litchidb.genomics.cn>, unpublished) using SOAPAligner (Version 2.21) allowing up to two nucleotide mismatches with the parameters of “-m 0 -x 1000 -s 28 -l 32 -v 2 -r 2,” which are specified on <http://soap.genomics.org.cn/soapaligner.html>. Clean reads mapped to reference, from multiple genes, were filtered and unambiguous clean reads

were remained. For gene expression analysis, the number of unambiguous clean reads for each gene was calculated and normalized to RPKM (Reads Per Kilo base per Million reads) (Mortazavi et al., 2008). Six paired-libraries including CK0 vs. CK1, CK0 vs. CK2, CK0 vs. CK3, CK1 vs. ETH1, CK2 vs. ETH2, and CK3 vs. ETH3 were used to analyze the differential gene expression, according to the method described in Audic and Claverie (1997). Two filter criteria were used to identify differentially expressed genes (DEGs): a four-fold change in transcript levels and a FDR (False Discovery Rate) value ≤ 0.001 among every comparison.

In order to eliminate the control background noise, take day 1 treatment data for example, we excluded the DEGs identified in CK0 vs. CK1 library from the DEGs identified in CK1 vs. ETH1 library, and the obtained data was recorded as ETH1/CK1. The other two time points were done as the same way, and the result was recorded as ETH2/CK2 and ETH3/CK3, respectively. Then the union of DEGs among ETH1/CK1, ETH2/CK2, and ETH3/CK3 was defined as ethephon-responsive genes. Finally, all ethephon-responsive genes were mapped to terms in GO and KEGG databases for functional and pathway-enrichment analysis. And those genes significantly enriched in GO term analysis ($FDR \leq 0.05$) or enriched in KEGG pathway ($Q\text{-value} \leq 0.05$) were screened to be the candidate genes involved in the fruit abscission process. Heat maps showing expression profiles were generated using the MultiExperiment Viewer (MeV, v4.9).

Quantitative Real-Time PCR

To validate the accuracy of our DTA profiles results, 41 randomly selected DEGs were evaluated by quantitative real-time PCR (qRT-PCR) after the ethephon treatment in litchi FAZ. The RNA samples for DTA analysis were also used for qRT-PCR. Gene-specific primer sequences were designed using Primer Premier 5.0 and listed in **Supplementary Data Excel File 1**. Purified total RNA (2 μg) from each sample was reverse-transcribed to synthesize cDNA by ReverTra Ace qPCR RT Master Mix with gDNA Remover (TOYOBO). Then, the cDNA was amplified using SYBR Green-PCR master kit (THUNDERBIRD SYBR qPCR Mix, TOYOBO) and LightCycler 480_II Real-Time PCR System (Roche). The PCR amplifications included the following condition: 95°C for 1 min, followed by 40 cycles of 95°C for 5 s, 55°C for 30 s and 72°C for 30 s. Dissociation curves were run to determine the specificity of the amplification reactions. The data were normalized using cycle threshold (Ct) value corresponding to two litchi reference genes, *EF-1 α* and *GAPDH* (Zhong et al., 2011). The relative expressive level of the target genes were calculated using the $\Delta\Delta\text{Ct}$ method. Duplicates from three biological replicates were used.

Results

Changes in Fruit Abscission Rate and Ethylene Production

CFAR and ethylene production in fruitlet were compared between the control and ethephon (ETH) treatment (**Figure 1**). The CFARs showed similar trends (**Figure 1A**), which gradually increased in the first day and had no visible difference. Two

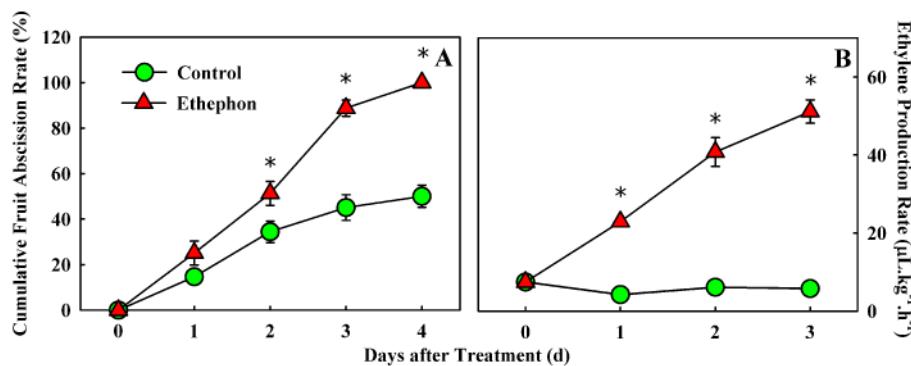


FIGURE 1 | Effect of ethephon treatment on fruitlet abscission (A) and ethylene production (B) in litchi. Each value represented the means of three biological replicates from nine different trees,

with the standard error (SE) indicated by vertical bars. Significant differences at 0.05 level are indicated with asterisk (*) according to t-test.

days after treatment, the CFAR in ETH-treated fruitlet was significantly higher than that in the control. Consequently, 100% of the fruitlet abscised by 4 d after ETH treatment, compared with a ~50% loss in the control, indicating that ETH treatment significantly accelerated fruitlet drop. In addition, a clear impact on ethylene production was also observed in ETH-treated fruitlet. Within 3 days of observation, ethylene production in the control fruitlet remained more or less flat and kept below $8 \mu\text{L kg}^{-1} \text{ h}^{-1}$. While ethylene production in the ETH-treated fruitlet increased rapidly and continuously, and achieved nearly a ten-fold higher level at day 3 than the control (Figure 1B). The increase in ethylene production suggested that ethephon application probably accelerates the fruit drop following the induction of ethylene production in fruitlet.

Digital Transcript Abundance Profile Analysis

To explore the transcriptional changes of litchi FAZ-enriched pedicel in response to ethephon treatment, seven digital transcript abundance (DTA) tag profiles of the control (CK0, CK1, CK2, and CK3) and ETH-treated samples (ETH1, ETH2, and ETH3) were sequenced (Table 1). After quality filtering, nearly 82 million clean reads were generated from the above libraries (10–12 million reads for each library). The tag sequences of the seven libraries were mapped to the litchi genome, and 87.11, 86.93, 86.69, 87.20, 87.53, 87.37, and 86.52% of all clean reads were obtained, respectively. These results showed that both the throughput and sequencing quality were high enough for further analysis.

Confirmation of Gene Expression Patterns by qRT-PCR

Forty-one randomly selected genes were analyzed by quantitative real-time PCR to verify their expression patterns (Figure 2). Among these, transcripts encoding the key enzymes involved in ethylene biosynthesis and signaling, such as ACS (L10030076), ACO (L10037576), ETR2 (U20306), EBF (U575), EIN3/EIL (U23712) and ERF (L10026303, L10050388, C2959.2, and U6276), were sharply increased at 1 d after treatment, which were consistent with the results analyzed by DTA (Figure 2).

TABLE 1 | Statistics of digital transcript abundance library sequencing and read mapping.

Libraries	Total reads	Total mapped reads*	Unique match reads
CK0	12,009,135	10,461,248 (87.11%)	4,491,205 (37.40%)
CK1	11,530,205	10,022,661 (86.93%)	4,400,232 (38.16%)
CK2	10,568,766	9,161,789 (86.69%)	4,003,146 (37.88%)
CK3	10,862,567	9,472,399 (87.20%)	4,147,267 (38.18%)
ETH1	12,477,700	10,921,142 (87.53%)	4,756,944 (38.12%)
ETH2	11,905,084	10,401,413 (87.37%)	4,516,857 (37.94%)
ETH3	12,284,279	10,628,560 (86.52%)	4,693,781 (38.21%)

*Number and percentage of reads mapped onto litchi genome (unpublished data). CK and ETH mean the untreated control and ethephon treatment, the numbers following the CK and ETH are the days after treatment.

Similarly, we verified the increased expression of genes encoding those enzymes related to cell wall degradation, TFs, cytokinin metabolism and ROS production, such as *BGLU* (U21052), *BXL* (L10010659), *XTH* (C5711.4 and U17815), *PE/PEI* (C5841.2, L10049394, and U1533), *PG* (L10048763), *HD-ZIP* (U10783), *NAC* (U13457), *WRKY* (U18045 and U9115), *CKX* (L10048833) and *Rboh* (L10001757), as well as the down-regulated genes involved in auxin transport and signaling, cytokinin signaling, ROS scavenging and calcium signaling, such as *PIN* (U11774), *AUX1* (U9940), *Aux/IAA* (C3897.1 and U9140), *SAUR* (C5333.1), *ILR1* (U1508), *AHK* (U11693), *AHP* (C4349.2), *ARRA* (C3809.1), *POD* (U20379 and U15492), *AO* (U10993), and *CML* (U2171 and U4839). The expression level of one auxin early responsive gene (*GH3*, C4818.1) and one calcium signaling gene (*CML*, U1498) were increased by ETH treatment, which was identical to the DTA results. One calcium transport gene (*CNGC*, L10064767) showed down-regulated at 1 d after treatment and increased at 3 d after treatment (Figure 2). However, the expression level of one gene encoding auxin response factor (*ARF*, L10058057) was contrasted to the DTA result (Figure 2). Thus, except one *ARF* gene, all the other 40 genes showed similar expression patterns as detected by the DTA, indicating that the DTA analysis results were effective.

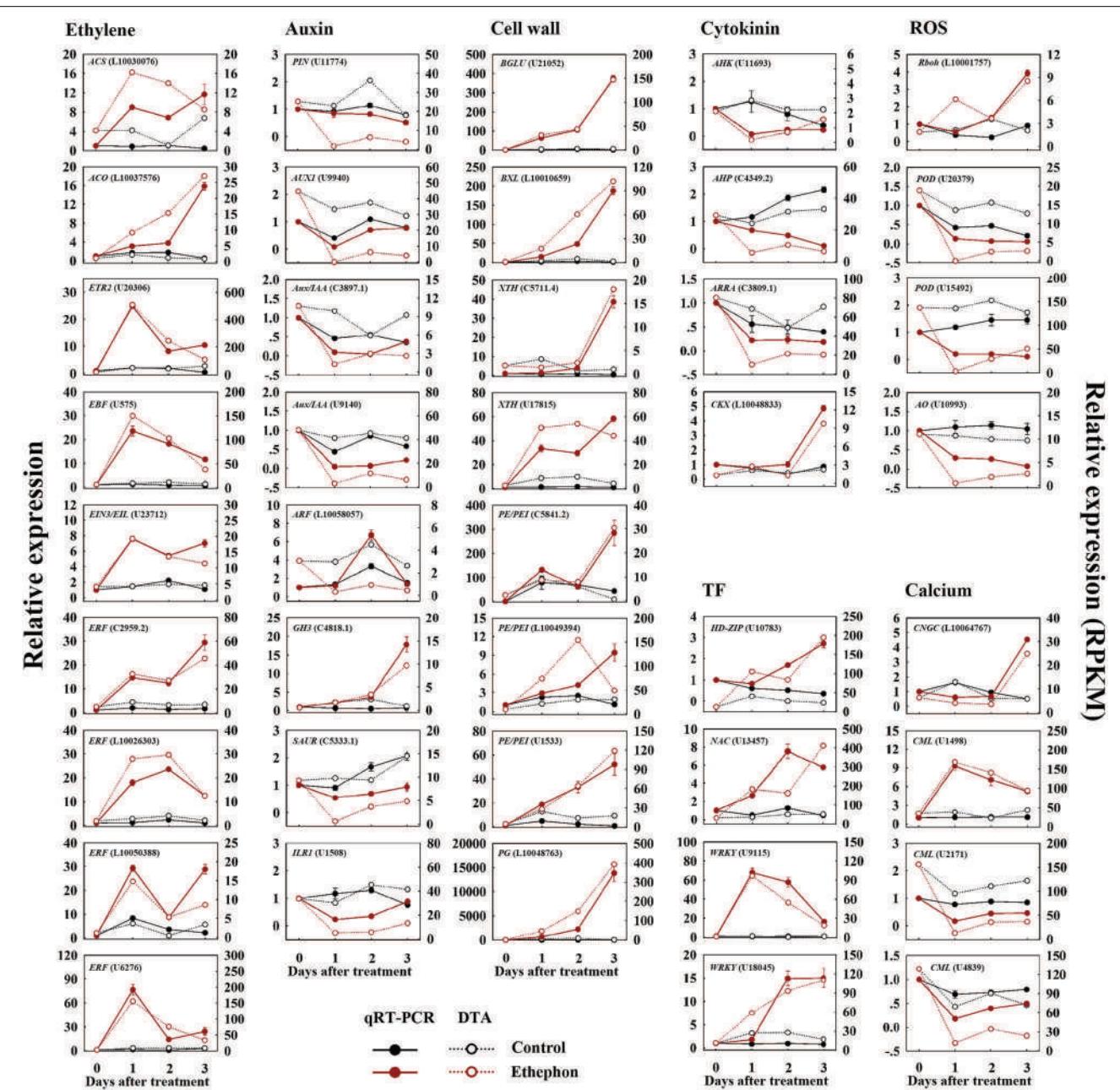


FIGURE 2 | Confirmation of candidate gene-expression pattern in FAZ-enriched pedicel during ethephon-induced fruit abscission.

qRT-PCR analysis (filled circles, left y-axis) of 41 selected genes at 0, 1, 2, 3 d after ethephon treatment. Relative expression values were normalized to the pre-treatment (0 d) expression value taken as 1. The data represent the mean values (\pm SE) of duplicate experiments from three independent

biological samples. Broken lines (open circle, right y-axis) show expression profiling of genes in the FAZ-enriched pedicel from the DTA data, and indicate the total read count in RPKM for each gene after normalization across the seven samples: CK0, CK1, CK2, CK3, ETH1, ETH2, and ETH3. CK and ETH mean the untreated control and ethephon treatment, the numbers following the CK and ETH are the days after treatment.

Transcriptome Responses during Fruit Abscission

After comparing three paired-libraries (CK1 vs. ETH1, CK2 vs. ETH2, and CK3 vs. ETH3), a total of 6167 genes were found to be significantly up- or down-regulated which named as ethephon-responsive genes (Supplementary Data Excel File 2).

Based on GO enrichment analysis, 3249 genes (53%) were divided into the three principal GO organization categories (Supplementary Figure 2A): biological process (2437 genes), cellular component (1459 genes) and molecular function (2825 genes). The enriched GO terms included carbohydrate metabolic process, photosynthesis, extracellular region, cell

wall, thylakoid, catalytic activity and oxygen binding. KEGG pathway enrichment analyses showed that a total of 3420 genes were assigned to the 118 related pathways, and the top 20 enriched pathways including plant hormone signal transduction (ko04075) and starch and sucrose metabolism (ko00500) were illustrated in **Supplementary Figure 2B**.

Analysis of the Candidate Genes Involved in the Fruit Abscission Process

A total of 2471 and 2344 genes were selected in GO ($FDR \leq 0.05$) and KEGG enrichment analyses ($Q\text{-value} \leq 0.05$) with a significant level, respectively. After eliminating duplicated, 2730 genes were identified as the candidate genes involved in the fruit abscission process regulated by ethephon (**Supplementary Data Excel File 3**). The up- and down-regulated genes accounted for 37.44% (1022 genes) and 62.56% (1708 genes) of them, respectively.

Based on their patterns of expression, these candidate genes could be classified into four groups, which consisted of genes with similar temporal patterns of expression kinetics (**Figure 3**). Group I included 1867 early-responsive genes whose expression were up- or down-regulated early at 1 d after treatment; Group II had 148 middle-responsive genes whose expression were not induced or suppressed until 2 d after treatment; Group III contained 258 late-responsive genes that were not regulated until 3 d after treatment; Group IV consisted of 457 constant-responsive genes that up- or down-regulated early and whose expression was maintained constant during the treatment. By hierarchical cluster analysis, each group could be subsequently divided into two to six clusters, for example, Group I included cluster 1A, 1B, 1C, 1D, 1E, and 1F which had 172, 261, 55, 776, 434, and 169 genes, respectively. In total, 723 up-regulated and 1601 down-regulated genes were found at 1 d after treatment, and 299 up-regulated and 107 down-regulated genes were found at 2 d or 3 d after treatment. These results showed that the majority (85.13%) of those candidate genes made a quick response to the ETH treatment in 24 h when no significant difference on fruit abscission rate was found between the control and the ETH treatment.

Except 101 genes encoding proteins of unknown functions, the other 2629 genes had unambiguous annotations. Those genes related to plant hormones, cell wall metabolism TFs, carbohydrate metabolism, ROS and calcium signaling were further analyzed as follows.

Genes Related to Plant Hormone Biosynthesis and Signaling Pathway

A total of 195 candidate genes were found related to plant hormone biosynthesis and signaling pathway (**Figure 4, Supplementary Data Excel File 4**). Of these, 124, 16, 17, and 38 genes belonged to the Group I, Group II, Group III, and Group IV, respectively. Sixty and fifty-five genes were related to auxin and ethylene, among them, 47 auxin-related genes were down-regulated and 39 ethylene-related genes were up-regulated. These genes should be closely associated with fruitlet abscission, including those encoding auxin efflux carrier component (PIN), auxin influx carrier (AUX1), AUX/IAA

protein, auxin response factor (ARF), SAUR family protein, GH3 protein, 1-aminocyclopropane-1-carboxylate oxidase (ACO), 1-aminocyclopropane-1-carboxylate synthase (ACS), AP2/ERF transcription factor and ethylene receptor (ETR), et cetera. Twenty-eight gibberellins (GA) related and 18 cytokinin-related genes were found, most of them were down-regulated, such as genes encoding gibberellin 20 oxidase (GA20ox), gibberellin receptor GID1, cytokinin hydroxylase (CYP735A), histidine kinase (AHK) and two-component response regulator (AARA), etc.. Eight of eighteen abscisic acid (ABA) related genes were up-regulated including those encoding zeaxanthin epoxidase (ZEP), abscisic acid receptor (PYR/PYL) and protein phosphatase 2C (PP2C), and the repressed genes mainly encoded 9-cis-epoxycarotenoid dioxygenase (NCED), carotenoid cleavage dioxygenase (CCD), and abscisic acid 8'-hydroxylase (CYP707). Moreover, six salicylic acid (SA) related genes encoded with salicylic acid-binding protein (SBP) and regulatory protein NPR1 were highly up-regulated, and five jasmonic acid (JA) related genes mainly encoded allene oxide cyclase (AOC) and jasmonate ZIM domain-containing protein (JAZ) were decreased. These results suggested that seven classes of plant hormones were involved in the process of fruitlet abscission induced by the ETH treatment. The most important hormones were ethylene and auxin, followed by GA, cytokinin and ABA. JA and SA ranked the last according to the number of DEGs.

Genes Encoding for Transcription Factors

Except for 58 genes putatively encoding TFs related to plant hormones, there were 127 candidate TFs belonged to diverse families including *ABI3/VP1*, *bHLH*, *BLH1*, *bZIP*, *GRAS*, *HD-ZIP*, *HSF*, *KNOX*, *LBD*, *MADS-box*, *MYB*, *NAC*, *WRKY*, *Trihelix*, and *zinc finger* (**Figure 5, Supplementary Data Excel File 5**). Of these, 51 and 76 genes were up- and down-regulated, respectively. And 78, 9, 14, and 26 belonged to Group I, Group II, Group III and Group IV, respectively. Remarkably, 71 out of 114 TFs in Group I and Group IV were down-regulated, indicating that most TFs were repressed at 1 d after the ETH treatment. There were more than 10 members in those families including *bHLH*, *MYB*, *WRKY*, *NAC*, *LBD*, and *HD-ZIP*. Most members of *bHLH* and *HD-ZIP* families were down-regulated, while 13 of 15 members in the family of *WRKY* were up-regulated, and all members in the families of *BLH1*, *MADS-box*, *KNOX*, and *Trihelix* were down-regulated. Moreover, 33 of the 51 up-regulated TFs were induced at 1 d after treatment, and the largest TF family was the *WRKY* (10 genes), followed by *MYB* (6 genes) and *NAC* (5 genes), implying that those genes from these TF families could be related with triggering the transcriptional chain reaction during ETH-induced abscission.

Genes Related to Cell Wall Biosynthesis, Degradation, Loosening, and Modification

A total of 208 genes including 56, 104, 21, and 27 genes related to cell wall biosynthesis, degradation, loosening and modification were found, respectively (**Supplementary Data Excel File 6**). Of these, 72 and 136 genes were up- and down-regulated, respectively. Of the up-regulated genes, there were 51 cell wall degradation, loosening and modification related transcripts

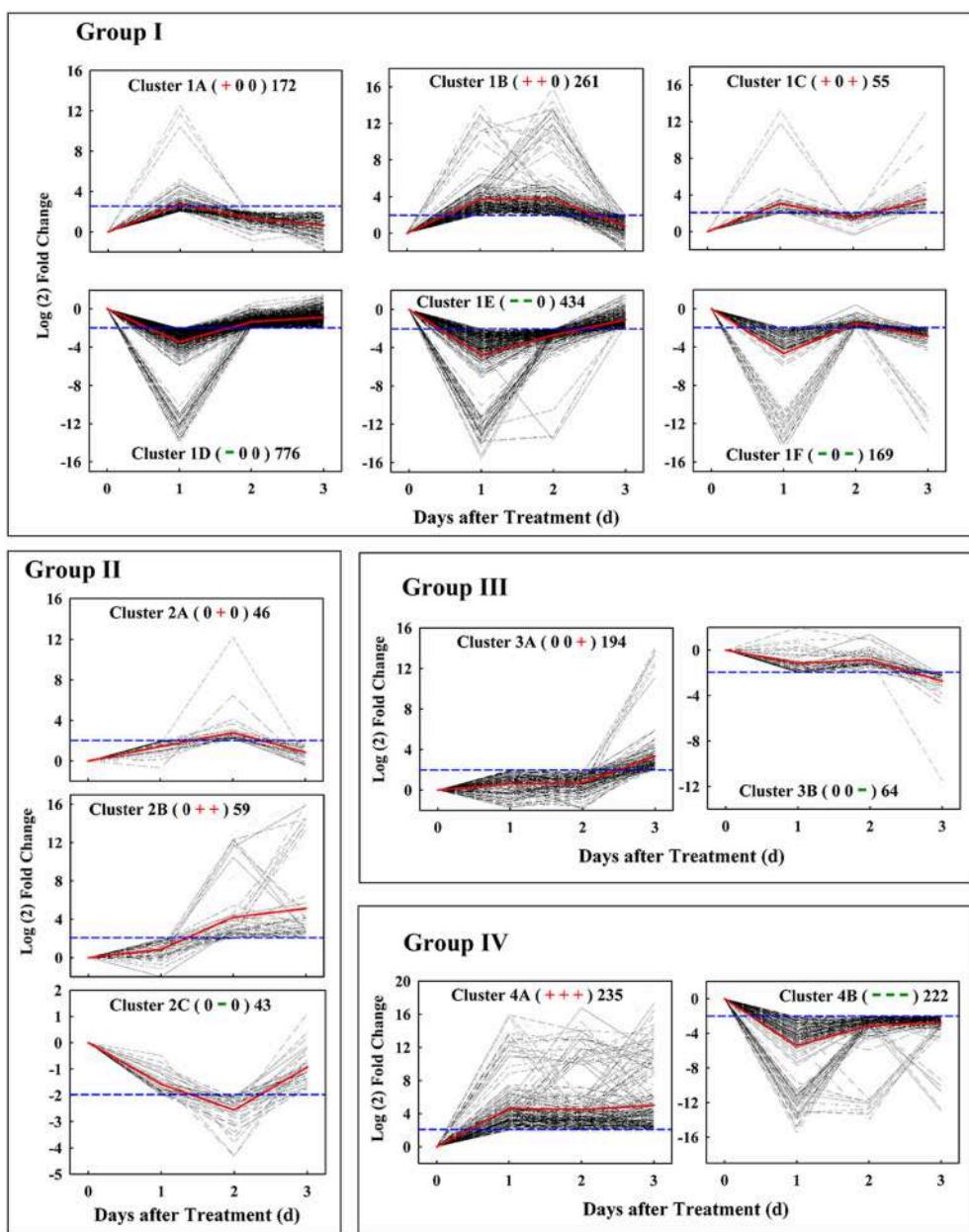
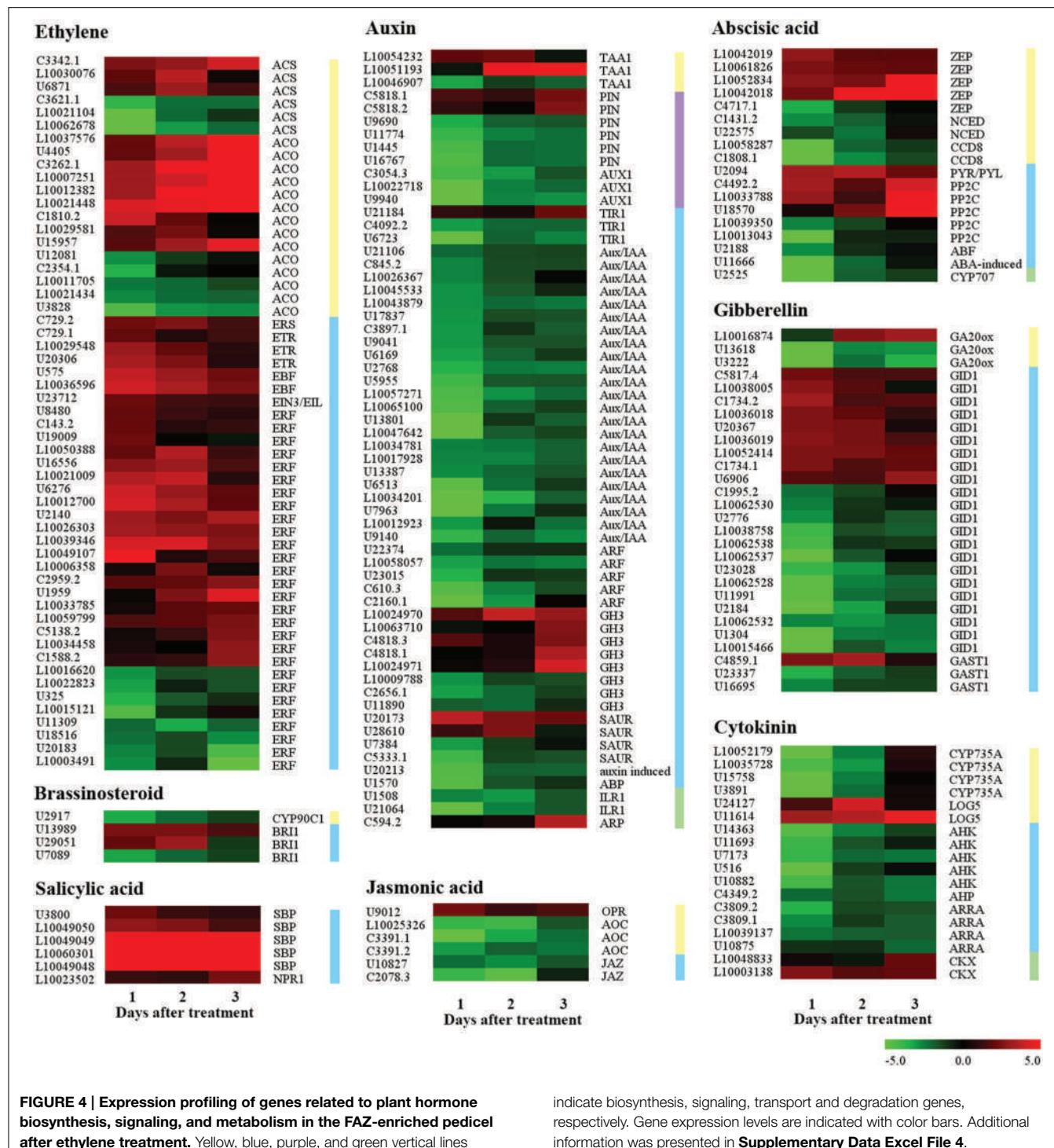


FIGURE 3 | Ethepron-responsive genes expression pattern obtained by kinetics-based clustering analysis. **Group I**, cluster of genes with early and transient changes; **group II**, clusters of genes modified in their expression until 2 d after treatment; **group III**, cluster of genes with expression kinetics exhibiting late changes; **group IV**, cluster of genes with persistent changes during the whole abscission process. The + and – signs

in bracket represent up- and down-regulated of genes, respectively, while 0 represents no change. The numbers on the right of bracket indicate the total numbers of genes in each cluster. All of these changes were based on a four-fold change criterion (\log_2 ratio) indicated by blue dotted line. Gray dotted line indicates the gene-expression levels and the average values of gene-expression level in clusters is shown by the red solid lines.

(Figure 6). Among them, 4 genes were related to callose degradation, like endo-1,3- β -glucosidases (*ENGs*) and β -1,3-glucanases (*BGN13s*); 5 genes were involved in cellulose degradation, such as endo-1,4- β -D-glucanases (*CELs*) and β -glucosidases (*BGLUs*); 11 genes like endo-1,4- β -mannosidase (*MAN*), xyloglucan endotransglucosylase/hydrolases (*XTHs*) and β -D-xylosidases (*BXLs*) were related to hemicellulose degradation; 13 genes such as polygalacturonases (*PGs*) and

pectate lyase (*PLs*) were associated with pectin degradation; 12 expansins (*EXPs*) related to cell wall loosening and 6 pectinesterase/pectinesterase inhibitors (*PE/PEIs*) genes were involved in cell wall modification. Of the down-regulated genes, there were 35 cell wall biosynthesis-related genes (Figure 6), including those encoding cellulose synthase, extensin, glycine-rich cell wall structural protein, UDP-glucuronate 4-epimerase and xyloglucan glycosyltransferase. There were not surprised



that those genes encoding enzymes mentioned above may be involved in the process of fruitlet abscission induced by ethephon. However, 101 down-regulated genes related to cell wall degradation, loosening and modification, and 21 up-regulated genes related to cell wall biosynthesis were also found in our study.

Genes Related to Photosynthesis, Carbohydrate, and Energy Metabolism

The expression of over 93% (103 out of 110 genes) genes involved in photosynthesis pathway were strongly decreased, especially repressed exclusively at 1 day after the ETH treatment, and most of them belonged to Group I (Supplementary Data Excel File 7).

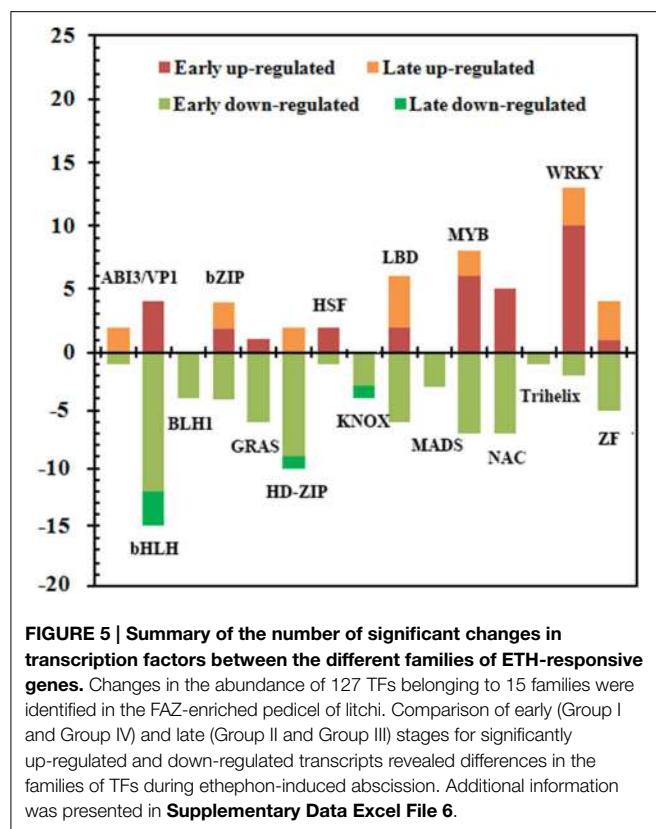


FIGURE 5 | Summary of the number of significant changes in transcription factors between the different families of ETH-responsive genes. Changes in the abundance of 127 TFs belonging to 15 families were identified in the FAZ-enriched pedicel of litchi. Comparison of early (Group I and Group IV) and late (Group II and Group III) stages for significantly up-regulated and down-regulated transcripts revealed differences in the families of TFs during ethephon-induced abscission. Additional information was presented in **Supplementary Data Excel File 6**.

The affected genes function in chlorophyll biosynthesis, thylakoid formation, chlorophyll a/b binding protein, light harvesting (PSI and PSII), electron transport and carbon fixation. These results suggested repressed expression of those encoding for chloroplast and light harvesting associated proteins by ethephon might lead to the process of chloroplast malfunction and photosynthesis inhibition.

Not surprisingly, the down-regulated photosynthesis related genes are linked with changes in the expression of genes in carbohydrate metabolism. A total of 137 candidate genes were found in this section (58 up-regulated and 79 down-regulated, **Supplementary Data Excel File 7**). Overall, 24 out of 32 genes involved in glycolysis and all members of the TCA cycle (7 genes), such as alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase and ATP-citrate synthase, were strongly down-regulated within Group I, indicating that the degradation of sugar was inhibited during the early ETH-induced abscission. However, an increased expression of seven genes associated with fructose and mannose degradation and four genes involved in galactose hydrolysis were also found during the late induction of abscission. These results suggested that sugar degradation was inhibited during the early induction but increased during the late induction. Moreover, our data also showed that 13 out of 17 genes encoding ATPase were repressed after the ETH treatment, especially down-regulated exclusively at 1 day post-treatment (**Supplementary Data Excel File 7**). These results indicated that ethephon might lead to the interdiction of ATP synthesis and the high energy supply.

Genes Related to ROS Response, Calcium Signal Transduction, and Protein Ubiquitination

A total of 88 transcripts belonging to the reactive oxygen species (ROS) response were preferentially regulated in the ETH-treated FAZ-enriched pedicels (**Supplementary Data Excel File 8**). Of these, 39 genes were up-regulated and the other 49 genes were down-regulated. Among the up-regulated genes (**Figure 6**), we found eight respiratory burst oxidase homolog proteins (*Rboh*) and one peroxisomal-(S)-2-hydroxy-acid oxidase (*GLO*) involved in ROS production, indicating a high ROS content might be produced in FAZ. Not surprisingly, 27 genes putatively encoding ROS scavenging enzymes of diverse families showed increased expression, and the most abundant transcripts encoding glutathione S-transferase (*GST*) and peroxidase (*POD*). However, the down-regulated ROS scavenging genes were also found, indicating that a competing activity occurred in the regulation of fruit abscission. In addition, 20 genes encoding the key enzymes related to ascorbate synthesis and metabolism, including L-ascorbate oxidase (*AO*) and inositol oxygenase (*MIOX*), showed an immediate decreased regulation at the 1 day post-treatment.

As an important signaling molecular, 52 transcripts related to calcium transport and perception displayed altered changes (**Figure 6, Supplementary Data Excel File 8**). Among them, 19 and 33 genes were up- and down-regulated respectively, and 46 of them belonged to Group I, indicating that most genes had instantaneous response to the ETH treatment. For example, two calcium influx transporters, *CNGC* (13 out of 18 genes) and *ANX* (2 genes), showed strongly decreased expression during the early induction. In contrast, 4 out of 6 transcripts encoding calcium efflux carrier proteins (PMCA) were increased. These results indicated that a role regulating calcium influx and maintaining calcium level in the FAZ may be associated with the beginning of abscission process. Moreover, two calcium responsive genes, *CML* (6 out of 10 genes) and *CDPK* (3 genes), were also repressed during the early induction.

A total of 40 genes involved in protein ubiquitination were found (**Figure 6, Supplementary Data Excel File 8**), and 32 of them belonged to Group I and IV, indicating that most genes had a quick response to the ETH treatment. Among them, 26 genes were up-regulated, including those encoding 26S proteasome, cullin, E3 ubiquitin-protein ligase, EIN3-binding f-box protein, U-box domain-containing protein and ubiquitin-conjugating enzyme E2. However, 14 down-regulated genes involved in protein ubiquitination were also found.

Discussion

Ethephon (2-chloroethylphosphonic acid) is a synthetic plant growth regulator discovered several decades ago, which acts by releasing ethylene when it penetrates plant tissues (Royer et al., 2006). The application of exogenous ethylene inducing fruit abscission has been observed in apple (Yuan, 2007; Kolaric et al., 2011), sweet orange (John-Karuppiah and Burns, 2010), sweet cherry (Smith and Whiting, 2010) and olive (Zahra, 2014). The data herein presented strengthen the direct observation that ethephon aggrandizes both the fruitlet abscission and ethylene

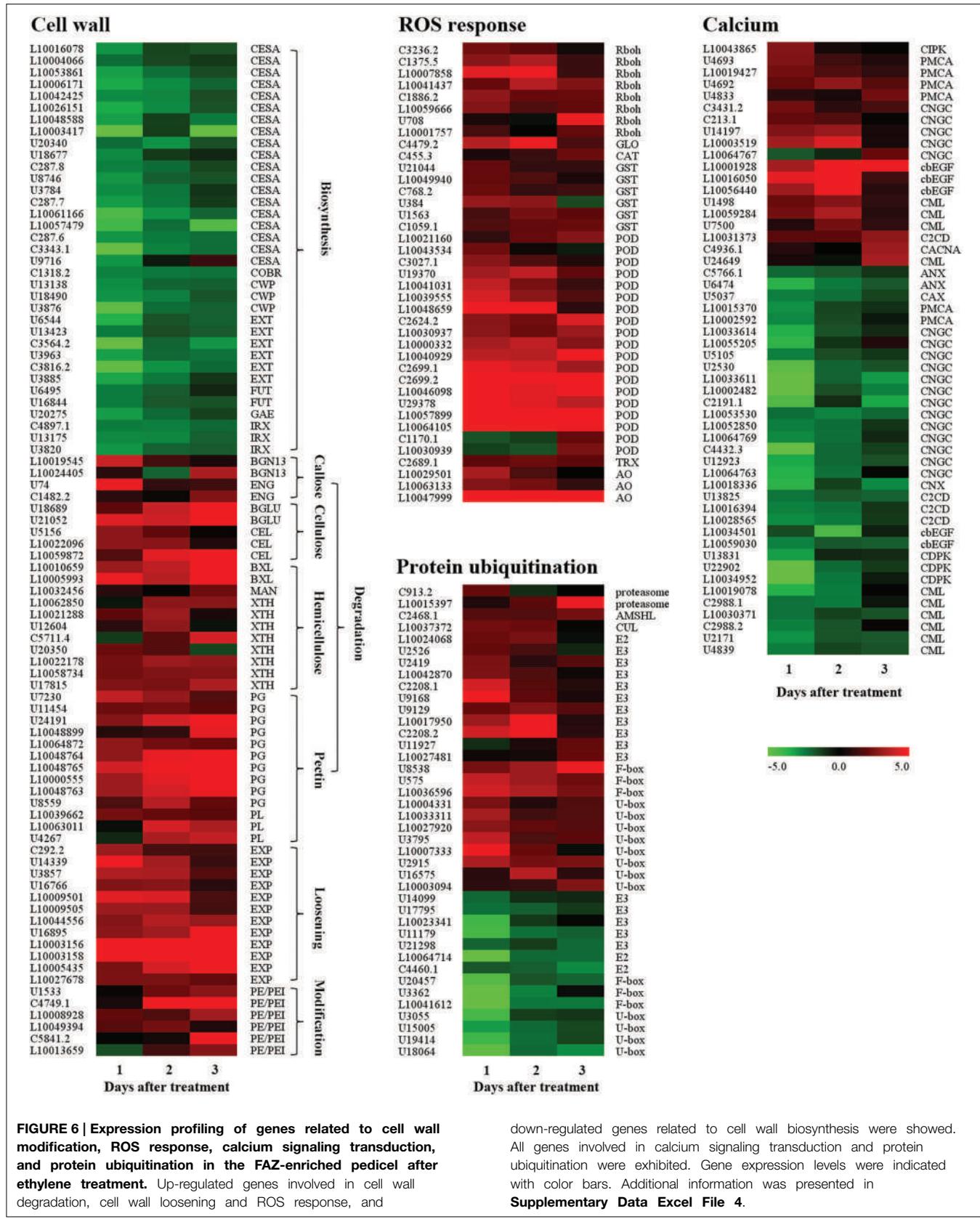


FIGURE 6 | Expression profiling of genes related to cell wall modification, ROS response, calcium signaling transduction, and protein ubiquitination in the FAZ-enriched pedicel after ethylene treatment. Up-regulated genes involved in cell wall degradation, cell wall loosening and ROS response, and

down-regulated genes related to cell wall biosynthesis were showed. All genes involved in calcium signaling transduction and protein ubiquitination were exhibited. Gene expression levels were indicated with color bars. Additional information was presented in **Supplementary Data Excel File 4**.

evolution in litchi. It is speculated that the mechanism of fruitlet abscission after treatment with exogenous ethephon may relate to the action of ethylene. However, a deep knowledge of the molecular events occurring in FAZ during fruit abscission induced by ethephon is still unknown. Although our previous study cloned a *LcPG1* gene and found its expression in FAZ was paralleled with the alteration of fruitlet abscission in litchi, induced by the ethephon treatment and inhibited by spraying 2,4-dichlorophenoxyacetic acid (2,4-D) (Peng et al., 2013). So this work mainly focused on genome-wide mining putative fruit abscission related genes regulated by ethylene in litchi.

Litchi fruit growth could be divided into two stages (Li et al., 2003). The first Stage constitutes about two thirds of the whole fruit growth cycle, which is the phase mainly characterized by the growth of pericarp and seedcoat; and the second Stage is the phase mainly characterized by the growth of embryo and the rapid aril growth. In the case of "Feizixiao" litchi used in our study, it needs 70–75 days from female flowering to a mature fruit, which can fluctuate some depending on the female opening date (Li et al., 2004). Fruit weight is about 25–30 g at maturity, but it is approximately 1.0 g when fruit develops at 25 days post anthesis (DPA) which is the time of the ETH treatment in this study. There were three to four waves of physiological fruit drop throughout fruit development in 70–90 days depending on cultivars (Yuan and Huang, 1988). Wave I, wave II, and wave III of abscission occurred around 1 week, 3 weeks and 6 weeks after anthesis, respectively. For a normal inflorescence of "Feizixiao" litchi, there are about 500–800 female flowers. Only 10% of them may set fruit successfully at 1 week after anthesis (wave I), and 30–50% of the surviving fruitlet will drop during wave II, after which no abscission will occur for the next 2–3 weeks. This study focuses on fruitlet abscission occurred at the second wave. The first 3 days after treatment might be coincidence with the peak of fruit drop wave II. It is not surprising that ~50% of the control fruitlet abscised between 25 and 28 DPA. Moreover, after 28 DPA, the remaining 50% of the fruitlet in the control treatment did not abscise over the next 2–3 weeks. ETH treatment, however, induced abscission of 100% of the fruitlet by 28 DPA. Thus, the ETH treatment largely magnified the second physiological fruit drop, which inducing a significantly higher rate of fruitlet abscission. This is exactly the biological effect expected for ETH. Moreover, ethylene production in fruit between the control and the ETH treatment had the substantial difference. Ethylene production in the control fruitlet remained more or less flat and kept below $8 \mu\text{l}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$ in the period of treatment, while which in ETH-treated fruitlet increased rapidly at 1 d after treatment and achieved nearly a ten-fold higher at 3 d when compared with the control. When the pH is above 4.0, ethephon slowly decomposes to release ethylene. The increase in ethylene production was the results of endogenous synthesis and ethephon release, which probably accelerates the fruit drop. In addition, the CFAR had no difference with the control in the first day after the ETH treatment, after then, the CFAR was sharply increased and significantly higher than that of the control. There were two obvious stages during the fruitlet abscission induced by ethephon: the early induction (0–1 d after treatment) that might induce acquisition of ethylene sensitivity and abscission

competence, and the late induction (1–3 d after treatment) that might lead to the execution of fruitlet abscission and formation of the defense layer.

A total of 6167 significantly DEGs were screened as ethylene-responsive genes and 2730 of them were identified as candidate genes involved in the fruitlet abscission process. Over 85% of the candidate genes displayed a significant transient change during the early ethylene-induction. It is generally accepted that ethylene operates as an activator, while auxin act as retardants (Roberts et al., 2002). In agreement with this supposition, 115 out of 195 candidate hormone related genes were involved in biosynthesis and signaling pathway of ethylene and auxin. These evidences were supported by the high expression levels of ethylene signal pathway related genes such as *ETR2*, *EBF*, *EIN3/EIL* and a class of *ERF* TFs, as previously demonstrated by John-Karuppiah and Burns (2010) in sweet orange fruit and leave abscission zone; and the repressed expression of transcript levels for auxin polar transport carriers (*PIN* and *AUX1*) and auxin responsive genes (*TIR1*, *Aux/IAA*, *ARF*, and *SAUR*). A decline in the abundance of auxin efflux carrier might be responsible for fruitlet abscission induced by shading and NAA in apple (Zhu et al., 2011) and mature-fruit abscission in melon (Corbacho et al., 2013). Moreover, Zhu et al. (2011) found that genes involved with cytokinin and gibberellic acid (GA) signaling pathways were down-regulated by shading and NAA in apple fruitlet FAZ. Similarly, a strongly decreased expression of large number of genes related to cytokinin and GA biosynthesis and signaling, such as *CYP735A*, *AHK*, *AHP*, *GA20ox*, and *GID1*, were also found in our study, probably implying that the metabolism of the cytokinin and GA in FAZ were affected in the early ethephon-promoted abscission process. On the other hand, it has been proposed that ABA and JA might be correlated with the abscission activation in citrus fruitlet (Gomez-Cadenas et al., 2000) or leaves (Agustí et al., 2009). They exhibited exactly the opposite results on mature fruit abscission, six of eight DEGs involved in ABA biosynthesis were up-regulated in melon (Corbacho et al., 2013), and six out of the seven DEGs were down-regulated in olive (Gil-Amado and Gomez-Jimenez, 2013). Our result was quite different from them, half of the eight DEGs involved in ABA biosynthesis showed increased transcript abundance during the ethephon-promoted fruitlet abscission in this study. All together, the mentioned above plant hormones were involved in the process of fruitlet abscission induced by the ETH treatment and the most important hormones were ethylene and auxin.

TFs are concerned as major switches of regulatory cascades during development, and the changes in the expression of such genes may affect various biological processes (Riechmann et al., 2000). A total of 185 different TF genes transcript such as *AP2/ERF*, *Aux/IAA*, *bHLH*, *MYB*, *WRKY*, *NAC*, *LBD*, and *HD-ZIP*, were affected by the ETH treatment. Those thought to be directly involved in ethylene and auxin signal transduction, such as *AP2/ERF*, *Aux/IAA*, and *ARF*, were already discussed before. The expression of most genes belonging to the family of *bHLH* was sharply down-regulation, and it was consist with the reports in the flower abscission zone in tomato after flower removal (Meir et al., 2010). Other up-regulated TFs in our work such

as NAC and WRKY, were previously reported to be involved in mature fruit abscission in melon and olive (Corbacho et al., 2013; Gil-Amado and Gomez-Jimenez, 2013). Many reports have shown that numerous characterized NAC and WRKY genes are involved in response to environmental stimuli and play various roles in response to biotic and abiotic stress (He et al., 2005; Jensen et al., 2010; Zhao et al., 2012). Thus, these up-regulated NAC and WRKY genes might be similar to ethylene- or stress-induced TFs found in other species (Yang et al., 2009; Jensen et al., 2010) and putatively involved in the downstream of ethylene signaling. Concerning MYB, Corbacho et al. (2013) reported that MYB was the most represent up-regulated TFs during the late mature-fruit abscission in melon, while only 7 members of the 15 affected MYB genes were induced in litchi. All these differential expression of genes encoding TFs belonging to different families might act as early regulators of the abscission induction, but the exact roles of these regulatory factors responding to ethylene induction remain to be further investigated.

ROS are versatile molecules related to a wide range of cellular processes, including programmed cell death, development, and hormonal signaling (Kwak et al., 2006). Previously, some reports supported a link between ROS and abscission. In tobacco, Henry et al. (1974) found that POD activity was increased during the ethylene-induced pedicel abscission. In tomato, delayed abscission of flowers and fruits was related to the increase of ROS-scavenging enzymes (Djanaguiraman et al., 2004). In pepper, Sakamoto et al. (2008) reported that H₂O₂ was involved in stress-induced petioles abscission, indicating that H₂O₂ acts downstream abscission signaling from ethylene. In ethylene-treated citrus leaves, a set of transcripts belonging to the ROS scavenging machinery have been reported to be over-represented in petioles rather than the laminar abscission zone (Agustí et al., 2008, 2009). Results in this study showed that a number of *Rboh* (role in ROS production) and genes encoding ROS scavenging enzymes were induced, suggesting the burst of ROS caused by ethephon treatment.

Calcium has been considered as an important intracellular messenger in plants and is essential for the maintenance of structural integrity of biomembrane and cell wall (Poovaiah and Rasmussen, 1973), and is also required for a variety of ethylene-dependent abscission processes (Raz and Fluhr, 1992). Xu et al. (2009) reported that direct application of calcium on tomato pedicel explants under ethylene would accelerate abscission but there are a number of reports describing that calcium delayed organ abscission (Poovaiah and Leopold, 1973; Beyer and Quebedeaux, 1974; Iwahori and Van Steveninck, 1988). In fact, Poovaiah and Rasmussen (1973) showed that ethephon treatment for bean leaf explants decreased calcium level in the AZ just prior to separation. The role of this element in organ abscission is still controversial. But how does the calcium signaling communicate with the fruit abscission? Our results showed that ethephon treatment up-regulated genes encoding calcium efflux carrier proteins (*PMCA*), down-regulated different genes encoding calcium influx carrier proteins such as cyclic nucleotide-gated channel (CNGC) genes and calcium responsive genes (*CML* and *CDPK*) in the litchi FAZ-enriched pedicel. These molecular results strongly suggested that regulating calcium

influx and maintaining calcium level in the FAZ might be associated with the onset of ethephon-promoted litchi fruitlet abscission process. It is speculated that ethylene might lead to a high extracellular calcium level in FAZ, and result in the deposition of calcium on cell wall and the deficiency on the cell inside.

Ubiquitylation-dependent proteolysis is a major event during both the induction and execution of cell death (Estelle, 2001), and could be triggered by H₂O₂ in tobacco (Vandenabeele et al., 2003). In ethylene-treated citrus leaves, a group of transcripts involved in the ubiquitin/proteasome system have been reported to be induced in both laminar abscission zone and petiolar cortical tissue (Agustí et al., 2008, 2009). The up-regulation of both ubiquitin-conjugating enzymes (E2) and ubiquitin-protein ligases (E3) after the ETH treatment in litchi FAZ-enriched pedicel, suggested that a similar proteolytic mechanism might be involved in ethephon-induced abscission. Also, two 26S proteasome components and a large number of proteins with F-box and U-box domain like potential E3 ligases were induced by ethephon. In *Arabidopsis*, abscission of floral organs is arrested with suppressed expression of a F-box protein (González-Carranza et al., 2007). These observations strongly suggested that a general proteasome-related mechanism might play a role in ethephon-induced abscission.

Zhu et al. (2011) found both shading and NAA treatment for apple tree resulted in a large number of photosynthesis-related genes were down-regulated in the FAZ. For instance, 90 out of the 94 DEGs were repressed in shading-treated FAZ. In our study, 103 out of the 110 DEGs involved in photosynthesis pathway were strongly decreased. These results also indicated that photosynthesis was one of the GO terms enriched in FAZ. The structural features of AZ cells were described by Sexton and Roberts (1982) as densely protoplasmic, with small intercellular spaces, containing large deposits of starch, and with a high density of branched plasmodesmata. Then, AZ cells should not contain photosynthetically active chloroplasts. However, hand-dissected AZ-enriched young and green fruitlet pedicel (see **Supplementary Figure 1**) used in our study should contain photosynthetically active chloroplasts, which is why so many down-regulated of photosynthesis-related genes were found. In order to uncover the abscission-associated metabolism of AZ cells we should take a laser capture microdissection (LCM) approach to obtain AZ-specific cells sample for performing an accurate study of the abscission events in the future. Thus, the roles of these genes on fruit abscission need to be further evaluated.

Some evidences supported a strong connection between the carbohydrate amounts available for the fruit and their probability of abscission (Yuan and Huang, 1988; Iglesias et al., 2003; Zhou et al., 2008). One of the reasons of abscission may be due to a lack of carbohydrate. Not surprisingly, ETH treatment also affected the carbohydrate and energy metabolism. Affected genes within these groups include those associated with glycolysis, TCA cycle and ATPase. These results indicated that ethephon might lead to the carbohydrate stress and the interdiction of ATP synthesis during the early abscission induction. Moreover, an increased ROS production may be linked to the inhibition of ATPase, as a

consequence to the mitochondrial damage (Roy et al., 2008), and the release of cytochrome c from mitochondria, which have been implicated as regulator of programmed cell death (Tiwari et al., 2002).

After the early induction, there was a lot of fruitlet dropped in paralleled with a continuous highly release of ethylene yield during the late induction (1–3 d after ETH treatment). Ethylene may act as the signal generated within the fruit, FAZ, or released from ethephon, through diffusion, triggering abscission event. It was supported by the abundant of several transcripts (*ACS*, *ACO*, *ERF*) involved in ethylene biosynthesis and transductive pathway. Several up-regulated genes were linked to ABA biosynthesis and signaling transduction, such as *ZEP* and *PP2C*. It is proposed that ABA might be corrected with the late stage of abscission process. Similarly, ethylene appeared to be a positive regulator of SA action during the abscission induction, since the expression level of *SBP* and *NPR1* genes were highly up-regulated, as found during melon mature-fruit

abscission (Corbacho et al., 2013). It assumed that SA might be as an endogenous signaling molecule for stress response or the formation of protective layer after AZ cell separation. Homologs of *LBD* and *WRKY* TFs were also highly expressed at 1–3 d after ETH treatment, showing similar change to that reported in tomato flower abscission zone (Wang et al., 2013). The *LBD* family played a possible role in lateral meristem initiation (Shuai et al., 2002), while the *WRKY* family hold central positions mediating the regulation of disease resistance (Robatzek and Somssich, 2001). These TFs might have functions in stress defense and the formation of protective layers after fruitlet abscission. For fruit to be shed, cell separation must occur in FAZ, and the abscission is paralleled with intercellular space increase and middle lamella lysis in the FAZ which is the result of the degradation and/or remodeling of cell wall (Lee et al., 2008; Bowling and Vaughn, 2011). There were 43 cell wall degradation and loosing related transcripts up-regulated and 29 cell wall biosynthesis related genes down-regulated, and most

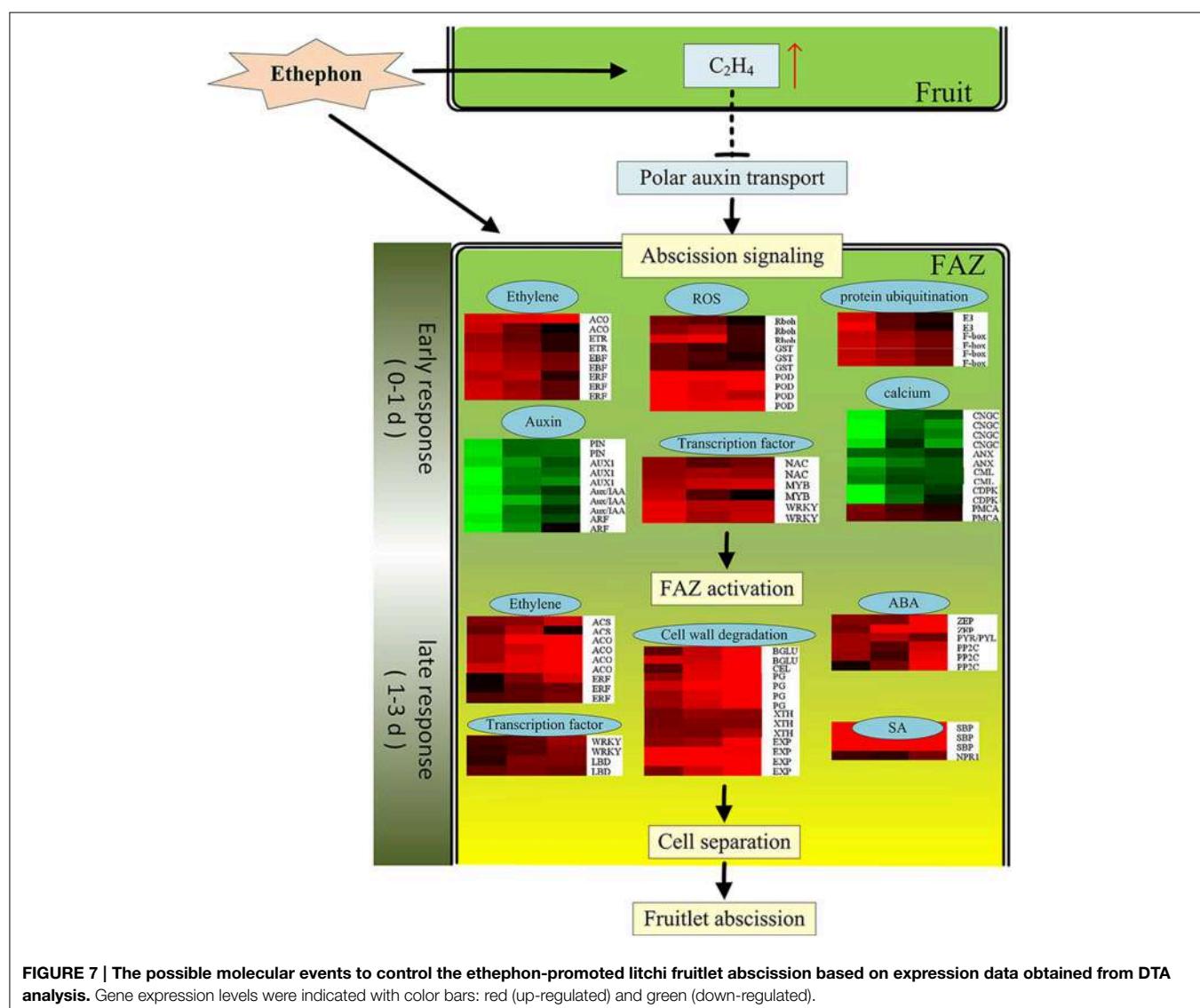


FIGURE 7 | The possible molecular events to control the ethephon-promoted litchi fruitlet abscission based on expression data obtained from DTA analysis. Gene expression levels were indicated with color bars: red (up-regulated) and green (down-regulated).

of them had significantly differential expression throughout the whole ethephon treatment, indicating that these genes might be involved in the process of fruitlet abscission induced by ethephon. Several researchers have already reported that the expression of genes encoding for cell wall-hydrolyzing enzymes was associated with abscission and also regulated by ethylene (Lashbrook et al., 1994; Kalaitzis et al., 1997; Roberts and Gonzalez-Carranza, 2009). However, 92 down-regulated genes related to cell wall degradation and loosing, and 15 up-regulated genes related to cell wall biosynthesis were also found in our study, which might be involved in the new cell wall synthesis and reconstruction for the formation of protective layers after fruitlet abscission.

This study only focus on the gene expression profile occurring in the FAZ-enriched pedicel during litchi fruit abscission induced by ethephon. In conclusion, a total of 2730 candidate genes were involved in the process of litchi fruit abscission induced by ethephon treatment. A preliminary molecular regulatory scheme was herein prompted out for litchi fruitlet abscission induced by ethephon based on our results (Figure 7). At the early beginning, the ethylene evolution in fruitlet was greatly increased by ETH treatment, which would suppress the synthesis and polar transport of auxin and trigger abscission signaling. At the same time, FAZ might perceive the abscission signals, and then, 1867 early-responsive genes were up- or down-regulated from 0 to 1 d after ETH treatment. The most affected genes included those related to ethylene biosynthesis and signaling, auxin transport and signaling, TFs, protein ubiquitination, ROS response, calcium signal transduction and etc... Then, a lot of genes related to cell wall degradation, TFs, ethylene, ABA and JA biosynthesis and signaling cascade were up-regulated. At the last, cell separation happened and the fruitlet abscission was enhanced. To the best of our knowledge, this study provides the first global monitoring of gene expression changes occurring in FAZ-enriched pedicel during litchi fruit abscission.

Author Contributions

JL was responsible for the overall concept and experimental design, and revising and finalizing the manuscript. CL carried out ethephon treatment, DTA data integration and analysis, performed qRT-PCR experiments, and drafted the manuscript. YW was responsible for bioinformatics analysis. PY and WM performed the ethephon treatment and sample collection. All the authors read and approved the final manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00502>

Supplementary Figure 1 | The pedicel of a litchi fruitlet showing the pre-existing fruit abscission zone (FAZ) with a visible sunken line (thick arrow). The section between the solid lines (~2 mm) was sample for DTA analysis.

Supplementary Figure 2 | Functional enrichment analysis for ethephon-responsive genes. **(A)** Enriched GO terms. Hierarchical tree graph of enriched GO terms in agriGO were used to simplify the analysis. The color bar shows the statistical significance, with enrichment significance level $FDR \leq 0.05$. **(B)** KEGG pathway enrichment analysis. The top 20 enriched pathway are showed. The color bar shows the statistical significance, with enrichment significance level $Q\text{-value} \leq 0.05$. The size of the circle is proportional to the number of genes in the KEGG pathway.

Supplementary Data Excel File 1 | Specific primers details for 41 genes selected for qRT-PCR. Representative genes selected for the analysis were those involved in plant hormone regulation pathway, cell wall modification, transcription factors regulation, ROS, and calcium signaling. All the gene ID, forward, and reverse primer sequences are included.

Supplementary Data Excel File 2 | List of 6167 differential expressed genes in the three pair-comparisons (CK1 vs. ETH1, CK2 vs. ETH2, and CK3 vs. ETH3). We used a false discovery rate ($FDR \leq 0.001$) and the absolute value of \log_2 ratio ≥ 2 as the threshold to judge the significance of transcript abundance differences, and then eliminated the control background influences (CK0 vs. CK1, CK0 vs. CK2, and CK0 vs. CK3).

Supplementary Data Excel File 3 | List of 2730 candidate genes involved in the ethephon-promoted fruitlet abscission process by GO ($FDR \leq 0.05$) and KEGG ($Q\text{-value} \leq 0.05$) enrichment analysis.

Supplementary Data Excel File 4 | Plant hormones-related genes induced or repressed in FAZ-enriched pedicel after ethephon treatment.

Supplementary Data Excel File 5 | Transcription factor genes induced or repressed in FAZ-enriched pedicel after ethephon treatment.

Supplementary Data Excel File 6 | Cell wall-related genes induced or repressed in FAZ-enriched pedicel after ethephon treatment.

Supplementary Data Excel File 7 | Photosynthesis, carbohydrate metabolism, and mitochondria-related genes induced or repressed in FAZ-enriched pedicel after ethephon treatment.

Supplementary Data Excel File 8 | ROS, calcium, and protein ubiquitination-related genes induced or repressed in FAZ-enriched pedicel after ethephon treatment.

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Cellular and Pectin Dynamics during Abscission Zone Development and Ripe Fruit Abscission of the Monocot Oil Palm

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The oil palm (*Elaeis guineensis* Jacq.) fruit primary abscission zone (AZ) is a multi-cell layered boundary region between the pedicel (P) and mesocarp (M) tissues. To examine the cellular processes that occur during the development and function of the AZ cell layers, we employed multiple histological and immunohistochemical methods combined with confocal, electron and Fourier-transform infrared (FT-IR) microspectroscopy approaches. During early fruit development and differentiation of the AZ, the orientation of cell divisions in the AZ was periclinal compared with anticlinal divisions in the P and M. AZ cell wall width increased earlier during development suggesting cell wall assembly occurred more rapidly in the AZ than the adjacent P and M tissues. The developing fruit AZ contain numerous intra-AZ cell layer plasmodesmata (PD), but very few inter-AZ cell layer PD. In the AZ of ripening fruit, PD were less frequent, wider, and mainly intra-AZ cell layer localized. Furthermore, DAPI staining revealed nuclei are located adjacent to PD and are remarkably aligned within AZ layer cells, and remain aligned and intact after cell separation. The polarized accumulation of ribosomes, rough endoplasmic reticulum, mitochondria, and vesicles suggested active secretion at the tip of AZ cells occurred during development which may contribute to the striated cell wall patterns in the AZ cell layers. AZ cells accumulated intracellular pectin during development, which appear to be released and/or degraded during cell separation. The signal for the JIM5 epitope, that recognizes low methylesterified and un-methylesterified homogalacturonan (HG), increased in the AZ layer cell walls prior to separation and dramatically increased on the separated AZ cell surfaces. Finally, FT-IR microspectroscopy analysis indicated a decrease in methylesterified HG occurred in AZ cell walls during separation, which may partially explain an increase in the JIM5 epitope signal. The results obtained through a multi-imaging approach allow an integrated view of the dynamic developmental processes that occur in a multi-layered boundary AZ

and provide evidence for distinct regulatory mechanisms that underlie oil palm fruit AZ development and function.

Keywords: fruit abscission, abscission zone, pectin, oil palm, cell wall

INTRODUCTION

Plant organ abscission is a highly regulated developmental process that results in the loss of various organs throughout the life cycle of the plant. The abscission process is complex with many overlapping points of regulation, and involves the integration of multiple external and internal signals that depend on the overall status of the plant (Roberts et al., 2002; Estornell et al., 2013). In particular, seed and fruit abscission are important to coordinate seed dispersal for plant reproductive success. If fruit are shed prematurely before seed development is complete, or too late in relation to seasonal climate changes, reproductive success can be jeopardized. For crop species, seed and fruit abscission are important traits to consider, if seed or fruit abscission occur too early or late, the economic consequences can be significant.

Central to the process is the role of the abscission zone (AZ) where cell separation occurs that leads to organ detachment. Cell separation that occurs during abscission is a tissue and cellular process, which involves the differentiation of the AZ located at the base of the organ to be shed. Generally, the plant AZ consists of one to several cell layers but can also consist of multiple layers as observed in the leaflet rachis of *Sambucus nigra* (common elder) with up to 30–40 layers (Osborne and Sargent, 1976). AZ cells are typically isodiametrically shaped with dense cytoplasms (Addicott, 1982; Sexton and Roberts, 1982; Roberts et al., 2002). Neighboring cells are joined together by the middle lamella composed primarily of pectin, the most structurally complex family of cell wall polysaccharides and a major component of primary walls of both monocots and dicots (Mohnen, 2008). Pectin, principally homogalacturonan (HG), is also the main component of the middle lamella between adjacent cells and is of paramount importance for cell adhesion and during cell separation (Willats et al., 2001a; Jarvis et al., 2003; Ogawa et al., 2009; Iwai et al., 2013; Daher and Braybrook, 2015). In addition, pectin derived oligogalacturonide degradation products can also act as signaling molecules, possibly through the action of ethylene (Baldwin and Biggs, 1988; Brecht and Huber, 1988; Campbell and Labavitch, 1991; Melotto et al., 1994; Ridley et al., 2001). However, the structural characteristics of pectin and how it functions during cell separation underlying organ abscission are not completely understood.

The methylesterification of HG plays an important role during plant development, can modulate the functionality of pectin, in particular for cell adhesion and for cell separation to occur (Willats et al., 2001b; Jarvis et al., 2003; Mouille et al., 2007). HG is thought to be synthesized in the Golgi complex, targeted through vesicles via the plasma membrane to the apoplast and finally inserted into the cell wall in a highly methylesterified form (Zhang and Staehelin, 1992; Atmodjo et al., 2013). After cell divisions, pectin undergoes demethylesterification at cell junctions where cell separation takes place for intercellular space formation (Willats et al., 2001b;

Jarvis et al., 2003). The demethylesterification of HG is catalyzed by pectin methylesterases (PME, EC 3.1.1.11), which modulate HG methylation status and consequently plant development (Wolf et al., 2009). It is believed that demethylesterification allows the formation of calcium (Ca^{2+}) cross-links and the “egg-box” pectin configuration between adjacent HG polymers, which can lead to the formation of rigid pectin gels or HG degradation by pectin degrading polygalacturonases (PGs, EC 3.2.1.15) (Grant et al., 1973; Cosgrove, 2005; Senechal et al., 2014). Indeed, PGs modify the texture and rigidity of the cell wall and also have roles during cell separation processes such as those controlling organ abscission (Hadfield et al., 1998; Ogawa et al., 2009; Swain et al., 2011).

The oil palm (*Elaeis guineensis* Jacq.) fruit has two types of AZs, one large multilayer primary AZ and up to four adjacent AZs that are less distinguishable. The primary AZ is in the boundary between the pedicel and mesocarp tissues at the base of the oil palm fruit, while the adjacent AZs are at the periphery of the primary AZ at the base of the outer whorl organs including the rudimentary androecium, tepals, and the bracteole (Henderson and Osborne, 1990, 1994; Henderson et al., 2001). The primary AZ of ripe oil palm fruit has high levels of un-methylesterified pectin proposed to contribute to the spatial specificity of cell separation (Henderson et al., 2001). Separation takes place first in the primary AZ while adjacent less distinguishable AZs separate only after the primary zone has separated, which suggested a signal generated in the primary AZ is necessary for adjacent AZ cells to separate (Henderson and Osborne, 1994). The primary AZ is larger and clearly more visible than the adjacent AZs, which are far less distinguishable. A previous study indicated that the primary AZ arises early during floral development, but more detailed information about the morphogenesis of this exceptionally large primary AZ is lacking (Henderson and Osborne, 1990).

Previous data showed the AZ response to ethylene depends on the stage of fruit development; the ripest fruit AZ responds quickest and function to allow rapid cell separation (Roongsattham et al., 2012). The objectives of this work were to examine in detail the intra- and extra-cellular changes during the development and function of the ripe fruit AZ to provide insight into the underlying mechanisms. We use histological and immunohistochemical methods combined with a multi-imaging approach including confocal, electron, and Fourier-transformed infrared microscopy to examine the cellular characteristics of the oil palm fruit AZ during development and abscission.

MATERIALS AND METHODS

Plant Material

Oil palm *E. guineensis* fruits (type *dura* of Deli Dabou origin) from CRA-PP Pobé plantation, Benin were used for classical histology experiments. Oil palm *E. guineensis* fruits (type *tenera*

clone C) from Golden Tenera Plantation in Krabi Province, Thailand were used for immunohistochemistry experiments. Fruit sampled at different stages of development were selected from bunches as described previously (Roongsattham et al., 2012).

Light Microscopy and Image Acquisition

Samples were fixed in 0.2 M phosphate buffer containing 2% (w/v) paraformaldehyde, 1% (w/v) caffeine, and 2% (v/v) glutaraldehyde 25% for a minimum of 2 days at 4°C as previously described (Buffard-Morel et al., 1992). Serial dehydration with ethanol (EtOH) from 30 to 100%, then 100% butanol/100% EtOH (v/v), and finally 100% butanol was performed for each sample and followed by impregnation and embedding in Technovit 7100 resin (Heraeus Kulzer). Semi-thin sections of 3 µm were cut using a microtome. Each section was stained with toluidine blue, ruthenium red, alcian blue (AB), AB, and periodic acid-Schiff (ABS). Ruthenium red is a commonly used dye to visualize pectin, which selectively binds to the intramolecular spaces of carboxyl groups of pectin (Sterling, 1970; Hou et al., 1999; Leroux et al., 2007). Toluidine blue is a metachromasia compound that stains lignin and phenols to bluish-green with a pH-independent covalent bonding and non-lignin cell wall components to reddish-violet (acid; e.g., pectin) and bluish-violet (neutral; Conrad, 2008). For ABS, sections were double-stained with Periodic Acid-Schiff (PAS) reagent, combined with AB. A description of the stains used in the study is included in Supplementary Table 1. Photomicrographs were taken with a Leica camera on a Leica (LEITZ DMRB) light microscope (x20/0.5; x40/0.7; and x100/1.3). Samples were taken and observations were made with fruit bases containing the AZ from at least three different fruit collected.

Electron Microscopy

The samples were prepared and analyzed as described previously (Verdeil et al., 2001). Samples taken from the central cell layers in the center of the primary AZ between vascular strand parenchyma cells (1 mm³ cubes of tissue) were fixed in a 0.05 M Sorensen buffer solution (pH 7.3) containing 2.5% glutaraldehyde and gently stirred for 16 h at 4°C. Cubes were then rapidly rinsed with distilled water (3 × 10 min), then post-fixed in 1% aqueous osmium tetroxide containing 3% sucrose for 2 h at 20°C in the dark. They were then dehydrated in an EtOH dilution series (30, 50, 70, and 90% EtOH; 10 min each) and finally for 15 min in pure EtOH; samples were then embedded in Epon EmBed 812 using an Automated Microwave Tissue Processor (Leica EM AMW). Ultrathin sections (thickness around 80 nm) were obtained with a Leica-Reichert Ultracut E ultramicrotome, and then stained with uranyl acetate in EtOH. Sections were then mounted on Ni-grids and examined with a Hitachi 7100 electron microscope.

Immunohistochemistry

Samples (base of the fruit including the AZ) were fixed in 1X Phosphate-buffered saline (PBS) buffer containing 4% (w/v) paraformaldehyde for a maximum of 16 h at 4°C in dark. After fixation, samples were washed twice in 1X PBS buffer with glycine (2%), then twice in 1X PBS buffer. The samples were

then dehydrated in EtOH (incubations in 50% EtOH for 1 and 2 h, then 70% EtOH for 1 and 2 h at RT) and samples were preserved in 70% EtOH at 4°C. Stored tissues were cut at 150 µm thickness with a vibratome (Microm HM 650V, Walldorf, Germany). The sections were placed in glass wells containing PBS solution (pH 7.4), then treated with blocking buffer (BB; 6% bovine serum albumin):PBS = 1:1 (500–1000 µl) and shook gently on a shaker for 3 h at room RT. BB:PBS:primary rat antibody (JIM5, JIM7, LM7, or LM8; Willats et al., 2000, 2004; Clausen et al., 2003) at 9:9:2 was prepared. The PBS-BB solution was removed and replaced with BB:PBS:primary rat antibody. Samples were agitated gently on a shaker overnight (18 h) at 4°C. After incubation, the BB:PBS:primary rat antibody solution was removed and replaced with PBS solution and shook gently on a shaker for 15 min, then repeat this step two times. After this step, all preparations were performed in the dark. Prepare BB:PBS:secondary antibody (Alexa Fluor® 546 Goat Anti-Rat, INVITROGEN, <https://www.lifetechnologies.com>) at 499:499:2. Alexa 546 was chosen because minimal autofluorescence was found in oil palm fruit tissue corresponding to the windows of spectral emission of this dye. The tissue observation was performed on a Zeiss LSM 510 Meta Confocal Microscope. Microscope imaging was performed at IGH, Montpellier RIO Imaging Platform (www.mri.cnrs.fr) with a multiphoton laser scanning Axiovert 200M 510 META NLO microscope (Carl Zeiss MicroImaging, Jena, Germany) using the multitrack image acquisition. Excitation was at 543 nm with laser power 65% for Alexa 546 and at 405 nm with laser power 7% for DAPI, a fluorescent nuclear stain that binds to A/T rich DNA repeats, with an emission peak of 460 nm (Kapuscinski, 1995). Sections were observed with an immersion objective C-Apochromat 40X/1.2 W and pictures were acquired using the ZEISS LSM image browser software (Carl Zeiss MicroImaging GmbH Standort Göttingen—Vertrieb Deutschland, Göttingen, Germany; www.zeiss.com). A description of the antibodies used in the study is included in Supplementary Table 1.

Cell Parameter Measurements

Cell parameter measurements were performed for cell width, cell wall width, and middle lamella width by using the ImageJ program (<http://rsb.info.nih.gov/ij/>). The cell width measurements were performed from the histology samples stained with toluidine blue or ruthenium red. The cell wall and the middle lamella width measurements were performed from the confocal microscope images from immunolocalization studies. The cell parameter data were statistically treated with one way ANOVA analyses and then Post Hoc tests were performed for multiple comparisons with DunnettT3 by using the Statistical Package for the Social Sciences (SPSS) Statistics 17.0 (<http://www-01.ibm.com/software/analytics/spss/>).

Fourier-Transform Infrared (FT-IR) Microspectroscopy and Degree of Esterification Measurements

The FT-IR microspectroscopy was performed as described previously using an IN10 Thermo Infrared Imaging system (Mouille et al., 2003). Sections of 50 µm were cut using a

vibratome. From the average spectra data for each sample, the degree of methylesterification (DME) was estimated using the formula $DME = A1740/(A1740 + A1630)$ described previously, where absorbance at 1630 cm^{-1} corresponds to the COO^- group and absorbance at 1740 corresponds to the carbonyl group from both COOH and COOCH_3 (Chatjigakis et al., 1998; Manrique and Lajolo, 2002; Gribaa et al., 2013). A total of 7–11 replicates for each sample were analyzed by FT-IR and used to quantify the DME. The A1630-value was obtained by the average between A1628 and A1632. Statistical significance of the DME between sample groups was calculated by ANOVA at $P = 0.01$.

RESULTS

Distinct Developmental Characters Accompany the Capacity for AZ Cells to Function in Ripe Fruit

In this study, we examined in detail the morphogenesis of the primary AZ compared to adjacent pedicel and mesocarp tissues. The results presented here are based on observations of at least three or more samples and the photos were selected to represent the most consistent features observed. Longitudinal sections through the base of the fruit were made to examine the cellular characteristics of the primary AZ compared with the adjacent mesocarp and pedicel tissues (Figures 1A–F). The primary AZ was easily observed at 30 days after pollination (DAP) in fruit sections stained with toluidine blue and consisted of small densely cytoplasmic cells as previously described (Figure 1A; Henderson and Osborne, 1990). At 30 DAP, vascular parenchyma tissues were not lignified in any of the tissues (Figure 1A). By 120 DAP, vascular bundles were differentiated and lignified in the pedicel and mesocarp, while lignification was interrupted in the AZ vascular bundles at both 120 and 180 DAP (Figures 1C,E, dots connected by white lines). As with a previous study, phenolic containing cells were observed near the primary AZ, but unlike the previous study, these specialized cells were also located within the primary AZ layers (Figures 1A,C,E, red arrows; Henderson and Osborne, 1990). The polyphenol-containing cells appeared larger by 120 DAP and were often found grouped in two or more cells (Figures 1C,E).

Longitudinal sections of 30 DAP fruit stained with DAPI revealed that the nuclei of the AZ layer cells were aligned, particularly in the developing vascular bundles at this stage, then became more visibly aligned throughout the primary AZ during development at 120 and 180 DAP (Figures 1B,D,F). At 30 DAP, recently divided cells in the mesocarp, AZ and pedicel tissues were observed (Figures 2A,D,G). In the AZ, the plane of recent cell division was periclinal (parallel to the outer fruit surface), while in the mesocarp and pedicel cell divisions were anticlinal (plane perpendicular to the outer fruit surface). At 120 DAP cell divisions were no longer evident, and cells appear elongated in all tissues, and AZ cells appeared narrower than mesocarp and pedicel cells (Figures 2B,E,H).

During development, AZ cells had dark staining intercellular spaces often located to the tips of cells suggesting concentrated areas of secretion related to cell wall biosynthesis activity

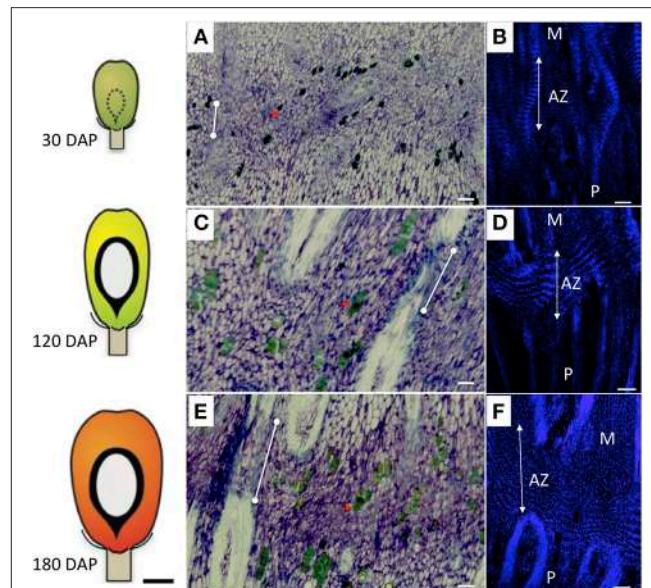


FIGURE 1 | Development of the AZ in the base of the oil palm fruit.

Longitudinal sections of oil palm fruit bases at 30 days after pollination (DAP, **A,B**), 120 DAP (**C,D**), and 180 DAP (**E,F**) were stained with toluidine blue (**A-C**) or DAPI (**B,D,F**). Schematic fruit diagrams to the left of photos correspond to the stages of development at which time the fruit tissues were collected (Tranbarger et al., 2011). The primary abscission zone (AZ) is depicted with dotted lines positioned between the mesocarp (M), and pedicel (P); red arrows, phenolic containing cells; dots connected by white lines, undifferentiated constricted vascular tissue; white lines with double arrows, nuclear alignment within AZ cell layers; Scale bars for photos are 100 μm ; Scale bar for schematic fruit diagrams is 10 mm.

(Figures 2E,F, 3). In contrast, mesocarp and pedicel cells had intercellular spaces that stain less intensively and appeared often to become regions of detachment that led to airspace formation (Willats et al., 2001b; Jarvis et al., 2003). Intercellular airspaces were rarely observed in the AZ cell layers (Figure 3). Also during development, dark staining particles in AZ cells appeared to be localized in the cytoplasm that became evident during ripening at 120 and 180 DAP. These intracellular particles appeared to contain pectin, based on coloration with both toluidine blue (Figures 2E,F) and ruthenium red staining (Figure 3). Similarly stained particles were not observed in either the mesocarp or pedicel cells at 120 or 180 DAP (Figures 2B,C,H,I, 3).

To complement this comparative study of the pedicel, AZ and mesocarp tissues, we measured several quantitative parameters of the cells including: cell width, cell wall width, and middle lamella width. The cell width measurement data from longitudinal sections revealed that at 30 DAP, the average width of a mesocarp cell was 28.29 μm and significantly increased to 36.67 μm at 120 DAP. In contrast, the average pedicel cell width was 30.94 μm at 30 DAP and did not significantly increase at 120 or 180 DAP while in the AZ, the average cell width was 16.05 μm at 30 DAP and significantly increased to 17.84 μm at 120 DAP and to a maximum of 21.87 μm at 180 DAP. Notably, the average AZ cell width was less than the other tissues at all developmental stages examined (Figure 3, Supplementary Tables 2–5).

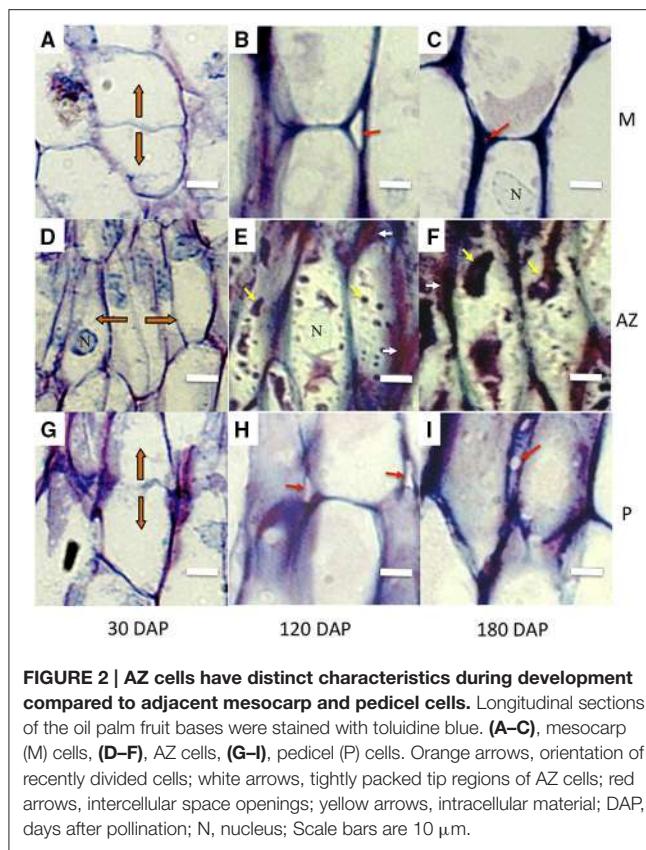


FIGURE 2 | AZ cells have distinct characteristics during development compared to adjacent mesocarp and pedicel cells. Longitudinal sections of the oil palm fruit bases were stained with toluidine blue. **(A–C)**, mesocarp (M) cells; **(D–F)**, AZ cells; **(G–I)**, pedicel (P) cells. Orange arrows, orientation of recently divided cells; white arrows, tightly packed tip regions of AZ cells; red arrows, intercellular space openings; yellow arrows, intracellular material; DAP, days after pollination; N, nucleus; Scale bars are 10 μm .

For the cell wall, the average mesocarp cell wall width was 0.59 μm at 30 DAP and significantly increased to 1.25 μm at 120 DAP and 2.59 μm at 180 DAP. In contrast, the average pedicel cell wall width was 1.05 μm at 30 DAP, did not change significantly at 120 DAP, but increased to 2.61 μm at 180 DAP. In contrast to these two adjacent tissues, the average AZ cell wall width was 0.91 μm at 30 DAP and significantly increased to 2.12 μm at 120 DAP, thicker than both the mesocarp and pedicel cells, which suggests more rapid cell wall biosynthesis occurs in the AZ between 30 and 120 DAP than the adjacent tissues. At 180 DAP, the AZ cell wall width was at a maximum of 2.58 μm , while all tissues display the maximum width and there was no statistically significant difference between the tissues at this stage (Figure 3, Supplementary Tables 6–9).

At 30 DAP the middle lamella width was too small to measure by our method at this early stage of development. At 120 DAP, the average mesocarp cell middle lamella width was 0.58 μm and significantly increased to 0.93 μm by 180 DAP. In the AZ, the average middle lamella width was 0.59 μm at 120 DAP and significantly increased to 0.94 μm by 180 DAP. In the pedicel, the average middle lamella width was 0.65 μm at 120 DAP and significantly increased to 0.99 μm by 180 DAP. Interestingly, the middle lamella width of each cell type was similar at each stage of development, and increased similarly between 120 and 180 DAP (Figure 3, Supplementary Tables 10–13).

To examine the ultrastructure changes that occur during AZ morphogenesis, electron microscopy was performed at 30 and

120 DAP. Thin longitudinal section preparations for electron microscopy analysis of the AZ of 30 and 120 DAP fruit stained reddish-violet with toluidine blue corroborating the presence of pectin material within AZ cells, and an increase cell wall width apparently rich in pectin, particularly at the tips of cells between AZ layers (Figures 4A,B). Electron microscopy analysis revealed a dramatic increase in cell wall width that occurs between 30 and 120 DAP, which corroborates the increase in cell wall width measured (Figures 3, 4A–D, 5A–D).

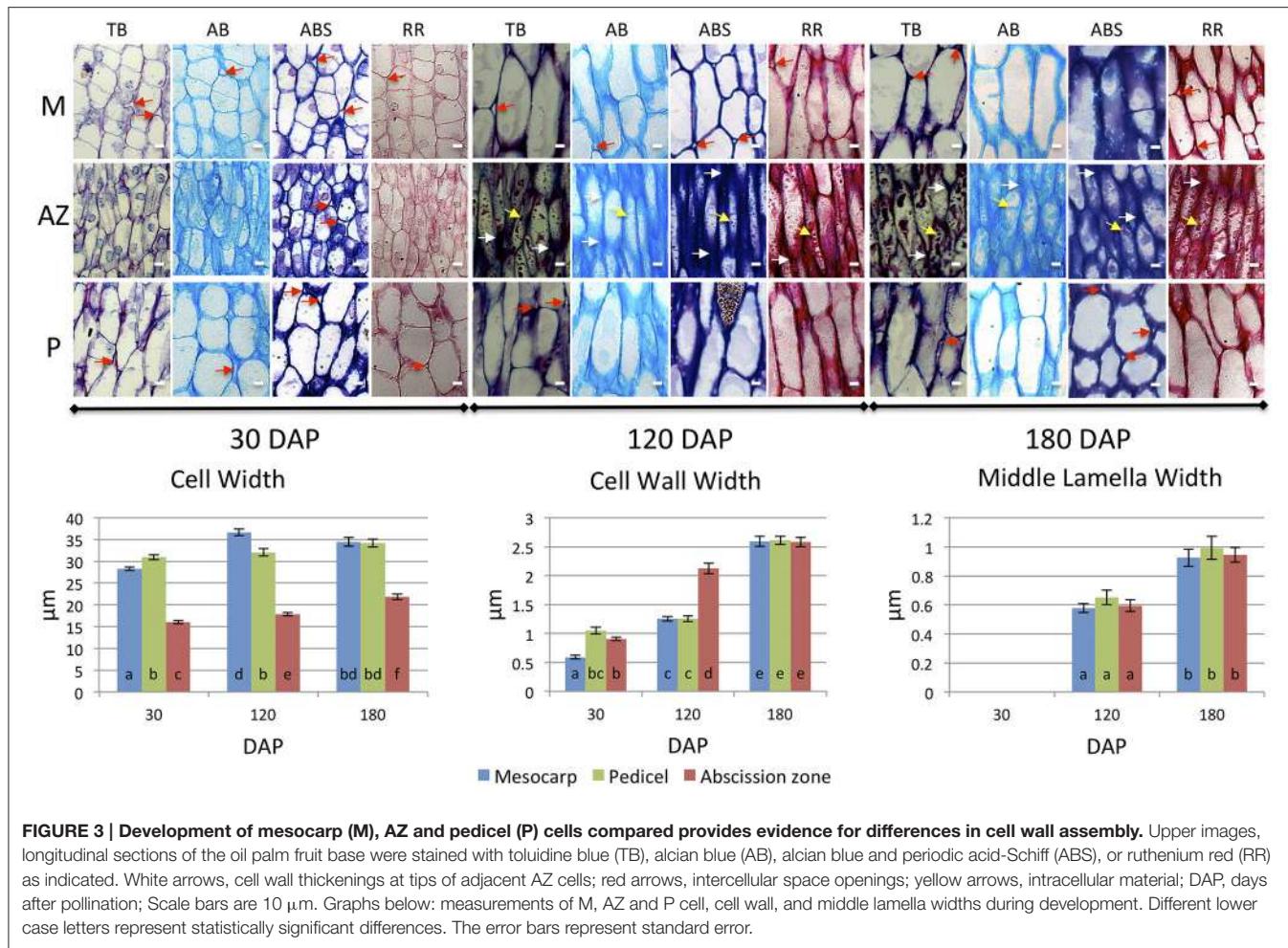
Accompanying these changes, AZ cell shape at 30 and 120 DAP cells were rectangular but more elongated at 120 DAP (Figures 4C,D). The AZ cell nuclei at 30 DAP were rounded or slightly elliptical, while those at 120 DAP were more amoeboid shaped (Figures 4E,F). Nucleoli could be observed at 30 DAP but were rarely found in 120 DAP (Figures 4E,F). At 30 DAP, nuclei with highly condensed chromatin were observed (Figure 4E). Interestingly, at the tip of the elongated AZ parenchyma cells, the cell wall was thickened with the appearance of multiple striated layers or ripples that could be either continuous with the middle lamella or the primary cell wall (Figure 5E). Finally, ribosomes, rough endoplasmic reticulum, mitochondria, and vesicles were observed at the periphery of the cytoplasm, in particular at the tip of the cells (Figure 5E), and a fibrous matrix can be found throughout the cell interior (Figures 4D,F, 5E). Taken together, these ultrastructural features suggest extensive cellular activity in the AZ elongated cell tips including high protein synthesis and metabolic activity possibly related to exocytosis.

Plasmodesmata (PD) in the cell walls at 30 DAP between cells in the same AZ layer (intra-layer PD) were frequently observed, but less frequent between cells in adjacent AZ layers (inter-layer PD, Figures 5A,C). PD appear to be grouped together in areas of cell wall constriction which became more pronounced by 120 DAP (Figures 5A–D). At 120 DAP, PD appeared less frequent but the constricted cell wall regions where PD were localized increased in width and were situated in the middle of the cell between cells of the same layer. The constricted regions with PD increased almost twofold between 30 and 120 DAP (Figures 5A–D). Nuclei were often closely associated to PD, while vesicle fusion to the plasma membrane could be observed adjacent to thickened cell walls at 120 DAP (Figure 5C).

Cellular Changes Occur in the AZ Cells of Ripe Fruit during Abscission

To assess the intra- and extra-cellular changes that occur in the AZ during abscission, we examined ripe fruit AZ cells before and after cell separation induced by ethylene (Roongsattham et al., 2012). Cell separation was commonly observed within AZ cell layers and not between the AZ layers and adjacent mesocarp or pedicel tissues (Figures 6A,C). In the example presented, cell separation occurred closest to the mesocarp, but this was variable and not always the case; separation can also be in the middle of the AZ layers or closer to the pedicel tissue, but was not observed between AZ/pedicel or AZ/mesocarp cells.

During and after cell separation occurs, no evidence for cell rupture or cell division was observed (Figure 6). One prominent characteristic after cell separation was the change



of toluidine blue staining from reddish-violet to bluish violet in the AZ cell walls, which suggests a change of cationic environment (i.e., Ca^{2+} and/or H^+) in this region that could be related to pectin modification in the AZ (Figures 6A–C,E,F). In contrast, pedicel and mesocarp cell walls stained dark with toluidine blue even after separation in the AZ occurred (Figures 6A–C). After separation in the AZ, the nuclei remained aligned and intact and there is no visible evidence of cell death based on the integrity of the nuclei (Figure 6D). Another prominent characteristic of AZ cells after separation was the change in the intracellular particles that accumulated in the AZ cells during development (Figures 2D–F, 3, 6A–C,E–L). Once separation occurred, the AZ cells appeared to have fewer particles as visualized by a decreased coloration with different histochemical stains (Figures 6A,C,E–L). Finally, cells in the AZ layers appeared to undergo tip elongation and there was evidence of exocytosis adjacent to separation sites (Figures 6F,H,J,L).

We then measured the changes in cell, cell wall and middle lamella widths after ethylene induced cell separation in 180 DAP fruit (Figure 6). The average cell width of mesocarp and pedicel cells did not significantly change in 180 DAP fruit after ethylene treatment. In contrast, the average cell width of AZ layer cells before ethylene treatment ($21.87 \mu\text{m}$) decreased significantly

in unseparated AZ cells ($17.99 \mu\text{m}$), and non-significantly in cells that separated (Figure 6, Supplementary Tables 14–17). The average mesocarp and pedicel cell wall width at 180 DAP (2.59 and $2.61 \mu\text{m}$, respectively) did not change significantly after ethylene treatment. In contrast, the average cell wall width ($2.58 \mu\text{m}$) decreased significantly after ethylene treatment in both the separation layer and unseparated AZ layer cells (1.76 and $1.84 \mu\text{m}$, respectively, Supplementary Tables 18–21). Finally, the average middle lamella width of mesocarp cells at 180 DAP ($0.93 \mu\text{m}$) decreased significantly ($0.52 \mu\text{m}$) after ethylene treatment. A similar decrease was observed in pedicel cells; the average width of the middle lamella was $0.99 \mu\text{m}$ and significantly decreased to $0.58 \mu\text{m}$ after ethylene treatment. In contrast, the average middle lamella width of the AZ cells was $0.94 \mu\text{m}$, and significantly increased after ethylene treatment to $1.49 \mu\text{m}$ in separated layer cells, while insignificantly increased to $1.10 \mu\text{m}$ in cells of the unseparated layers (Figure 6, Supplementary Tables 22–25). The increase in the AZ cell middle lamella width suggests this area is undergoing expansion prior to cell separation. The results reveal distinct and dynamic changes occur in the AZ cells in association with the cell separation and abscission process in contrast to adjacent mesocarp and pedicel tissues.

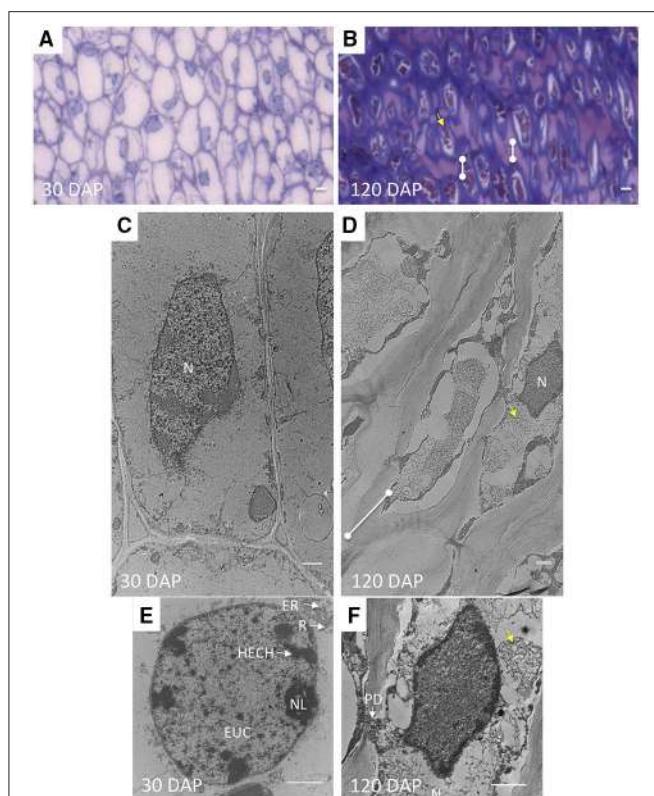


FIGURE 4 | The shape and characteristics of AZ cells and nuclei change during development related to the acquisition of abscission competence. (A,C,E) longitudinal ultra-thin sections of AZ cell layers at 30 DAP and (B,D,F) 120 DAP prepared for transmission electron microscopy (C–F), semi-thin longitudinal section of the same samples stained with toluidine blue (A–B). N, nucleus; HECH, heterochromatin; EUC, euchromatin; NL, nucleolus; R, ribosome; ER, rough endoplasmic reticulum (ER); dots connected by white lines, cell wall expansion at cell tips; yellow arrows, fibrous matrix or intracellular particles. Scale bars in (A) and (B) are 10 μ m; Scale bars in (C–F) are 1 μ m.

FT-IR Microspectroscopy Reveal Changes in Methylesterification Status Occur in AZ Cell Walls during Cell Separation

To examine specific changes that occur in the AZ cell walls during cell separation in more detail, FT-IR microscopy was used to compare unseparated and separated AZ cells (Figure 7). The spectra revealed that mesocarp cells had higher absorbance at 1731–1749 (a range that corresponds to both the COOH and methylester carbonyl group) than either separated or unseparated AZ cells, which suggested a higher esterification in mesocarp cells (Chatjigakis et al., 1998; Manrique and Lajolo, 2002; Gribaa et al., 2013). Furthermore, unseparated AZ cells had a higher absorbance at 1731–1749 than separated cells in both the distal (close to mesocarp) or proximal (close to pedicel) AZ separation layer cells, which suggested a higher esterification in unseparated cells. From the spectra data, the DME was estimated (see Material and Methods for description) that revealed a significant decrease in DME occurs within separated AZ cells in the distal layers after separation (Figure 7, Graph inset).

The Jim5 Epitope Signal Increases Prior to Cell Separation and is High at Cell Edges and Cells that Have Undergone Separation

The classical histology, electron microscopy, and cell parameter analyses described above provide evidence for changes in the cell wall of AZ layer cells during ethylene-induced abscission, in particular changes related to pectin. We used the antibodies JIM5, JIM7, and LM7 to distinguish the cellular localization of partially methylesterified HG, and LM8 to recognize xylogalacturonan (XGA), another form of pectin that is associated with detached cells (Knox et al., 1990; Clausen et al., 2004; Willats et al., 2004). For each experiment, the fluorescent nuclear stain DAPI was used to ensure the region examined was the AZ by the observation of the nuclear alignment of the AZ layer cells (Figures 1B,D,F). The immunohistological controls included incubation without primary antibodies, with secondary antibodies with the different tissues examined (Supplementary Figure 1).

The signal intensity that corresponds to the JIM5 epitope in the AZ from ripe fruit (180 DAP) after 3 h with air treatment was very low (Figures 8A,B). In contrast, by 3 and 6 h after ethylene treatment, the signal obtained with JIM5 increased on the inner side of primary cell wall (Figures 8C–F). By 9 h after ethylene treatment when separation had occurred, the signal increased dramatically at sites of separation of AZ cells on both the mesocarp (distal) and pedicel (proximal) sides (Figures 8G–J). Signals on the inner side of the primary cell wall and in cytoplasm could be detected, in addition to line like signals that arise from deeper within the AZ cells and/or cell layers. A film was made to give a three-dimensional overview of the JIM5 signal in AZ after separation at 9 h (Supplementary Movie 1). The film allows visualization of the high JIM5 signal along the separated cell surfaces, in addition to the lower signals deeper in the AZ layers where cells have not yet separated. By 12 h after ethylene treatment, the signal at the separated primary cell wall of separation layer cells of both the mesocarp and pedicel sides remained high, while the line like signals were lost from the mesocarp side (Figures 8K–N). In samples where both separated and non-separated cells could be observed at 9 h with ethylene treatment, the signal was not only observed on the exposed separated cell surfaces, but also appeared to increase in non-separated cells which may indicate the future separation sites (Figures 8O,P). In addition, the signal intensity was seen all along the separated cell layer surface (Supplementary Figure 2). Moreover, the signals at possible future separation sites within the non-separated zone were quantified using the profile option of the Zeiss LSM image browser software and the results suggested that indeed the JIM5 epitope increased in certain AZ cells prior to separation, and may predict the future sites of cell separation (Supplementary Figure 3).

No JIM7 epitope signal was detected in the AZ of 180 DAP fruit at 3 h without ethylene treatment or at 3 and 6 h with ethylene treatment (Supplementary Figures 4A–F). By contrast, at 9 h with ethylene treatment and with separation occurring, the signal increased at the separated primary cell wall of separation layer cells on both mesocarp and pedicel side (Supplementary Figures 4G–J). Similar to the signal at 9 h, the signal at 12 h with ethylene treatment at the separated primary cell wall of

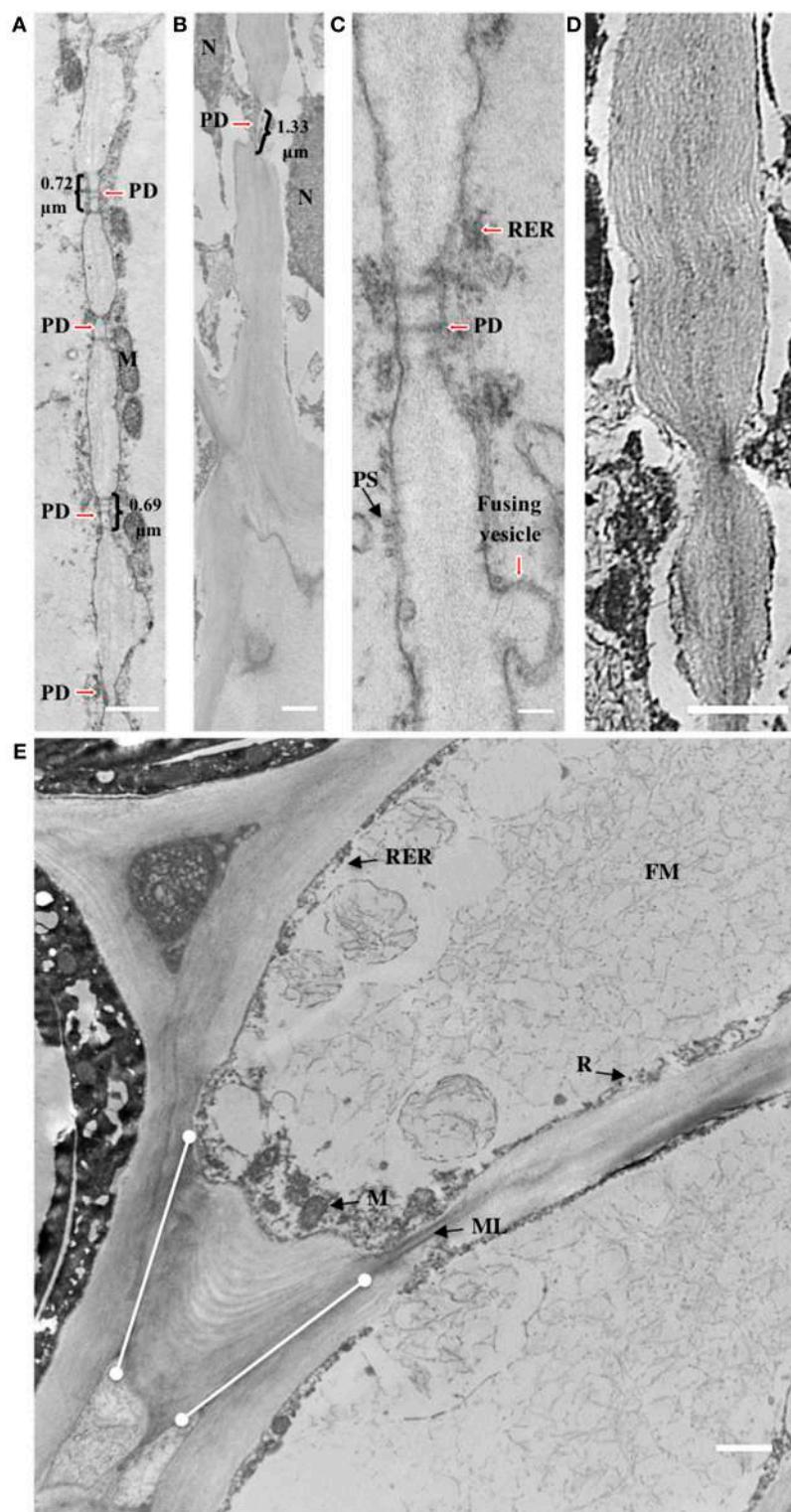


FIGURE 5 | Cell wall striations appear and plasmodesmata increase in size during development of the AZ layer cells. (A,C) 30 DAP; (B,D,E) 120 DAP; PD, plasmodesmata; R, ribosome; M, mitochondria; ML, middle lamella; FM, fibrous matrix material; PS, polysome; dots connected by white lines, cell wall expansion layers at cell tips. Scale bars in (A–E) are 1 μm and 0.1 μm in C.

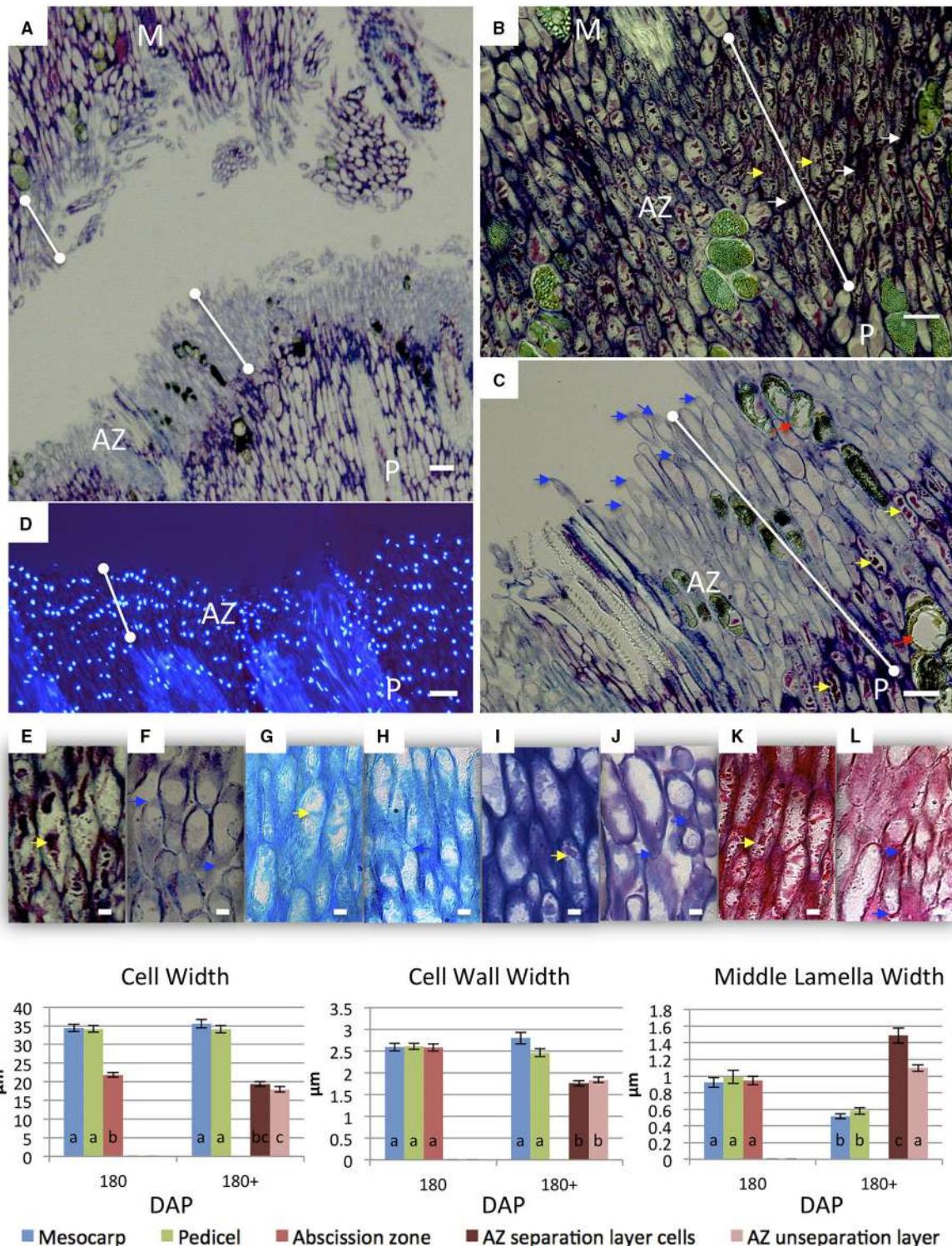


FIGURE 6 | Changes in AZ cells of ripe fruit occur during cell separation induced by ethylene treatment. Longitudinal sections of the oil palm fruit before (B,E,G,I,K) and after 9 h of ethylene treatment (A,C,D,F,H,J,L) cell separation in the AZ stained with toluidine blue (A–C,E,F), DAPI (D) alcian blue (G,H), alcian blue and periodic acid-Schiff (I,J), or ruthenium red (K,L). AZ before and after cell separation; red arrows, degraded phenolic containing cells; white arrows, intercellular space thickenings; yellow arrows, intracellular material; blue arrows, tip elongation with evidence of secretion adjacent separation sites; dots connected by white lines, abscission zone (AZ); M, mesocarp; P, pedicel; Scale bars (A–D) are 100 μm ; Scale bars (E–L) are 10 μm . Graphs below: measurements of M, AZ and P cell, cell wall, and middle lamella widths before and after cell separation in the AZ. Different lower case letters represent statistically significant differences. The error bars represent standard error.

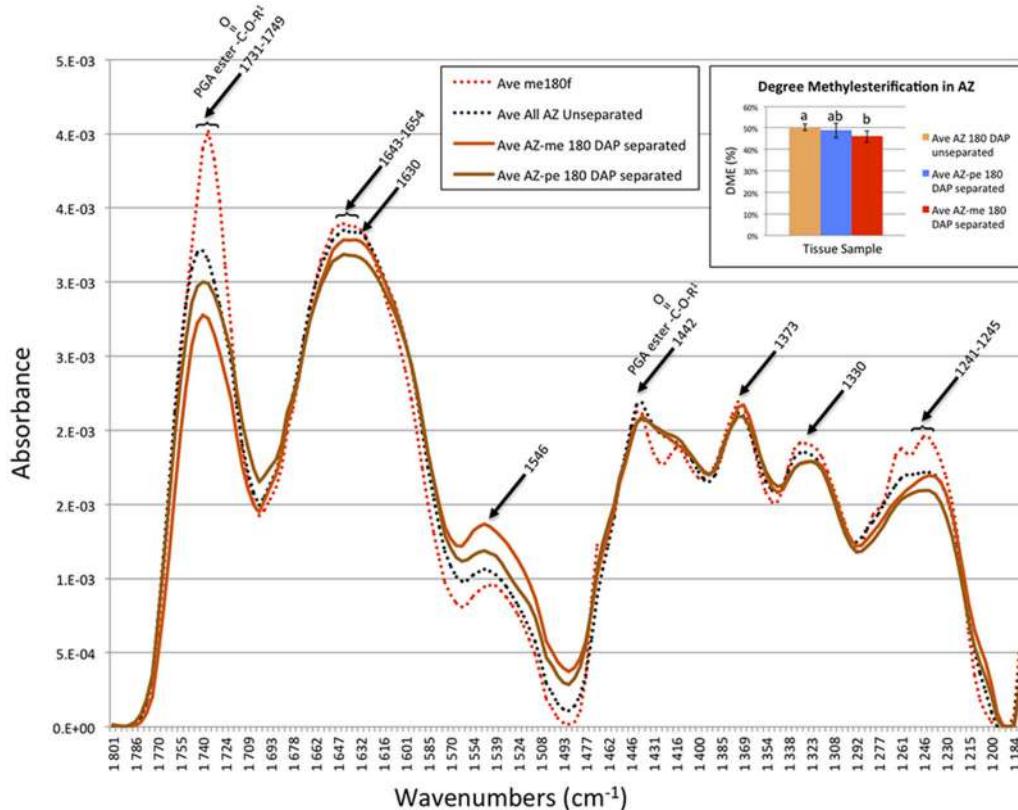


FIGURE 7 | FT-IR microscopy spectra suggest a decrease in the degree of pectin methylesterification (DME) occurs during cell separation in AZ cells closest to the mesocarp. Ave, average absorbance from 11–29 replicates; PGA, polygalacturonic acid; me, mesocarp cell; AZ-me, separated cells in the AZ closest to the mesocarp; AZ-pe, separated cells in the AZ closest to the pedicel. Graph inset, the degree of pectin methylesterification ($A_{1740}/[A_{1740} + A_{1630}]$) in the oil palm AZ cells during separation calculated from the FT-IR spectroscopy data (see Section Materials and Methods). Error bars indicate standard error. Mean values followed by different letters are statistically different.

separation layer cells in both mesocarp and pedicel side of AZ remained high (Supplementary Figures 4K–N). However, the signal interior to the separation face decreased on the mesocarp side while the signal on the pedicel side remained high. In contrast to the JIM5 epitope, the JIM7 epitope did not reveal an increase in cells prior to separation, only in separated cells (Supplementary Figures 2, 4O,P).

The signal corresponding to the LM7 epitope in the AZ of all samples (3 h without ethylene treatment, 3, 6, 9, 12 h with ethylene treatment) was very low and not associated with cell separation (Supplementary Figures 5A–N).

The signal corresponding to the LM8 epitope after 3 h without ethylene treatment was undetectable but becomes stronger after 9 and 12 h of ethylene treatment and only after AZ cell separation (Supplementary Figures 6A–N). By contrast, the pattern of the signal observed was spotted and appeared to be associated to the vascular tissue cells (Supplementary Figures 2I–L, 6G–N). In comparison, the JIM5 and JIM7 epitopes were observed all along the separation surfaces on both the mesocarp and pedicel sides of the AZ (Supplementary Figures 2A–H). Notably, the JIM5 epitope appeared to increase earlier than either the JIM7 or the LM8.

DISCUSSION

AZ Specific Regulation during Development Targets Cell Wall Biogenesis and Results in Anatomy Specialized for Intra-AZ-layer Cell–Cell Communication

The duration of development from the early to ripe stages at which time the fruit abscise and are shed from bunches is variable and depends on the genetic material (Fooyontphanich et al., in press). In the current studies, the final stages of ripening ranged from 160 to 190 DAP when the fruit could be observed to detach naturally in the field. During ripening, ethylene production begins to increase by 120 DAP in the mesocarp, and is thought to be the signal that initiates and regulates oil palm fruit abscission (Henderson and Osborne, 1994; Tranbarger et al., 2011). Indeed, several studies have shown that ethylene induces cell separation in the oil palm fruit AZ, while the response to ethylene is developmentally dependent; ripe fruit AZ have the highest capacity to respond to ethylene (Henderson and Osborne, 1994; Roongsattham et al., 2012). A primary objective of this study was to identify cellular characters of the oil palm ripe fruit AZ that correspond to the capacity to respond and function

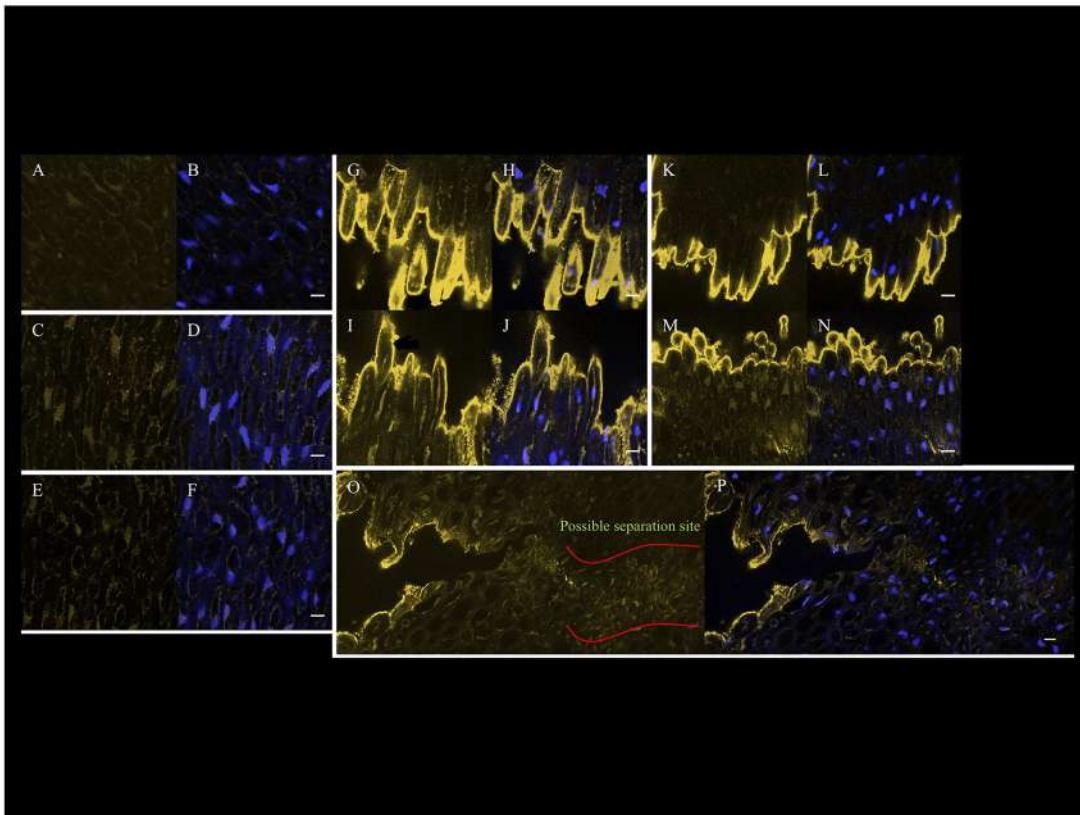


FIGURE 8 | Confocal immunohistological images of the JIM5 epitope signal that increases in the AZ cells prior to and after cell separation on the surfaces of separated cells of 180 DAP fruit. **(A)** JIM5, and **(B)** JIM5 and DAPI AZ 3 h without ethylene treatment. **(C)** JIM5, and **(D)** JIM5 and DAPI AZ 3 h with ethylene treatment. **(E)** JIM5, and **(F)** JIM5 and DAPI AZ 6 h with ethylene treatment. **(G)** JIM5, and **(H)** JIM5 and DAPI AZ at M side at 9 h with ethylene treatment and separation occurring. **(I)** JIM5, and **(J)** JIM5 and DAPI AZ at P side at 9 h with ethylene treatment and separation occurring. **(K)** JIM5, and **(L)** JIM5 and DAPI AZ at M side at 12 h with ethylene treatment and separation occurring. **(M)** JIM5, and **(N)** JIM5 and DAPI AZ at P side at 12 h with ethylene treatment and separation occurring. **(O)** JIM5, and **(P)** JIM5 and DAPI of samples where both separated and non-separated cells can be observed at 9 h with ethylene treatment. Scale bars are 10 μ m.

in response to ethylene. During oil palm AZ development, we observed several cellular features that arise during development with timing that corresponds with the capacity for AZ function and response to ethylene.

During early fruit development, the cell division plane in the AZ layer cells is opposite to that observed in both adjacent tissues; periclinal (parallel to the outer fruit surface) in the AZ, and anticlinal in both the pedicel and mesocarp cells. This suggests that AZ expansion is under distinct regulation compared with adjacent tissues from an early stage. Consequently, periclinal cell divisions in the AZ may give rise to the unusual alignment of nuclei between adjacent cells within each AZ layer. Nuclear alignment appears to originate early during vascular tissue differentiation in the base of the fruit then extends across the primary AZ during development. To the best of our knowledge, this is the first observation of nuclear alignment in an AZ. In contrast, no nuclear alignment is observed in the tomato pedicel AZ where cells appear to undergo cell death during the abscission process (Bar-Dror et al., 2011). In the oil palm fruit AZ, our analysis found no evidence for nuclear degradation well after cell separation took place. Interestingly the AZ cell nuclei are

closely associated with PD, which could provide an efficient intra-AZ-layer structural capacity to communicate signals and coordinate transcriptional responses, analogous to the observed role for PD in defense signaling (Sager and Lee, 2014; Lee, 2015). Indeed, the timing of PD structural changes between 30 and 120 DAP corresponds to the period when AZ cells acquire the capacity to respond to abscission signals, and may provide the structural anatomical basis to coordinate signals that promote a rapid intra-AZ-layer transcriptional response that leads to cell separation.

The observations that cell wall constrictions are associated with groups of PD between adjacent cells, and vascular cell walls are less lignified within AZ layers, suggests that cell wall biosynthesis and differentiation are under specific control in AZ cells. Indeed, we also show cell wall expansion occurs more rapidly in the AZ than in adjacent mesocarp and pedicel cells, and that this expansion occurs in a polarized manner at the tips of AZ cells (Figures 3, 5). Importantly, the increase in cell wall biosynthesis between 30 and 120 DAP corresponds to the period during which AZ acquire the capacity to respond to ethylene (Roongsattham et al., 2012). Overall, these observations indicate

that the control of AZ cell wall biosynthesis is coordinated differentially compared with adjacent tissues, which may be related to the spatial capacity of AZ cells to separate. Differential cell wall construction in the AZ associated with PD development may provide a structural basis for strong intra-layer cell-cell communication required to coordinate abscission signals, while differential cell wall expansion between cell layers may provide the appropriate apoplastic environment for cell separation to take place, analogous to the cell separation that occurs during intercellular space formation (Willats et al., 2001b; Jarvis et al., 2003). Finally, less lignified and differentiated vascular cells in the AZ makes them more susceptible to pectin dissolution by PG. Taken together, AZ cell layer differentiation is controlled by distinct regulation that gives rise to cellular characters that may be important for the capacity to undergo cell separation.

The oil palm AZ consists of a very large primary multi-cell layer AZ that forms a boundary between two fruit tissues with distinct functions; the pedicel for support of the fruit and vascular connection to the bunch, and the mesocarp which has an unusually high capacity for lipid biosynthesis and storage (Tranbarger et al., 2011). As discussed above, we provide evidence that the AZ is under distinct development controls compared to adjacent tissues. Indeed it is known from dicot model species that AZ development and function are highly regulated processes (Estornell et al., 2013). Arabidopsis, boundary AZs are under the control of specific transcription factors including BLADE-ON-PETIOLE1/2 (BOP1/2), ASYMMETRIC LEAVES1 (AS1), and HAWAIIAN SKIRT (HWS) which determine the differentiation, placement and function of floral organ AZs (Gonzalez-Carranza et al., 2007; McKim et al., 2008; Gubert et al., 2014). In the tomato, flower and fruit pedicel AZ differentiation is controlled by a completely different basis including a complex of MADS-box transcription factors (Butler, 1936; Mao et al., 2000; Nakano et al., 2012; Ito and Nakano, 2015). While the molecular basis for these AZ types may be different, it is clear that in both cases the development and function of the AZ is tightly linked; disruption of development affects the capacity to function in the promotion of cell separation and ultimately organ abscission. The current study highlights that strong spatial regulation leads to differential cell wall biosynthesis and assembly processes (e.g., inhibition of the cell wall lignification of vascular bundles across the AZ and extensive cell wall biogenesis at the tips of AZ cells) in the oil palm ripe fruit AZ. Given that the oil palm fruit AZ is multi-cell layered and very large compared to other plant AZs, it provides an interesting experimental system to examine in detail the cellular and biochemical processes that take place during cell separation and abscission, in particular for a monocot fruit species.

Pectin Biosynthesis, Metabolism, Esterification Status, and Other Cell Wall Parameters in the Oil Palm AZ are Regulated Differently than Adjacent Tissues

Previous studies revealed that oil palm fruit AZ cell walls are rich in unmethylated pectin and that PG activity and the

EgPG4 transcript is highly expressed in the AZ in response to ethylene (Henderson and Osborne, 1994; Henderson et al., 2001; Roongsattham et al., 2012). These features were proposed as a mechanism to specify cell separation to the AZ in the oil palm fruit base. In the current study, we provide several lines of evidence for the importance of pectin and pectin methylesterification status for the abscission process, and provide a link between ethylene and effect on the AZ cell walls. Firstly, during AZ development, we observed an increase in intracellular particles that stained strongly for pectin with ruthenium red. Furthermore, these particles decreased in separated AZ cells after abscission, suggesting a functional importance for the abscission process. Secondly, we observed an increase in the JIM5 epitope by 3 h after ethylene treatment localized to cells in the AZ region before separation. Once separation occurs, the JIM5 epitope increases dramatically on both proximal and distal AZ layer cell surfaces that have undergone cell separation. A previous study described nodular structures thought to be pectin material that appear on the separated cell surfaces (Henderson and Osborne, 1990). The current study provides further evidence that the material that accumulates on the separated cell surfaces is pectin. JIM5 binds strongly to HG, the most abundant pectin polymer of galacturonic acid, with a relatively low methylesterification status and binds weakly to unesterified oligogalacturonides (Willats et al., 2000; Clausen et al., 2003). In parallel experiments, we did not see the same pattern of epitope signal with JIM7, which only increased after cell separation. JIM7 recognizes higher order methylesterified HG than JIM5 (Willats et al., 2000; Clausen et al., 2003). In addition, the absence of an increase in the LM7 signal rules out the low methylesterified epitope (four contiguous unesterified galacturonic acid residues adjacent to or flanked by residues with methylester groups) recognized by both JIM5 and LM7, which leaves the moderately methylesterified epitope (three contiguous unesterified galacturonic acid residues adjacent to or flanked by residues with methylester groups) or unesterified HG as the main epitopes detected by JIM5 in AZ cells prior to and after cell separation. Finally, the LM8 epitope consists of a region of highly substituted xylose in xyloglacturonan (XGA) that is observed in regions of cell separation and complete cell detachment (Willats et al., 2004). In the current study, the LM8 epitope does not increase in cells prior to separation, but is mainly localized to vascular strand cells that have separated, suggesting changes to xyloglacturonan are important for these cell types to separate in the oil palm AZ.

Several studies have examined the cell wall structure before, during and after cell separation in different species undergoing various organ abscission processes (Ueda and Nakamura, 2000; Lee et al., 2008; Bowling and Vaughn, 2011; Iwai et al., 2013). During *Azolla* branch abscission, the JIM5 epitope signal disappeared in the fracture surface of the separated cell (Ueda and Nakamura, 2000). Similarly, during impatiens leaf abscission, a loss of the JIM5 (unmethylesterified HG) epitope signal was observed in the cell wall/middle lamella at the face of the separation surface of separated cells (Bowling and Vaughn, 2011). In contrast, during the induction of AZ differentiation in the poinsettia leaf base, a demethylesterification of HG was inferred by the differential signals observed with JIM5

(high signal for unmethylesterified HG) and JIM7 (low signal for partially methylesterified HG) in the AZ cell walls and on the cells distal to the AZ 7 days after induction (Lee et al., 2008). The results suggested the possible involvement of PMEs for the activation of the AZ. However, the study of poinsettia leaf abscission did not examine events after cell separation, and in addition, changes in other cell wall components such as RGI, hemicellulose, and lignin could be more important than pectin (Lee et al., 2008). In tomato, no change in JIM5 or JIM7 was observed during flower or fruit abscission, while increases in xyloglucan, pectic galactan, and arabinan were observed during flower abscission (Iwai et al., 2013). Notably, no changes in signals from any antibodies tested in the previous study were observed during tomato fruit abscission.

In the current study, we provide evidence for a preferential accumulation of JIM5 labeled HG at the edges of separated cells. During development, we observed rough endoplasmic reticulum, mitochondria, and vesicles, in addition to striated cell wall formation at the tips of AZ cells. Together, these results suggest that during development AZ cells undergo polarized oriented cell wall building activity, which culminates with the secretion of HG (JIM5 signal) during abscission in a polarized manner. These functions could either be a part of the mechanism for the abscission process, or part of the defense mechanism to protect the scar after separation. However, these two possible functions are not mutually exclusive and could be linked. Indeed, several lines of evidence suggest an active role for HG in the abscission process. First, the JIM5 epitope increases in the AZ prior to separation and the signal continues to increase after AZ cells separate. Second, pectin accumulation detected in AZ cells prior to separation decreases after separation takes place, suggesting pectin is secreted during abscission. We also observed a high JIM5 signal on both the distal and proximal separated AZ cells, which is not consistent solely with a role for defense, given that a defense response would not be expected in the distal AZ cells of the shed fruit. Finally, a significant decrease in the DME in separated AZ cells closest to the mesocarp suggests that methyl ester groups are being actively removed from HG, possibly by PME activity, in these AZ cell layers, and could explain the high JIM5 signal observed prior to and after AZ cell separation.

During abscission, cell elongation and expansion have been observed but it is unclear whether they are an active part of the abscission process (Wright and Osborne, 1974; Butenko et al., 2003; Patterson and Bleecker, 2004). The current results provide evidence for cell elongation accompanied by a decrease in cell and cell wall width, and an expansion of the middle lamella width in AZ cells that separate. While these changes are significantly quantified in the AZ cells that separate, similar changes do not occur in mesocarp or pedicel cells, again highlighting the differential regulation that controls AZ cell

function and fate. Interestingly, during pollen tube and root hair tip growth the polar secretion of HG plays key roles in the elongation process (Rounds and Bezanilla, 2013). The tip growth model proposes that methylated HG is secreted into the wall where it undergoes de-methylesterification by PME, which allows the de-methylesterified HG to form Ca^{2+} crosslinks that results in more rigid cell walls. In the current study, we provide evidence that functional components for tip growth are present in the AZ cells; cell elongation, tip oriented pectin biosynthesis and a decrease in methylesterification, all associated with cells that have undergone separation. While in the tip growth systems cells are elongated, in AZ cells elongation may be a source of HG secretion that plays an active role in cell separation.

AUTHOR CONTRIBUTIONS

TT and FM devised and participated in all aspects of the study. TT, CJ, and ST coordinated the logistics for study. TT, FM, PR, and CJ performed the ethylene experiments and collected samples for studies. PR, KF, MC, and JV performed the immunolocalization studies. PR, MC, and JV performed the light microscopy and electron microscopy analyses. GM and PR performed the FT-IR microscopy analysis. ST, CJ, PR, and PA participated in the data analysis and critically read the manuscript. TT, JV, FM, and PR participated in writing the article. All authors read and approved the final submitted manuscript.

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SUPPLEMENTARY MATERIAL

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Two abscission zones proximal to *Lansium domesticum* fruit: one more sensitive to exogenous ethylene than the other

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Longkong (*Lansium domesticum*) fruit grows in bunches and is also sold as bunches. Individual fruit can separate from the bunch both before and after commercial harvest. The fruit has two separation sites. The first is located between bracts on the stem and the fused sepals (separation zone 1: SZ1) and the second between the fused sepals and the fruit (separation zone 2: SZ2). True abscission occurred at both zones. We investigated whether the two zones were active at different stages of development and if they were differentially sensitive to ethylene. Abscission occurred in the SZ1 in very young fruit (fruit still at the ovary stage), during early fruit development (5 weeks after full bloom; WAFB), and in ripe and overripe fruit (15–17 WAFB). Abscission did not spontaneously occur in the SZ2, but by the time the fruit was fully ripe, 15 WAFB, and later, a slight mechanical force was sufficient to break this zone. In fruit bunches severed from the tree at 5, 8, and 13 WAFB, break strength (BS) in SZ1 decreased much more after exogenous ethylene treatment than that in SZ2. Ethylene induced abscission in the SZ1, but not in SZ2. At 5, 8, and 13 WAFB, treatment with 1-methylcyclopropane (1-MCP; an inhibitor of ethylene perception) had a small effect on BS in the SZ1 and no effect in the SZ2. It is concluded that abscission in the SZ1 was much more sensitive to ethylene than that in the SZ2. In intact plants SZ1 reacts to endogenous ethylene, e.g., as a result of stress, while SZ2 apparently allows animals to remove the ripe fruit from the tree with minimal force.

Keywords: abscission, ethylene, fruit drop, *Lansium domesticum*, longkong, 1-methylcyclopropene, ultrastructure

Introduction

Longkong (*Lansium domesticum* Correa) is a tropical fruit of the Meliaceae family. The pulp of longkong is consumed fresh. The peel contains oleoresin, which is used to counter diarrhea, while the crushed seeds are used to cure fever. The fruit is widespread throughout South East Asia (Yacob and Bamroongrugsu, 1992; Paull, 2004). Currently longkong is only traded within the South East Asian region. Shipping the fruit to distant markets must be done by air, to reduce the problem of fruit abscission.

There has been some discussion about nomenclature, e.g., whether *Lansium domesticum* is synonymous with *Aglaia dookoo*. Muellner et al. (2005) separated *Aglaia* from *Lansium* whereby the former has 1–3 ovary locules, while *Lansium* has five. Fruit called langsat is also *L. domesticum*,

but has a thinner fruit peel than longkong. Two fruit called duku and duku-langsat, are also *L. domesticum*; with a morphology similar to that of longkong and langsat (Song et al., 2000). Lim (2012) gave a detailed description of the various fruit in the langsat-longkong group.

Longkong fruit are berries which develop on inflorescences, bunches contain about 25–40 fruit. The ripe fruit is round, about 3–5 cm in diameter. When immature, the peel is green and the fruit pulp is white. When ripe, the peel turns yellow and the pulp becomes translucent with a juicy texture and a typical smell. The fruit pulp is then sweet with a slightly sour tone (Paull, 2004).

Longkong fruit is non-climacteric, i.e., it does not produce a peak in respiration rate or in ethylene production, and fruit ripening is not sensitive to exogenous ethylene. Normally, the fruit is harvested before full maturity. If sold on local markets, recommended harvesting is about 15 weeks after full bloom (WAFB) (Lichanporn et al., 2009). When intended for markets at greater distances, the fruit should be harvested at 13 WAFB. Fruit harvested at 15 WAFB shows a high percentage of fruit abscission (Taesakul et al., 2012).

A few species reportedly contain more than one fruit abscission zone. In citrus, abscission zones were found at the base of the pedicel, and between the sepals and the fruit. The pedicel is defined as a stem that attaches single flowers/fruit to a bigger stem of the inflorescence/fruit bunch (The stem or branch that holds a group of pedicels is called a peduncle). After exposure to ethylene, increased cell wall degrading enzyme activity was found in both abscission zones (Goren, 1993a,b; Burns, 1997). Tomato fruit also has two abscission zones (Biain de Elizalde, 1980), while peach has three (Rascio et al., 1985).

Longkong has sessile fruit, hence the pedicel is absent. The stem branches of the inflorescence/fruit bunch are called peduncles. A separation zone was found between the peduncle and the sepals (SZ1) and a second between the sepals and the fruit (SZ2; Taesakul et al., 2012). Preliminary experiments showed that in the absence of exogenous ethylene, drop of mature fruit occurred when using a small mechanical force, at the SZ2. When exogenous ethylene was present, fruit drop occurred solely at the SZ1.

We studied the separation process at the two sites in more detail. We describe anatomical changes during flower and fruit development, using light and electron microscopy and measured the break strength (BS) at the separation zones. Both ethylene and 1-MCP (which inhibits ethylene perception) were applied to test the role of ethylene in this process.

Materials and Methods

Plant Material

Bunches of longkong fruit (*Lansium domesticum* Corr.) were severed from 5 to 10 randomly selected trees, which were 12 years old and had similar canopy size and apparent health. The trees grew in an orchard in Wang Saem District of Chanthaburi Province (Thailand). One bunch was randomly severed per tree every 2 weeks, starting 3 WAFB and ending 17 WAFB. The means of average daily temperature and relative humidity during the 4 months fruit growth period were $28 \pm 2.5^\circ\text{C}$ and $85 \pm$

10%, respectively (Figure S1). Bunches were placed in perforated plastic crates ($36 \times 60 \times 33$ cm) lined with paper, and were held at ambient temperature for 1–3 h. Crates were then transported to the laboratory, using an air-conditioned van at $25\text{--}27^\circ\text{C}$. Transport took 5 h. Upon arrival the fruit was held dry in the crates, at $24\text{--}26^\circ\text{C}$ for about 3 h until use.

Physical Properties During Fruit Development

In 10 fruit from each of the ten bunches we determined fresh weight, length, diameter of the fruit, and diameter of the fused sepals, using a digital scale (TANITA 1579, Tokyo, Japan) for weight, and Vernier calipers (Mitutoyo 532-121, Kanagawa, Japan) for length.

Flower Structure and the Presence of Abscission Zones in the Flowers

An Olympus 302990 stereo-microscope (Tokyo, Japan) with two Olympus TGHM lateral light sources were used to assess flower structure and the presence of abscission zones in the flowers. Pictures were recorded using a Dinoeye AM423X digital eyepiece (Taipei, Taiwan ROC) connected to a computer.

Abscission Zone Morphology and Ultrastructure

Longkong bunches were severed from the trees at 3, 8, and 13 WAFB and separated into two groups. One group was evaluated using light microscopy, the other using scanning electron microscopy.

Cell Size

The two separation zones were removed by cutting, using a sharp razor blade. A small part of the peduncle remained attached at the proximal side of the SZ1. A small part of the fruit remained attached at the distal side of the SZ2. The isolated parts were then cut in the middle between SZ1 and SZ2. The segments were cut longitudinally, fixed in 3.7% (v/v) formaldehyde, 50% (v/v) ethanol, and 5% (v/v) acetic acid (FAA) solution, embedded in paraffin, cut into $16 \mu\text{m}$ thick slices, stained with Fast-green and Safranin-O and mounted on glass slides for observation using light microscopy (Axistar plus, Carl Zeiss, Aalen, Germany). Cell sizes in the peduncle at the proximal side of the SZ1, in the SZ1, in the SZ2, and at the distal side of the SZ2 were determined using an ocular micrometer. Three samples (replicates) were used per treatment; nine cells were measured in each sample.

Ultrastructure, Ethylene Treatment

Eighteen longkong bunches were divided into two groups. One group was used as control. The second group was exposed to $1 \mu\text{L L}^{-1}$ ethylene for 12 h. After the ethylene treatment, the fruit were forcefully removed from the fused sepals. The sepals were also forcefully removed from the peduncle. The tissues near the separation site were collected and were dipped immediately in 2% glutaraldehyde in 0.05 M sodium phosphate buffer overnight, then treated for 2 h with 2% osmium tetroxide. The samples were dehydrated in an ethanol series, and were critical-point dried with CO_2 , and coated with 60 nm of gold/palladium. Observations were made using scanning electron microscopy (JEOL JSM-S410LV, Tokyo, Japan). Three samples were used for each stage.

Break Strength and Abscission

During Fruit Development on the Tree

The number of abscised fruit was determined both during development on the tree and after the harvest of almost ripe fruit. Abscission and BS were observed in bunches severed from the trees 3–17 WAFB. Abscission from the trees was low, as indicated by the low number of abscission zone scars. Abscission was determined by the drop of fruit during transportation from the trees to the laboratory. BS was determined using a push-pull force gauge (John Chatillon and Sons, Greensboro, NC), following the method of Gersch et al. (1998). To determine the BS at SZ1, the peduncle was attached to a drill clip which was mounted to the end of the push-pull force gauge. For SZ2 BS, the peduncle was removed using a razor blade. The fused sepals were attached to the drill clip. BS was determined by slowly pulling the sepal or the fruit, straight from the abscission planes, to separate them from the peduncle or the sepal. Care was taken not to pull the fruit at an angle under the abscission plane. The number of replicates was 25, consisting of five bunches (each cut from a different tree) and five fruit per bunch, at each sampling time.

During Fruit Storage

Longkong bunches were severed from five trees at 5, 8, and 13 WAFB, and brought to the laboratory. Bunches were divided into three groups. The first group was used as the control, the second group was exposed to $1 \mu\text{L L}^{-1}$ ethylene for 12 h and the third was treated with $1 \mu\text{L L}^{-1}$ 1-MCP (EthylBloc, Floralife Inc., Walterboro, SC) for 6 h. Treatments occurred at 25°C . Fruit was then stored at 25°C for 4 days. The BS and fruit drop at SZ1 and SZ2 were determined at intervals, as described. The number of replicates was 25, consisting of five bunches (each cut from a different tree) and five fruit per bunch, at each sampling time.

Statistical Analysis

Analysis of variance was performed on all data. Mean separation was carried out using paired *t*-test and Duncan's multiple range test (SPSS 15 program, Landau and Everitt, 2004). Differences at $P < 0.05$ were considered to be significant.

Results

Abscission of Young Ovaries

In the field many young ovaries were found to abscise. Figures 1, 2A show the position of the ovary, and the structure of the flower. The flower had five fleshy petals, fused at their base (Figures 1A–C,L, 2A). The petals of flowers that had just opened were off-white with creamy shades (Figure 1A). Fused with the inner base of the petals was a ring of very thick, fused filaments (Figures 1L, 2A), bearing ten anthers (Figure 1L, white arrows). Subtending the petals were five green sepals, which are also fused (Figures 1A–H,L, 2A). The sepals were distal to three bracts (Figures 1C,I–K).

The following stages of flower development were distinguished: (a) flower opening, (b) petals plus stamens separate from the flower, (c) stigma separates from the flower, (d) ovary or young fruit may separate from the flower. In Figures 1L, 2A the petals plus stamens had separated from the rest of the flower,

but had not yet fallen off. In Figure 1D the petals and fused stamens had fallen. An apparent abscission zone at the petal base is indicated by arrowheads in Figure 1L. Initially, separated petals plus stamens can be removed by little mechanical force, as they were still surrounded by the sepals (Figures 1D,L, 2A). The petals were still whitish by the time of their separation. If the separated petals and stamens did not fall early (thus remain kept in check by the sepals), they showed discoloration to brown (Figure 1B), followed by desiccation (Figure 1C). After the fall of the petals plus stamens the female reproductive organs were visible (Figures 1D–H). The top of the stigma was flat, somewhat irregularly shaped at the edges (Figures 1D,F). The style was short and thick. The stigma + style separated at their junction with the hypogynous ovary. It usually fell off after slight discoloration to brown (Figures 1G,H). The ovary was situated on top of, and was fused with, the sepals (Figures 1D–H,L, 2A). This entire structure (ovary and sepals) could abscise, whereby the abscission zone was localized at the proximal side of the sepals, which was the distal side of the three subtending bracts (Figures 1I–K). The localization of this abscission zone (SZ1), after abscission, is indicated in Figures 1I,K. The three bracts are indicated and the abscission area is shown by arrows. It should be noted that the flower as well as the fruit were situated under an angle less than 90° with respect to the peduncle (Figures 1J, 2B).

Position of the Two Separation Zones of the Fruit and Fruit Development

Abscission of ovaries (very young fruit) occurs at SZ1; it usually takes place in less than 5% of the ovaries on a bunch. The ovaries abscise together with the subtending sepals. A longitudinal section of SZ1 and the future separation zone SZ2 are indicated in Figure 2A. SZ1 is located just below the ovary, but had apparently not become fully developed by the time of ovary abscission. SZ2 extends, at its circumference, into the separation zone of the petals plus stamens. Thus, the SZ1 was located between the bracts on the peduncle and the fused sepals, while the SZ2 was between the sepals and the fruit. Figure 2B gives a scheme of the position of the two separation zones in full-grown fruit. As the fruit is sessile (has no pedicel), only the sepals separate the ovary from the distal part of the bracts.

The length and diameter of longkong fruit increased almost linearly from 3 to 15 WAFB, while fruit weight increased exponentially. The diameter of the fused sepals (0.4 cm by week 3 after full bloom) only slightly increased during this period (Table S1). Fruit development included a change of the peel from green to yellow by about 10 WAFB (Figure 3).

Cell Size in and Ultrastructure of the Separation Zones

Cell Size

At 3 WAFB, the cells in the peduncle above the SZ1 were about $32 \mu\text{m}$ long. Cell size increased to $44 \mu\text{m}$ by 13 WAFB. At 3 WAFB the cells in SZ1 were considerably smaller ($20 \mu\text{m}$) than in those in the peduncle. Their size had increased to about $30 \mu\text{m}$ by 13 WAFB. In SZ2 the cells were about the same size as in SZ1 (Table S2).



FIGURE 1 | Abscission of floral parts, including young ovaries, in longkong (*Lansium domesticum*). (A) Longkong flower with fused petals (p) above the sepals (s) at opening stage; **(B)** Flower at senescence stage, the fused petals had already abscised from the fused sepals; **(C)** Flower when the fused petals had desiccated but remained on the flower; **(D)** Flower when the fused petals had already fallen off, exposing the ovary (ov), style (sty), and stigma (sti); **(E)** Close up of the style and stigma; **(F)** Top view of the stigma; **(G)** Desiccation of the stigma; **(H)** Ovary after stigma

abscission; **(I)** Base of the flower showing one bract (br) and the SZ1; **(J)** Young fruit on top of 2 bracts; **(K)** After flower abscission, showing the remaining 3 bracts, arrows point at SZ1; **(L)** Longitudinal section of a flower showing the separation of the fused petals from the base of the ovary, arrows indicate anthers, arrowheads show the abscission zone of the petals and the fused stamens (fi, filament). Note abscission of (1) petals and attached ring of stamens, (2) of the stigma plus short style, and (3) the ovaries plus sepals. See text for explanation. Bars in all pictures equal 1 mm.

Ultrastructure

All cells in SZ1 at 3 and 8 WAFB had ruptured when the fruit together with the fused sepals (calyx) were pulled off the

bunch. At these periods rupture occurred irrespective of ethylene treatment (Figures 4A,B,D,E). By contrast, at 13 WAFB and no ethylene treatment, half of the cells had separated (Figure 4C).

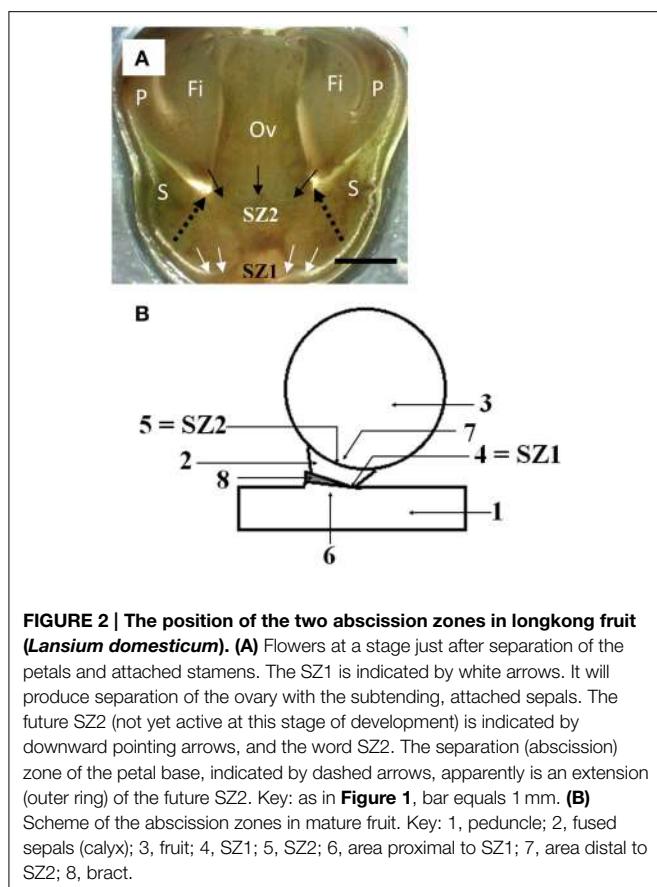


FIGURE 2 | The position of the two abscission zones in longkong fruit (*Lansium domesticum*). (A) Flowers at a stage just after separation of the petals and attached stamens. The SZ1 is indicated by white arrows. It will produce separation of the ovary with the subtending, attached sepals. The future SZ2 (not yet active at this stage of development) is indicated by downward pointing arrows, and the word SZ2. The separation (abscission) zone of the petal base, indicated by dashed arrows, apparently is an extension (outer ring) of the future SZ2. Key: as in Figure 1, bar equals 1 mm. (B) Scheme of the abscission zones in mature fruit. Key: 1, peduncle; 2, fused sepals (calyx); 3, fruit; 4, SZ1; 5, SZ2; 6, area proximal to SZ1; 7, area distal to SZ2; 8, bract.

After ethylene treatment at 13 WAFB all cells showed smooth separation (Figure 4F).

At 3 WAFB all cells in the SZ2 had ruptured when the fruit were pulled from the fused sepals, irrespective of ethylene treatment (Figures 4G,J). At 8 WAFB about half of the cells in the SZ2 showed smooth separation, while the remainder of the cells ruptured (Figures 4H,K). This was observed irrespective of ethylene exposure. At 13 WAFB, most of the cells had smoothly separated in the control (Figure 4I). All cells smoothly separated after ethylene treatment (Figure 4L).

Fruit Drop and BS in Intact Plants

By 3 WAFB and later, fruit abscission from the trees was low, as only a small number of abscission zone scars were observed. Abscission was determined by the drop of fruit during transportation from the trees to the laboratory. Figure 5A shows that, in the season investigated, fruit drop at the SZ1 took place during 5–7 WAFB (immature fruit with green peel) and during 15–17 WAFB (mature with full yellow peel). No fruit drop was found at the SZ2 during fruit development on the trees (Figure 5A). The BS at SZ1 and SZ2 increased from 3 to 9 WAFB (Figure 5B). The BS at SZ1 slightly decreased from 9 to 15 WAFB (Figure 5B). By contrast, the BS at SZ2 decreased sharply from 9 to 15 WAFB (Figure 5B).

Effects of Ethylene and 1-MCP

Effects of ethylene and 1-MCP were determined using bunches that were severed from the trees at 5, 8, and 13 WAFB. Bunches picked at 5 WAFB, and kept at 25°C, fruit drop was low in untreated controls, higher after ethylene treatment, and lower than the controls in the 1-MCP treatment (Figure 6A). In bunches severed at 8 WAFB, and kept at 25°C, fruit drop in the controls was 20% by day 4. After ethylene treatment it was about 30% by day 4 (Figure 6C). When bunches were taken at 13 WAFB, and kept at 25°C, fruit drop in the controls was absent by day 4 (Figure 6E), and was still very low by day 7 (data not shown). In fruit exposed to 1 µL L⁻¹ ethylene, fruit drop was more than 20% by day 4 (Figure 6E), and more than 40% by day 7 (data not shown), while 1-MCP treatment had no effect (Figure 6E). At any of the picking dates no spontaneous fruit drop during storage was found at the SZ2, in controls, ethylene treatment, and 1-MCP treatment (data not shown).

BS was determined at 5, 8, and 13 WAFB. Each ethylene treatment resulted in a rapid drop in BS at the SZ1 (Figures 6B,D,E). By contrast, ethylene had only a slight effect at SZ2 (Figures 6B,D,E). Treatment with 1-MCP (carried out on bunches severed at 5 and 13 WAFB) had little effect, both at SZ1 and SZ2 (Figures 6B,D,E).

Discussion

Several separation zones were observed in the flowers and fruits of longkong: one at the base of the style, one at the base of the petals and attached stamens, one between the fused sepals and three bracts on the peduncle (SZ1), and one between the sepals and the fruit (SZ2). Separation of the petals and stamens occurred together. This was apparently due to true abscission as small cells were observed, but the separation process was not studied in detail hence it cannot be excluded that the tissue was torn. Tearing off instead of abscission has been described in petals, stamens, and styles in some species. This tearing is due to the mechanical forces generated by the growing fruit (Reiche, 1885; Wacker, 1911; van Doorn and Stead, 1997).

SZ1 and SZ2 of longkong fruit contained cells that were much smaller than the adjacent cells. Abscission zones usually contain small cells, but the presence of small cells is by itself inadequate proof of true abscission. After fruit drop at SZ1 (either without exogenous ethylene or after ethylene treatment) the zone showed smooth separation rather than cell breakage. This strongly suggests that SZ1 is a true abscission zone. After slight mechanical force is exerted on ripe longkong fruit, this leads to fruit drop at SZ2. In addition, most cells were easily separated, strongly indicating that an abscission process was in progress. Nonetheless, as the fruit did not abscise spontaneously, a number of cells apparently still needed to be broken. It was previously suggested that there was no true abscission zone at SZ2 (Taesakul et al., 2012). This is correct insofar the zone did not show spontaneous abscission, but at the same time not true because most of the zone cells showed smooth cell walls (Figure 4L) after separating the fruit from the bunch at 13 WAFB. The data show that middle lamella separation (abscission) occurred in many cells at SZ2.

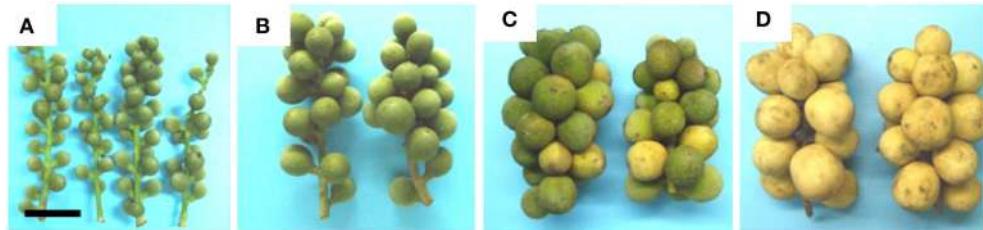


FIGURE 3 | Morphology of longkong fruit (*Lansium domesticum*) bunches. (A) 5 WAFB, **(B)** 7 WAFB, **(C)** 10 WAFB, **(D)** 13 WAFB. Color break occurred at 10 WAFB. Bar in **(A)** is 5 cm. The bar also applies to **(B–D)**.

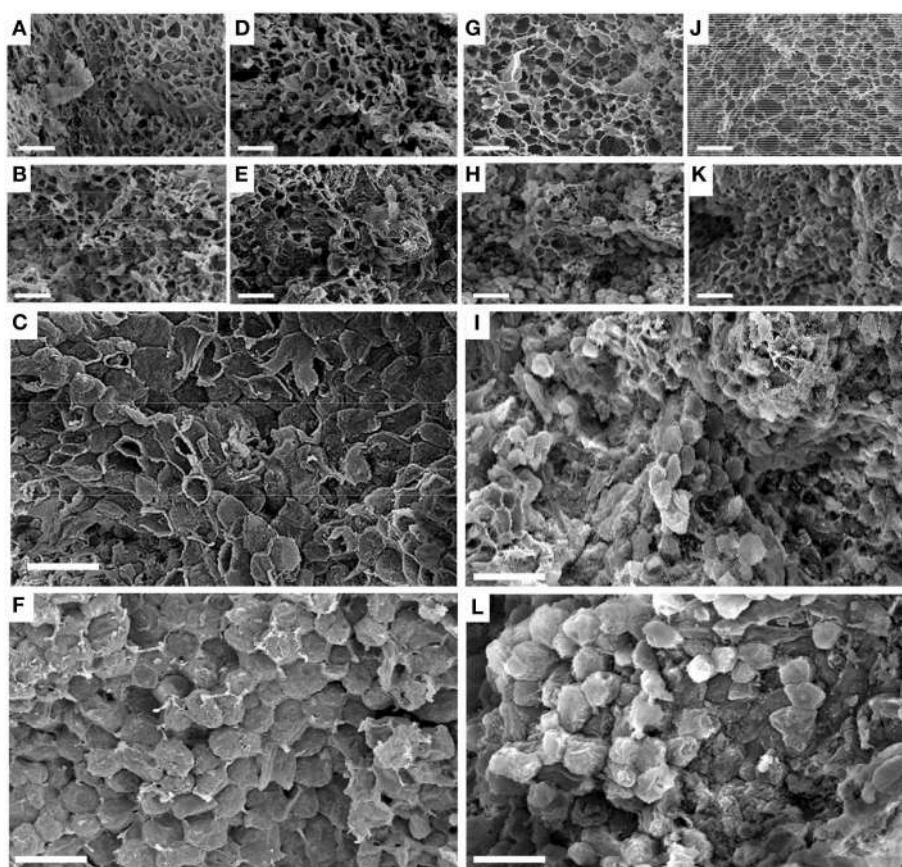


FIGURE 4 | Scanning electron micrographs of cells in longkong fruit (*Lansium domesticum*). (A–F) Morphology of the SZ1 after the fruit had been forcefully removed from the peduncle. **(G–L)** Morphology of the SZ2 after the fruit had been forcefully removed

from the fused sepals, either without **(A–C,G–I)** or after **(D–F,J–L)** $1 \mu\text{L L}^{-1}$ ethylene for 12 h. Times of harvest were 3 **(A,D,G,J)**, 8 **(B,E,H,K)**, and 13 **(C,F,I,L)** WAFB. Bars in all pictures equal 100 μm .

In many abscission zones the vascular bundle is the only part not undergoing wall separation (Roberts et al., 2002), but this does not hamper abscission. This may also be true for SZ1. However, because of the lack of spontaneous abscission even by 15 WAFB, SZ2 seems to contain more cells that have not undergone cell separation than SZ1 by the time of abscission at that zone.

During fruit development on the trees, the BS at SZ1, at 15 WAFB, was clearly higher than SZ2, but fruit drop and abscission

was higher at SZ1 (Figure 5). The results seem conflicting, but they could be explained either by tearing off of the fruit due to growth, or by normal abscission. We found smooth abscission scars, which seems to exclude tearing off of the fruit. The explanation of the apparent discrepancy may possibly be that a subset of the fruit was attacked by pests such as fungi, causing a local increase in ethylene production and thus abscission. This fruit then dropped, hence their BS must have been close to zero.

However, the majority of the fruit that remained was healthy, thus had high BS. This means that the average BS, determined in the remaining fruit, was high. However, one may ask why fruit dropped at SZ1 and not at SZ2, while both zones were equally exposed to fungal-induced ethylene. The explanation might be that SZ2 is less ethylene-sensitive. The difference in ethylene sensitivity between SZ1 and SZ2 is shown in **Figure 6**, where harvested fruit was exposed to ethylene.

After ethylene treatment of fruit harvested at the harvest time for local consumption (i.e., 15 WAFB) the BS at SZ1 had decreased significantly. The cells that had separated in the SZ1 showed smooth cell walls, indicative of a natural abscission processes. These data indicate that SZ1 contains an abscission zone that is normally sensitive to ethylene, similar to the zones in many other fruit, for example tomato (Tabuchi et al., 2000).

The anatomical data on longkong separation zones are similar to those in citrus (Goren, 1993a,b). One citrus fruit abscission zone (called A) is located at the proximal part of the pedicel, which is similar to the location of SZ1 in longkong. A more distal abscission zone in citrus is located between the sepals (calyx) and the fruit (called zone C), which is similar to the position of the SZ2 in longkong. The main difference between longkong and citrus is the closer proximity of the two abscission zones in longkong, as longkong has no pedicel. In longkong the two abscission zones are located at both sides (distal and proximal) of the fused sepals. Similarly, tomato has two abscission zones in the fruit stem. A proximal zone is located between the pedicel and peduncle (comparable to SZ1), and a distal zone between fruit and pedicel (slightly comparable to SZ2; Blain de Elizalde, 1980). In peach fruit stems three abscission zones have been found, the most distal one is at the junction of the fruit with the pedicel (slightly similar to SZ2 in longkong), and two others are in the pedicel (Rascio et al., 1985).

During growth on the trees, ovary and fruit drop occurs during three periods. Very young fruit (ovaries) abscised just after the petal life span had terminated. Young fruitlets abscised about 5 WAFB. Mature fruit abscised at about 15 WAFB. Similar abscission periods have been described for several other fruit. In *Citrus lemon*, for example, a period of massive abscission results in loss of flowers and ovaries. About a week later, fruit having a diameter similar to that of full-grown peas abscised. The third period of abscission occurred in the spring, reducing the number of growing fruitlets, which were by then about the size of a golf ball. This is similar to what is called June fruit drop in many species in the Northern Hemisphere (Goren, 1993a,b; Iglesias et al., 2006). Citrus fruit remained on the tree until over-mature, but seems to eventually abscise.

Flower abscission and early fruit abscission is usually not determined by the absence of pollination/fertilization but by competition between pollinated flowers and between fertilized ovaries. Abscission of fertilized flowers and very young fruit was found to be regulated by the availability, to the organ, of mineral nutrients or carbohydrates (Stephenson, 1981; Racskó et al., 2006; McFadyen et al., 2012). Abscission of the ovaries in longkong also does not seem related to the absence of fertilization, as longkong fruit is thought to be predominantly parthenocarpic (Bernardo et al., 1961). Ovary abscission could possibly therefore relate to

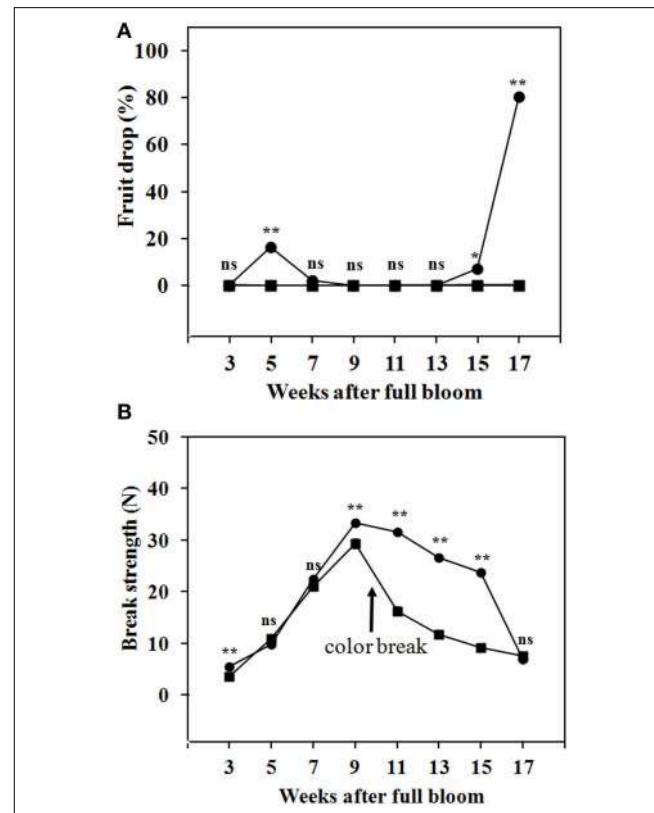


FIGURE 5 | Fruit drop and BS of longkong fruit (*Lansium domesticum*) during development on the tree. (A) Fruit drop at the SZ1 (●) and the SZ2 (■). (B) BS at the SZ1 (●) and SZ2 (■). It should be noted that in these tests no mechanical force was exerted on the fruit. A slight mechanical force (about 10 N) will lead to drop by breakage at the SZ2, from 3–17 WAFB onward. Data are means of 25 replicates. *, ** Significant difference at 0.05 and 0.01 respectively, ns not significant using paired t-test.

competition. Similarly, it could be suggested that abscission of longkong fruit at the immature stage is mainly due to competition (Stephenson, 1981; Wright, 1989; Iglesias et al., 2006).

The longkong ovaries are fused with the subtending sepals. Ovaries therefore abscise together with the sepals, at an abscission zone that is located between the sepals and three subtending bracts (thus at SZ1). We found that (a) abscission of ovaries in longkong flowers occurred only at SZ1, (b) abscission of immature fruit by 5 WAFB took place only at SZ1, and (c) spontaneous abscission of ripe fruit (not the fall after slight mechanical force) was also restricted to SZ1. Only after a slight mechanical force was exerted on ripe (mature) fruit, was abscission found at SZ2. This is quite different from citrus, where abscission of the ovaries and that of very small fruit, i.e., during 6–8 weeks after fruit set, took place exclusively at abscission zone A (similar to SZ1), while by the time of the June abscission period abscission zone A had become progressively inactivated while abscission zone C (similar to SZ2) began to operate and later on all abscission took place at abscission zone C. Only during 2–3 intermediate weeks is abscission found at both sites (Goren, 1993a,b; Iglesias et al., 2006). The data on longkong also differ from those in tomato and peach. Blain de Elizalde (1980) reported that small immature tomato

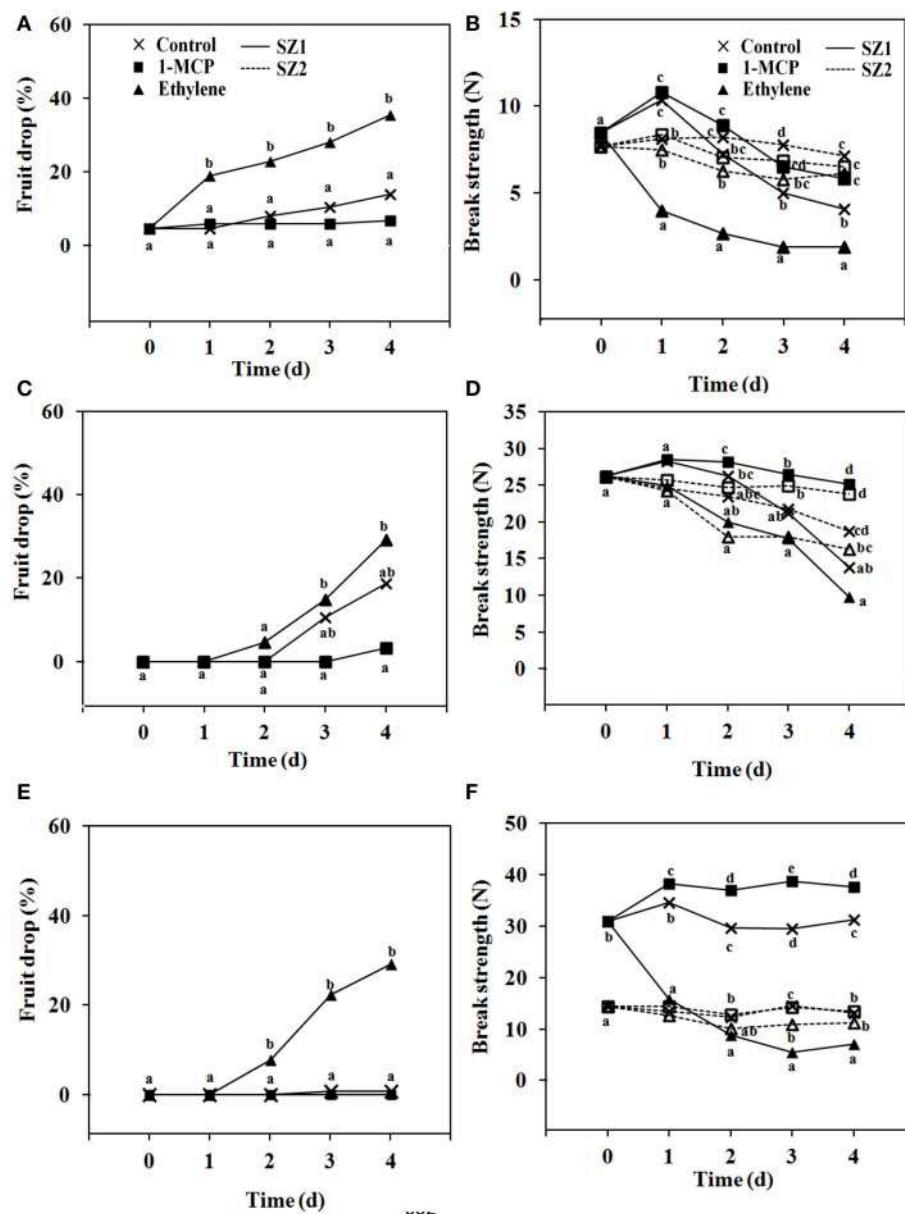


FIGURE 6 | Fruit drop and BS of longkong fruit (*Lansium domesticum*). **(A,C,E)** Fruit drop and **(B,D,F)** BS after harvest at 5 **(A,B)**, 8 **(C,D)**, and 13 **(E,F)** WAFB. Fruit drop refers to the SZ1 after treatment with air (\times), $1\text{ }\mu\text{L L}^{-1}$ 1-MCP for 6 h (\blacksquare) or $1\text{ }\mu\text{L L}^{-1}$ ethylene for 12 h (\blacktriangle), and storage at 25°C for 4 days. No fruit drop was found at the SZ2, at 5, 8, or 13 WAFB. BS refers to the SZ1 (—) and the SZ2 (.....) of fruit treated (\blacktriangle , \square) or not treated (\times , \blacksquare)

with $1\text{ }\mu\text{L L}^{-1}$ ethylene for 12 h, or treated with $1\text{ }\mu\text{L L}^{-1}$ 1-MCP (\blacksquare , \square) for 6 h. Fruit was stored at 25°C for 4 days. Both with regard to fruit drop and BS only minor further changes were found between day 4 and 7. Note that the data on the SZ2 of day 13 WAFB of the control and the treatment with 1-MCP overlap. Data are means of 25 replicates. Data on the same day with the same letter are not different significant at $P = 0.05$, DMRT.

fruit abscised at the proximal zone (similar to SZ1) while ripe fruit showed abscission at the distal zone (slightly similar to SZ2). In peach Rascio et al. (1985) found that the abscission zone at the junction of the fruit and the pedicel (similar to SZ1) is responsible for abscission of floral buds, open flowers, and very young fruit. Two abscission zones in the pedicel become activated later on, one during early June drop, the other during late June drop.

At 5, 8, and 13 WAFB we observed increased abscission at SZ1 after ethylene treatment while SZ2 did not respond to ethylene by increasing abscission. Ethylene only induced a small decrease in the BS at SZ2 (where abscission of mature fruit took place, after slight mechanical force only), but did not induce abscission at SZ2. These data show that SZ1 was highly sensitive to ethylene, whereas SZ2 was only slightly sensitive, not enough to induce

abscission. This is quite different from tomato fruit where ethephon (ethylene) treatment increased the abscission of ripe fruit but not of small immature fruit (Biaín de Elizalde, 1980). In citrus ethylene increased abscission in both abscission zone A (although only until week 6–8 after fruit set, and no longer thereafter) and abscission zone C (Goren, 1993b; Iglesias et al., 2006). This is also different from our findings in longkong fruit.

In citrus it depends on the developmental stage of zone A whether ethylene induces abscission in this zone or not (Goren, 1993b; Iglesias et al., 2006). The present data on longkong show that exogenous ethylene was also not able to induce abscission in the SZ2, even though the BS became slightly lower after the ethylene treatment. SZ2 was considerably less sensitive to exogenous ethylene than the SZ1. It is therefore possible that the SZ2 is relatively insensitive to endogenous ethylene. This needs to be determined in further experiments.

After a short transportation period (from the field to the laboratory) of bunches harvested at 13–15 WAFB, fruit drop during transportation was low. In the present tests (Figure 6), which lasted only 4 days, no fruit drop was found in controls harvested at 13 WAFB. By contrast, in other (unpublished) postharvest storage simulation experiments in the laboratory, using fruit harvested at 13 WAFB, abscission mainly increased after day 4 of storage, and varied between and 10 and 70% by day 10. Drop occurred mostly at SZ1 with about 5% at SZ2. High rates of abscission might be due mainly to ethylene production of fruit infected by fungus. An additional practical test (also unpublished data) found that fruit harvested at 13 WAFB and undergoing long sea transportation in a container (7 days at 18°C) showed about 70–80% fruit drop by the end of transportation, increasing to 100% after transfer to ambient temperature for 2 days. Most of the drop (about 95%) occurred at SZ1. These data also suggested that fruit infected with fungus produced relatively high ethylene concentrations in the closed container, inducing abscission at SZ1. This abscission can largely be prevented, as it was previously reported that a 6 h treatment with 1 $\mu\text{L L}^{-1}$ 1-MCP drastically reduces fruit drop at the SZ1 (Taesakul et al., 2012).

A hypothesis might be proposed as to the possible function of the two separation zones in longkong fruit. As the separation at SZ1 by 13 WAFB was significantly promoted by exogenous ethylene, this zone could possibly function in response to changes in endogenous ethylene levels or to changes in ethylene sensitivity. It could therefore be responsive to factors that cause physiological stress. Infection of longkong fruit with fungi, of which *Phomopsis* sp. and *Lasiodiplodia theobromae* are the most important

(Kaewsorn and Sangchote, 2003), might induce such a stress. SZ2 was much less affected by the ethylene treatment than SZ1. Separation at SZ2 had already started before the change in peel color (Figure 6B), as indicated by the presence of smooth cell walls after pulling the fruit from the fused sepals (Figure 4H). By 15 WAFB the BS at SZ2 was quite low, although not low enough for spontaneous abscission. This low BS possibly allows animals to remove healthy and ripe fruit from the tree, using only slight mechanical force.

It is concluded that longkong ovary and fruit drop on the tree only occurred at SZ1, which contains an abscission zone that is highly sensitive to exogenous ethylene. By contrast, fruit drop at SZ2 was not induced by treatments with exogenous ethylene, even though cell separation in this zone had already started by the time of fruit coloration, and was slightly increased by the ethylene treatment. Incomplete cell separation at SZ2 seems to explain why no spontaneous fruit drop occurred at this zone. By 15 WAFB only a small mechanical force induced full separation at SZ2. The two zones are thus functionally different: one shows abscission in response to an increase in exogenous ethylene concentrations while the other does not. We suggested a hypothesis to account for the presence of two abscission zones with different characteristics.

Author Contributions

PT did the experimental work, carried out data analysis, and drafted the manuscript; WD interpreted the data and revised the manuscript; JS designed the work, acquired funding, supervised experiments, interpreted the data, and was involved in revising the manuscript. The three authors approved the final version and agree to be accountable for all aspects of the work.

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Supplementary Material

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Burst of reactive oxygen species in pedicel-mediated fruit abscission after carbohydrate supply was cut off in longan (*Dimocarpus longan*)

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Cutting off carbohydrate supply to longan (*Dimocarpus longan* Lour.) fruit by girdling and defoliation or by detachment induced 100% abscission within a few days. We used these treatments to study the involvement of reactive oxygen species (ROS) in fruit abscission. Girdling plus defoliation decreased sugar concentrations in the fruit and pedicel and depleted starch grains in the chloroplasts in the cells of abscission zone. Prior to the occurrence of intensive fruit abscission, there was a burst in ROS in the pedicel, which peaked at 1 day after treatment (DAT), when H₂O₂ in the abscission zone was found to be chiefly located along the plasma membrane (PM). H₂O₂ was found exclusively in the cell walls 2 DAT, almost disappeared 3 DAT, and reappeared in the mitochondria and cell walls 4 DAT. Signs of cell death such as cytoplasm breakdown were apparent from 3 DAT. The burst of ROS coincided with a sharp increase in the activity of PM-bound NADPH oxidase in the pedicel. At the same time, activities of antioxidant enzymes including superoxide dismutase (SOD), catalase, and peroxidase (POD) were all increased by the treatment and maintained higher than those in the control. Accompanying the reduction in H₂O₂ abundance, there was a sharp decrease in PM-bound NADPH oxidase activity after 1 DAT in the treated fruit. H₂O₂ scavenger dimethylthiourea (DMTU, 1 g L⁻¹) significantly inhibited fruit abscission in detached fruit clusters and suppressed the increase in cellulase activity in the abscission zone. These results suggest that fruit abscission induced by carbohydrate stress is mediated by ROS. Roles of ROS in regulating fruit abscission were discussed in relation to its subcellular distribution.

Keywords: fruit abscission, carbohydrate stress, reactive oxygen species, plasma membrane-bound NADPH oxidase, cellulase, longan

Introduction

Carbohydrates serve as the “hard currency” in plants, representing the costs for various biological functions including growth, maintenance, and defense. Fruit are net importers of carbohydrates from the tree reserves or leaf photosynthesis (Mehouachi et al., 2000; Hieke et al., 2002; Iglesias et al., 2003). Fruit trees generally produce more fruitlets than they can support to harvest, and fruit abscission

is a normal physiological event during fruit development due to a self-regulatory mechanism to reduce fruit load (Bangerth, 2000). However, under adverse conditions, such as shading (Yuan and Huang, 1988; Zhou et al., 2008; Li et al., 2013), high temperatures (Atkinson et al., 2001; Gazit and Degani, 2002), or abrupt temperature fluctuations (Yang et al., 2010), this mechanism may cause excessive fruit abscission. According to Lakso et al. (2006), environmental factors affect fruit abscission based on the carbohydrate supply–demand balance, and higher carbohydrate availability reduces sensitivities to abscission-inducing stresses or fruit-thinning chemicals. Mehouachi et al. (1995) suggested the existence of a threshold carbohydrate concentration in citrus below which fruit shedding was intensified.

There is limited evidence on how a shortage of carbohydrates initiates the activity of the abscission zone leading to fruit shedding. Gómez-Cadenas et al. (2000) found that defoliation increased the concentration of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) and abscisic acid (ABA). They suggested that these hormones participate in the self-regulatory mechanism that adjusts fruit load depending on the availability of carbohydrates. Iglesias et al. (2006) observed high ethylene evolution in the fruit and the pedicel and intensive fruit abscission after the fruit stalk was girdled. While the hormone ethylene is a well-known hormone that triggers fruit abscission (Taylor and Whitelaw, 2001; Meir et al., 2010), there is much less information about roles of reactive oxygen species (ROS) in abscission regulation.

Reactive oxygen species is involved in the responses of plants to stresses and is generated by a number of mechanisms including NADPH oxidation catalyzed by plasma membrane (PM)-bound NADPH oxidase (Cheeseman, 2007; Tripathy and Oelmüller, 2012). Reports about the roles ROS in fruit abscission have been inconsistent. Lai et al. (2001) found that H₂O₂ reduced wax apple abscission under low temperatures, while Ueda et al. (1991) reported that H₂O₂ stimulated abscission of bean petioles under high light levels independent of ethylene. Sakamoto et al. (2008) showed that H₂O₂ was involved in salt-induced abscission of pepper petioles and that it acted downstream of ethylene in signaling abscission. Botton et al. (2011) suggested that an increase in sugar concentration in the cortex of apple fruit serves as an initial senescing signal that induced H₂O₂ and ethylene production, causing abortion of seed, reduction in IAA export and fruit drop.

Longan (*Dimocarpus longan* Lour.) is a tropical fruit tree that generally sets heavily and requires thinning to produce large fruit, although natural fruit abscission occurs during fruit development. The fruit are borne in multi-fruit panicles. Unlike apple, whose fruit abscission is highly predictable with obvious dominant central fruit and weak side fruit, longan fruit within a panicle are similar in size and vigor and it is difficult to predict which and when fruit will abscise. In this study, longan fruit clusters were starved for carbohydrates by girdling and defoliating, or detaching the fruit clusters. These treatments induced 100% fruit abscission within a few days, providing a convenient experimental system to study signals involved in fruit abscission. Using this system, we examined the occurrence and roles of ROS in regulating abscission under carbohydrate stress.

Materials and Methods

Materials and Treatments

The study was carried out during the mid stage of fruit development (50–60 days after anthesis), after the early wave of fruit drop had ended and before the rapid aril (flesh) growth initiated. The on-tree experiments involved girdling and defoliation treatments, which were performed on 12- to 14-year-old “Chuliang” trees at the South China Agricultural University or Dongguan Agricultural Research Center. The off-tree experiments used detached fruit clusters harvested from these trees.

Effectiveness of girdling plus defoliation in inducing fruit abscission was examined. Twenty bearing shoots from different positions of the canopy, each with more than 20 leaves and one terminal fruit cluster bearing 40–50 fruit were selected from a tree. They were randomly allocated to four treatment groups, each with five replicates consisting of five bearing shoots as the experimental plots: no girdling or defoliation (control); or girdled at a width of 5 mm at around 60 cm from the fruit cluster base and defoliated to leave 0, 5, or 10 top leaves above the girdle. The number of fruit on each panicle was counted every day until 5 days after treatment (DAT), when all fruit in the “0 leaf” group had been shed. Since girdling plus complete defoliation induced 100% fruit drop, we adopted this treatment for the other on-tree experiments in later seasons. Twenty bearing shoots with a similar sized terminal panicle were selected from different positions of five trees ($n = 5$, one tree as one experimental block) and girdled and defoliated 50 days after anthesis as mentioned above, and 20 untreated panicles with similar fruit load from each tree used as controls. Fifteen of them were used for daily sampling, and five of them used for tracing abscission through daily counting of fruit in each panicle. Fifty fruit per treatment from each tree were collected every day and dissected into fruit and pedicel for analyses of sugars and enzyme activities.

For the off-tree experiment, our initial trial showed that detached fruit desiccated quickly and never shed. However, if the peduncle of the fruit cluster was inserted into distilled water immediately after detachment from the tree to prevent desiccation, intensive fruit abscission occurred in a few days. Therefore, water-fed detached fruit clusters each with over 20 fruit were used to examine the effects of the H₂O₂ scavenger dimethylthiourea (DMTU) on abscission and cellulase activity in the abscission zone. The treatment used DMTU solution (0.1%, w/v) to replaced distilled water used in the control group. All the clusters were placed in an incubator (RXZ-0450, Jiangnan, Nibo, China) at 28°C, 85% relative humidity, and a 12/12 dark-light cycle with a light intensity of 40 $\mu\text{mol m}^{-2}\text{s}^{-1}$ during light period. Five clusters in each treatment were set for daily recording of fruit abscission, and five other clusters were set for sample taking. Pedicels were collected from four to five fruit in each cluster every day for measurement of cellulase activity in the abscission layer. The experiments had five replicates consisting of samples from the five clusters.

Sugar Contents in the Fruit and Pedicel

Fruit and pedicel samples were taken from the on-tree experiment. Sugars were extracted from fruit or pedicel tissues of known

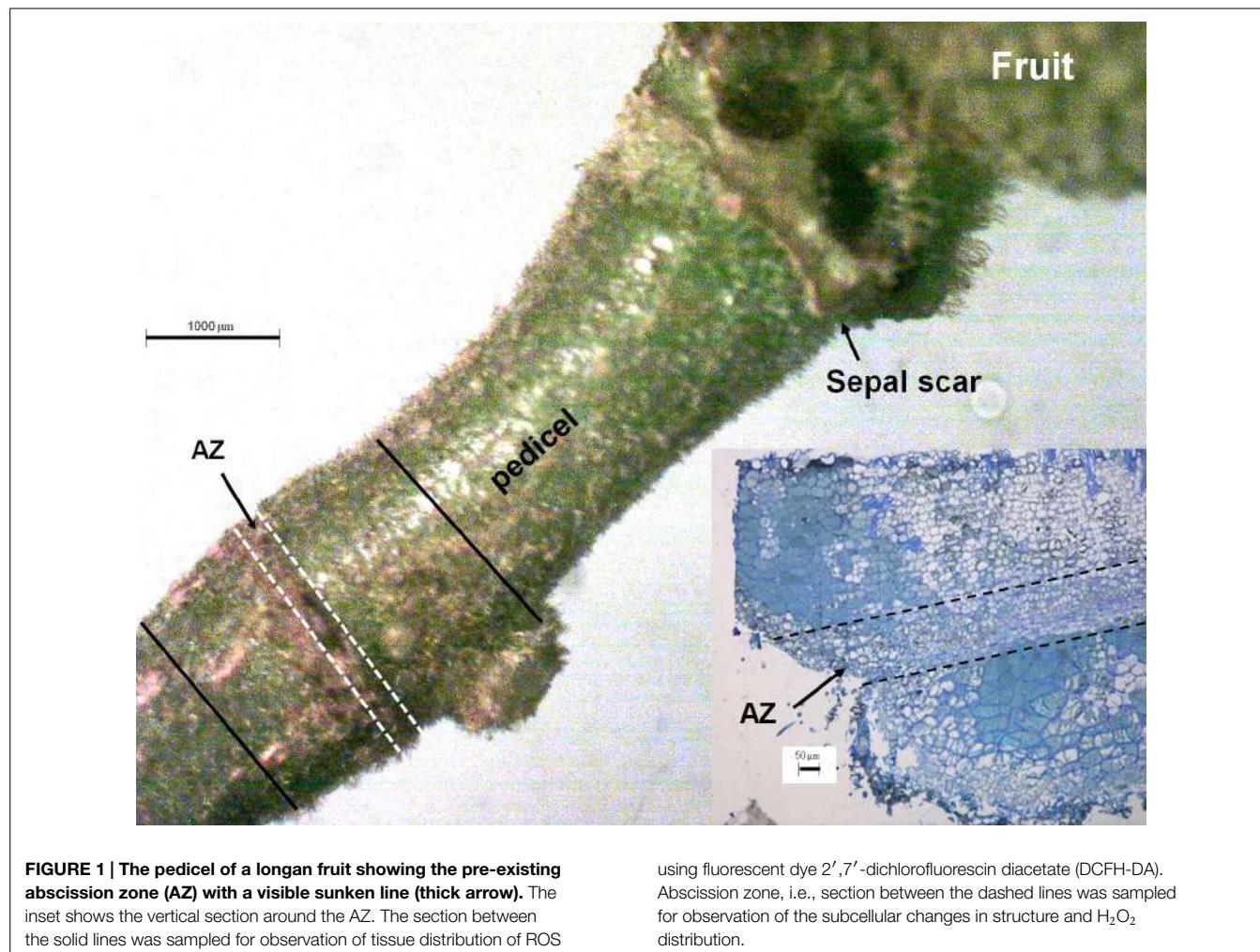


FIGURE 1 | The pedicel of a longan fruit showing the pre-existing abscission zone (AZ) with a visible sunken line (thick arrow). The inset shows the vertical section around the AZ. The section between the solid lines was sampled for observation of tissue distribution of ROS

using fluorescent dye 2',7'-dichlorofluorescin diacetate (DCFH-DA). Abscission zone, i.e., section between the dashed lines was sampled for observation of the subcellular changes in structure and H₂O₂ distribution.

weight with 90% (v/v) ethanol solution and analyzed using high performance liquid chromatograph (HPLC) according to Wang et al. (2006).

Observation of ROS Occurrence Around the Abscission Zone in the Pedicel by Confocal Microscopy

The occurrence and tissue distribution of ROS was analyzed using the H₂O₂ fluorescent probe 2',7'-dichlorofluorescin diacetate (DCFH-DA; Coelho et al., 2002). DCFH-DA is converted into DCFH that reacts with one-electron oxidizing species including •OH generated from Fenton reduction of H₂O₂ instead of with H₂O₂ *per se* and generates fluorescence-emitting DCF* (Kalyanaraman et al., 2012). Therefore, the method indirectly reflects the abundance of H₂O₂. The fluorescent dye is generally considered as an intracellular ROS probe, as it requires intracellular esterase to release the reactive DCFH (Afri et al., 2004). However, the enzyme is present apoplastically in plants (Cummins and Edwards, 2004). Hence, the dye is able to probe both intracellular and apoplastic ROS in plants.

For this analysis, we chose a separate longan tree of "Chuliang" and girdled and defoliated three different bearing shoots each day

from 50 days after anthesis until day 5, when fruit samples at different DAT together with the non-treated control fruit were collected. As a result, we could observe samples at different DAT on the same day, avoiding the influence of fruit age.

The abscission zone of the longan pedicel is easily identified (Figure 1). Segments of pedicel about 2 mm long including the abscission zone (between the solid lines in Figure 1) were excised, and 0.1-mm-thick vertical sections cut by hand. The sections were evacuated with a syringe in 3 mL of loading buffer (10 mmol L⁻¹ Tris, 50 mmol L⁻¹ KCl, and 50 μmol L⁻¹ DCFH-DA, pH 7.2) with 10 μL of Triton X-100, and incubated for 30 min in the dark at 25°C. The sections were then rinsed three times with loading buffer without DCFH-DA, placed on a slide and observed under a confocal microscope (Leica TCS SP2, Mannheim, Germany), with the excitation beam at 488 nm and the emission beam at 543 nm.

Subcellular Distribution of H₂O₂ in Cells of the Abscission Zone

The study was carried out with pedicels from the on-tree experiment mentioned in the section above. Pedicel disks about 0.2 mm thick comprising the abscission zone (section between dashed

lines in **Figure 1**) were cut into slices about 0.2 mm wide and 1 mm long. The samples were prepared for observation of the subcellular distribution of H₂O₂ using the cerium chloride (CeCl₃) precipitation method (Bestwick et al., 1997). The ultra-thin sections of the abscission zone were observed under a Philips FEI-TECNAI 12 transmission electron microscope (Eindhoven, Holland).

Determination of Plasma Membrane-Bound NADPH Oxidase in the Pedicel

Purified plasma membranes were isolated from pedicel tissues by aqueous two-phase partitioning (Liu et al., 2009). Membrane protein content was estimated by the Coomassie blue G-250 protein assay using bovine serum albumin (BSA) as a standard. PM-bound NADPH oxidase was measured based on NADPH-dependent O₂⁻ generation (Gestelen et al., 1997) using nitro-blue tetrazolium (NBT) dye, which is converted to monoformazan by O₂⁻. This reduction was detected spectrophotometrically at 530 nm. The reaction mixture consisted of a Tris buffer (50 mmol L⁻¹ Tris-HCl, pH 7.4, 250 mmol L⁻¹ sucrose, 20 mM DTT, 0.1 mmol L⁻¹ NBT and 0.1 mmol L⁻¹ NADPH) with or without superoxide dismutase (SOD; 50 units mL⁻¹). NBT reduction by O₂⁻ was calculated from the difference in the absorbance increase rate between the presence and absence of SOD.

Assays of Catalase (CAT), Superoxide Dismutase (SOD), and Peroxidase (POD) in the Pedicel

Pedicel tissue of known fresh weight (0.5 g) was ground into powder in liquid nitrogen added with 0.02 g PVPP. The powder was washed into a centrifuge tube with 2.5 mL ice-cold phosphate buffer solution (50 mmol L⁻¹, pH 7.5) containing 0.1 mmol L⁻¹ EDTA and 0.3% (v/v) Triton X-100, and centrifuged at 13,000 × g for 10 min at 4°C, and the supernatant was used as the crude enzyme. The protein content in the crude enzyme was determined using Coomassie blue G-250 as mentioned above.

The analysis of CAT was conducted using an oxygen electrode (Zhang and Qu, 2003). The substrate solution was a fresh 100 mmol L⁻¹ H₂O₂ solution. Two milliliters of this substrate was transferred into the reaction well of a Hansatech Oxygraph system. When the oxygen signal in the solution had stabilized, 50 µL of crude enzyme was injected into the reaction well. The rate of oxygen release was recorded and used to calculate enzyme activity.

SOD activity was determined using a commercial assay kit provided by Nanjing Jiangcheng Bioengineering Institute. With xanthine-xanthine oxidase as the superoxide generator, SOD activity was quantified by the percentage of inhibition of nitroblue tetrazolium (NBT) reduction, which was recorded by optical density at 530 (OD₅₃₀) nm. One unit enzyme activity was regarded as the inhibition of 50% of NBT reduction in 10 min. Enzyme activity per mg protein in the tissue was calculated.

POD activity was determined by guaiacol method, where 0.05 mL of the crude enzyme was added to 1 mL of 50 mmol L⁻¹ phosphate buffer solution (pH = 7.0) containing 30 mmol L⁻¹ H₂O₂ and 5 mmol L⁻¹ guaiacol, and increment of absorbance at 470 nm (OD₄₇₀) was recorded. An increment of 0.01 in OD₄₇₀ per second was regarded as one unit of enzyme activity.

Assay of Cellulase Activity in the Abscission Zone by Gel Diffusion

Wall-degrading cellulase in the abscission zone was analyzed by using tissue blotting and gel diffusion (Bourgault and Bewley, 2002). The substrate was sodium carboxymethylcellulose dissolved in McIlvaine buffer, pH 4.8 at 0.1% (w/v). To prepare assay gels, 100 mL of substrate was combined with 1.2 g agarose (1.2% w/v) in a 250 mL flask, boiled, cooled to approximately 65°C, and 6 mL of solution poured into a pre-warmed (65°C) set of petri dishes (60 mm in diameter). After the gel had cooled down to room temperature, 1 mm pedicel segments were cut out crosswise along the abscission line (**Figure 1**), and placed on the gel with the cut surface facing down. The gel with samples was incubated for 18 h at 40°C, stained for 30 min in 0.2% Congo red dye, washed for 3 min in water and then in 1 mol L⁻¹ NaCl for 3 min, and fixed for 5 min in 5% acetic acid. The diameters (mm) of the transparent spots created by cellulase activity on developed gel were used to indicate relative cellulase activity.

Statistical Analyses

Unless otherwise specified, the experiments were set out in a randomized block design using five individual trees as experimental blocks (*n* = 5). Student's *t*-tests and least significant difference (LSD) multiple range tests (*P* < 0.05) were carried out using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA).

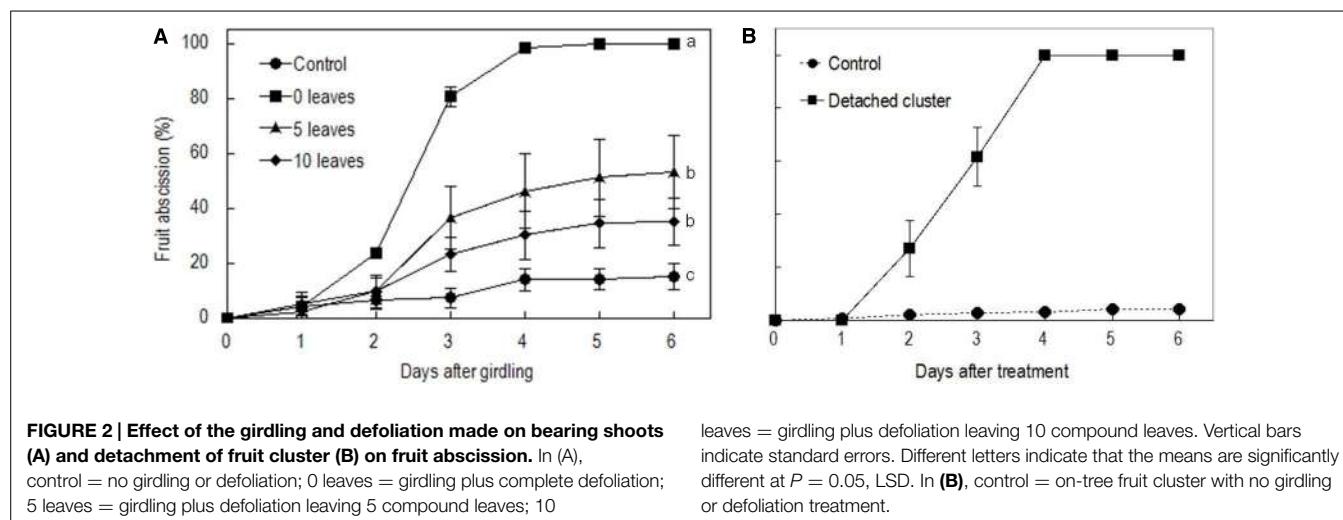
Results

Effects of Girdling and Defoliation and Detachment Treatments on Fruit Abscission

Girdling and defoliation increased fruit abscission compared with the rate in the control, and it also increased with the rate of defoliation (**Figure 2A**). The results suggest that the presence of source leaves determines the intensity of fruit abscission after girdling. Hence, the intensive abscission after girdling and defoliation was not a result of a wounding effect of the treatment but a result of reduced photosynthate supply due to reduced leaf number. All fruit were shed within 5 days after the treatment with girdling and complete defoliation (referring to girdling plus defoliation hereafter). The result was repeatable in the experiments in the following seasons (result not shown). Since fruit abscission in this treatment was completely predictable, we used it to study the occurrence of ROS and its related enzymes during fruit abscission. Similarly, water-fed detached clusters shed all their fruit within 5 days (**Figure 2B**).

Effects of Girdling Plus Defoliation on Sugar Contents

Sucrose was the major sugar in both the pedicel and the fruit. Girdling plus defoliation significantly decreased the contents of sugars in both parts compared with the controls (**Figure 3**). Sucrose showed a greater and faster decrease than glucose and fructose after the treatment. The pedicel lost nearly 50% total sugars, especially sucrose within 1 DAT, and sugar decrease was faster than in the fruit. The result confirms a carbohydrate stress is created by girdling plus defoliation.



Occurrence of ROS in the Pedicel After Girdling Plus Defoliation Treatment

In the pedicel of the control fruit, DCF fluorescence was more concentrated in the phloem and the cambium (**Figure 4A**), indicating constitutive production of ROS in these tissues. As revealed by CeCl₃ precipitation, H₂O₂ was found exclusively in the cell walls in the cells of the abscission zone (**Figure 4F**). There was an abrupt increase in ROS 1 DAT, when strong DCF fluorescence was found in tissues along the abscission zone (**Figure 4B**). Under transmission electron microscope, H₂O₂ was located along the plasma membrane as well as on the cell walls in the cells of the abscission zone (**Figures 4G,M**). No H₂O₂ was observed in the chloroplasts or mitochondria (**Figure 4M**). The result suggests that *de novo* H₂O₂ production by a membrane-bound mechanism was activated by the treatment. At 2 DAT (**Figure 4C**), DCF fluorescence became much weaker than that on day 1. However, the abscission layer maintained high levels of fluorescence, where H₂O₂ was exclusively found in the cell walls (**Figure 4H**). Thereafter, H₂O₂ level decreased drastically (**Figures 4D,E,I,J**). Throughout the experiment, no CeCl₃ precipitation was observed within the cytoplasm or nucleus but at 4 DAT there was some CeCl₃ precipitation in the mitochondria as well as in the cell walls (**Figures 4L–P**).

In addition to changes in abundance and subcellular distribution of H₂O₂, some ultra-structural changes in the cells were also observed after girdling plus defoliation treatment. Starch grains observed in the chloroplasts on day 0 (**Figure 4L**) had disappeared by day 1 (**Figure 4M**). By day 3, cytoplasm breakdown had become apparent with the loss of plasma membrane integrity and disappearance of vacuole boundary (**Figures 4I,J**), which are signs of cell death.

Changes in ROS Metabolism Enzymes in the Pedicel After Girdling Plus Defoliation

Coinciding with the changes in the abundance of ROS, the activity of PM-bound NADPH oxidase in the pedicel increased drastically within 12 h after girdling plus defoliation, peaked around 24 h after the treatment, and then decreased sharply although

remained higher than the control (**Figure 5A**). The treatment also induced significant increase in SOD activity (**Figure 5B**), which converts superoxide anion radical generated by PM-bound NADPH oxidase into H₂O₂. From 24 to 72 h after the girdling plus defoliation, the activities of H₂O₂-scavenging enzymes, i.e., CAT (**Figure 5C**) and POD (**Figure 5D**) were significantly higher in the treated pedicel than in the control. The two enzymes displayed opposite trends, CAT decreasing while POD increasing during fruit development.

The above results show that carbohydrate stress induced an endogenous ROS burst around the abscission zone and increased the activities of both ROS generating and scavenging enzymes.

Effects of DMTU on Fruit Abscission and Cellulase Activity in the Abscission Zone

DMTU, which erased the H₂O₂ burst (**Figures 6A,B**), significantly suppressed fruit abscission in detached clusters (**Figure 6C**), suggesting H₂O₂ has an essential role in regulating fruit abscission. Cellulase activity in the abscission zone of the control detached fruit increased over time (**Figure 7**). The increase was significantly inhibited by DMTU (**Figure 7**), which agreed with its effect on fruit abscission.

Discussion

Girdling is a common horticultural practice to promote flowering and fruit set, and also serves as a useful tool for physiological study of shoot behavior when it is isolated from other plant parts in terms of carbohydrate exchange (Goren et al., 2004). However, the effect of girdling on fruit set varies depending on the availability of source leaves above the girdle (Hieke et al., 2002; **Figure 2A**). When the supply of carbohydrates to longan fruit was cut off by girdling and complete defoliation, all the fruit abscised in a few days (**Figure 2A**). Similarly, detached fruit cluster deprived of carbohydrate supply shed all their fruit within 5 days (**Figure 2B**). Unlike the results obtained by Botton et al. (2011), who found an increase in sugar content in abscising apple fruit and suggested sugar increase as an early senescence signal in

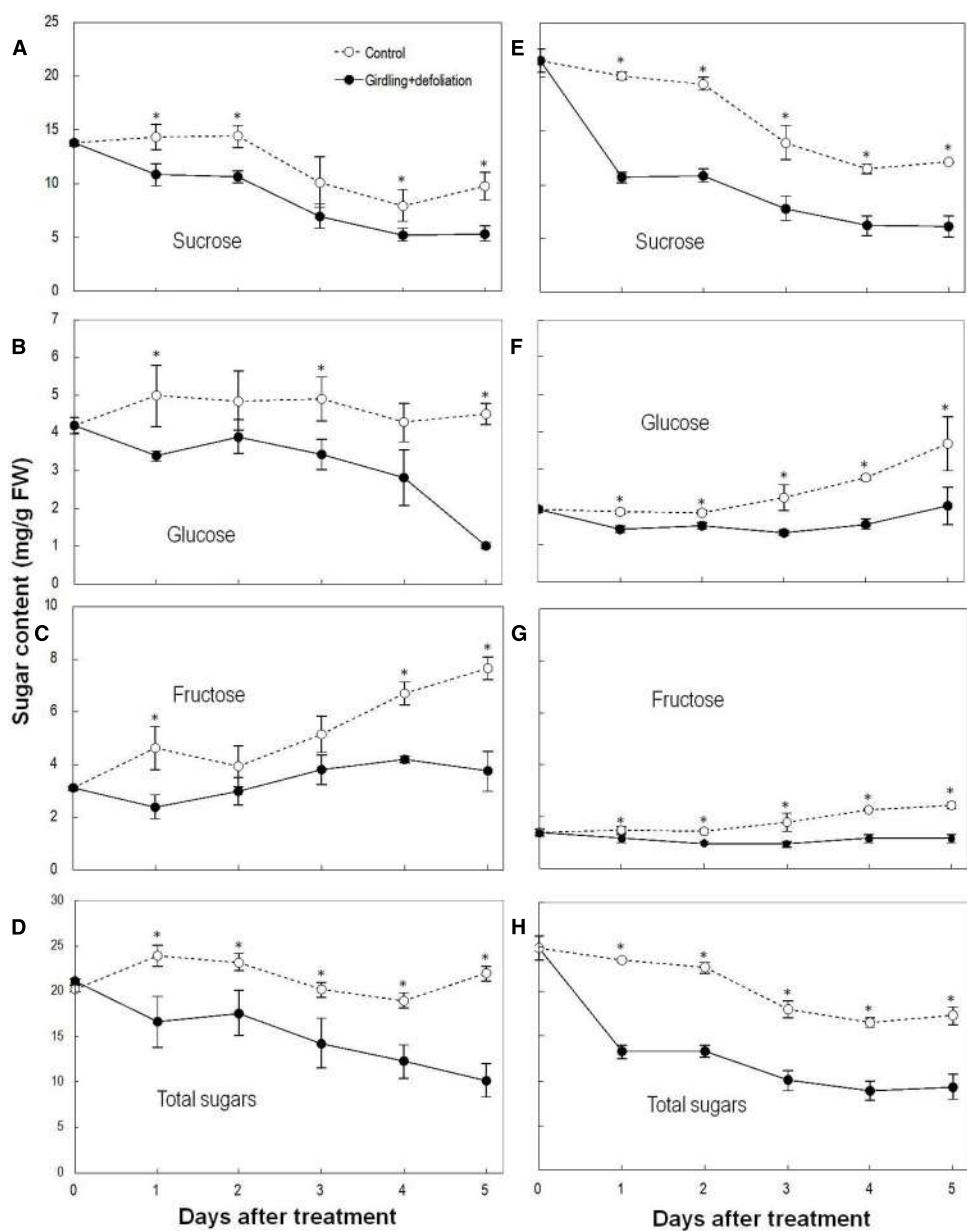


FIGURE 3 | Effect of girdling plus defoliation on the concentrations of sucrose, glucose, fructose, and total sugars in fruit (A–D) and pedicel (E–H) of longan. Vertical bars indicate standard errors of means. Control and

treatment results are indicated by dashed and solid lines, respectively. Asterisks indicate that the paired means were significantly different at $P = 0.05$, Student's t -test ($n = 5$).

fruit that triggers abscission, girdling plus defoliation treatment induced a significant drop of all major sugars (Figure 3) and loss of starch (Figure 4) in the fruit and/or pedicel. Hence, the treatment generated a carbohydrate stress that triggered abscission. Our study was the first to explore the occurrence of ROS in response to carbohydrate stress and its role in fruit abscission.

The Occurrence of ROS Induced by Carbohydrate Stress

Plant cells constantly produce ROS during the processes of aerobic metabolism. Several mechanisms in different cell compartments

such as chloroplast, mitochondria, peroxisomes, plasma membrane, and cell walls are involved in ROS generation (Cheeseman, 2007; Sharma et al., 2012; Tripathy and Oelmüller, 2012). Results in this study showed that H₂O₂ occurred exclusively in the cell walls in the pedicel from non-starved fruit (day 0 sample; Figure 4F), suggesting wall-bound mechanisms were involved in normal generation of ROS. ROS is restricted to a homeostatic level in the normal cells due to the presence of ROS-scavenging mechanisms including various antioxidant molecules and enzymic processes. However, stresses such as drought, chilling, salinity, metal toxicity, UV irradiation, and pathogen attack activate the

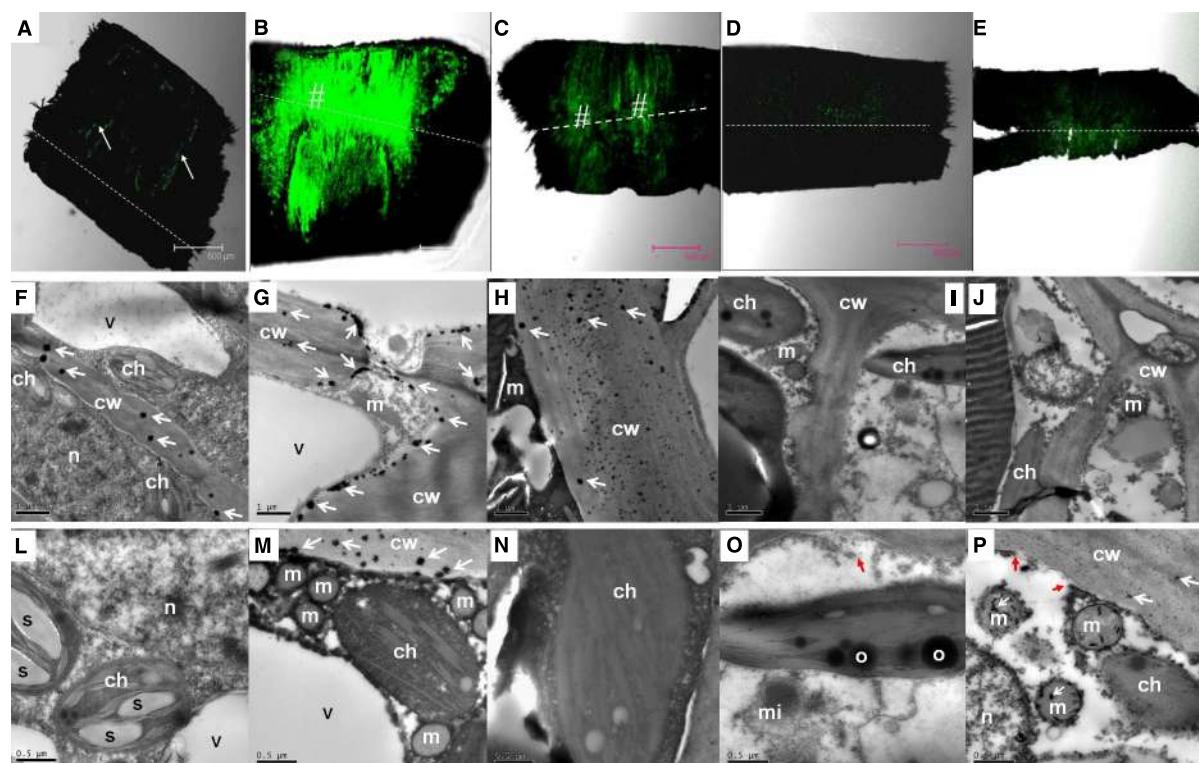


FIGURE 4 | Changes in the distribution of H₂O₂ as revealed by DCF fluorescence under a confocal scanning microscope in the pedicel (A–E) and by CeCl₃ precipitation in the abscission zone under a transmission electron microscope (F–P) after girdling plus defoliation treatment.

A,F,L: samples at day 0 (control), with occurrence of H₂O₂ that was more concentrated in the cambium (arrow in **A**) and exclusively located in the cell walls (arrows in **F**); **B,G,M:** samples of 1 day after treatment (DAT). Note increase in fluorescence yield compared with that at Day 0 (**B** vs **A**) and apoplastic location of H₂O₂ along the plasma membrane (arrows) and the cell walls (arrows in **G** and **M**). **C,H,N:** samples of 2 DAT. Note fluorescence

reduced compared with 1 DAT (**C** vs **B**) and H₂O₂ occurred exclusively in the cell walls (arrows in **H**). **D,I,O:** samples of 3 DAT, with fluorescence weaker than in the earlier samples and no observable occurrence of H₂O₂ in the cell walls and other cell parts. Note apparent breakdown of cytoplasm and plasma membrane (red arrow in **O**). **E,J,P:** samples of 4 DAT, with reappearance of H₂O₂ in the cell walls and mitochondria (white arrows in **P**) and breakdown of plasma membrane (red arrows in **P**). Dashed lines indicate the abscission layer. The symbol “#” indicates the abscission zone with relatively high H₂O₂. cw, cell wall; ch, chloroplast; m, mitochondrion; mi, microsome; n, nuclear; o, oil drop in chloroplast; s, starch grains in chloroplasts; v, vacuole.

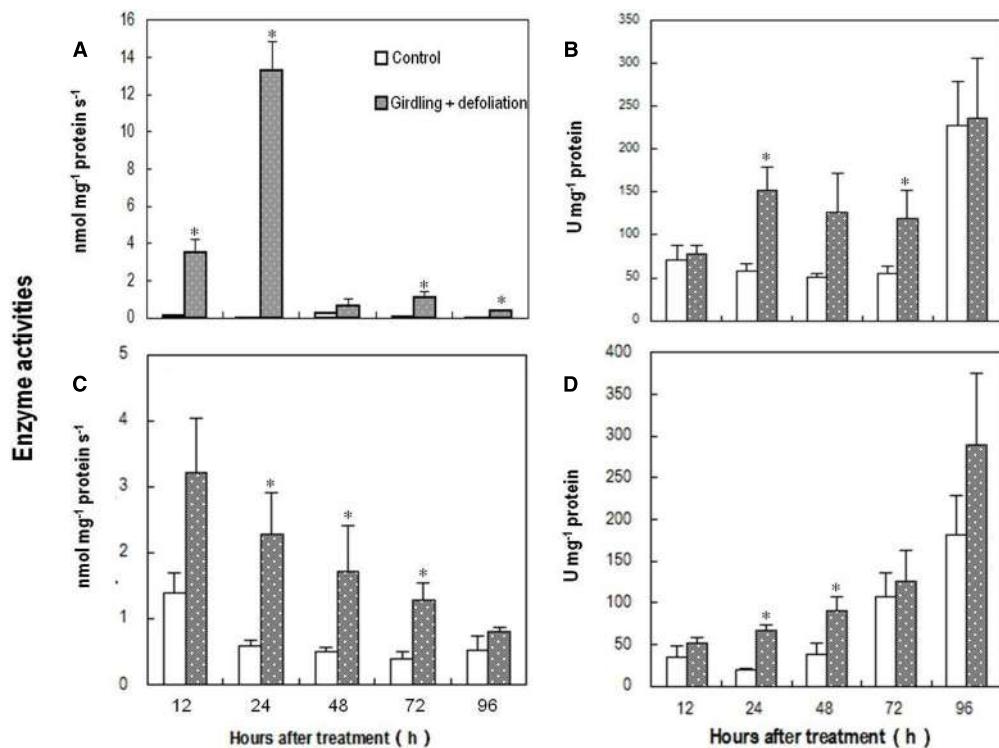
generation mechanisms and cause a burst of ROS (Lee et al., 2004; Sakamoto et al., 2008; Sharma et al., 2012). Our results provided direct evidence showing a ROS burst that occurred in response to carbohydrate stress (Figure 4). The strongest DCF fluorescence reflecting massive accumulation of one-electron oxidizing species, e.g., •OH occurred in the abscission zone at 1 DAT (Figure 4B), when H₂O₂ was chiefly distributed apoplastically along the plasma membrane (Figure 4G). In addition, no accumulation was found in the chloroplasts, mitochondria, or other cell parts, suggesting the outbreak of ROS caused by carbohydrate stress was generated by a membrane-bound mechanism. This coincided with a sharp increase in the activity of PM-bound NADPH oxidase (Figure 5A). Hence, this enzyme appears to be responsible for ROS generation under carbohydrate stress. A similar conclusion was obtained by Sakamoto et al. (2008) who found ROS accumulation generated by NADPH oxidase in the abscission zone of pepper leaves under salt stress. Interestingly, carbohydrate stress treatment also up-regulated ROS scavenging enzymes including SOD, catalase, and POD (Figure 5). The results clearly show that the ROS burst induced by carbohydrate stress

was not a result of deactivation of scavenging mechanisms but a result of increased PM-bound NADPH oxidase.

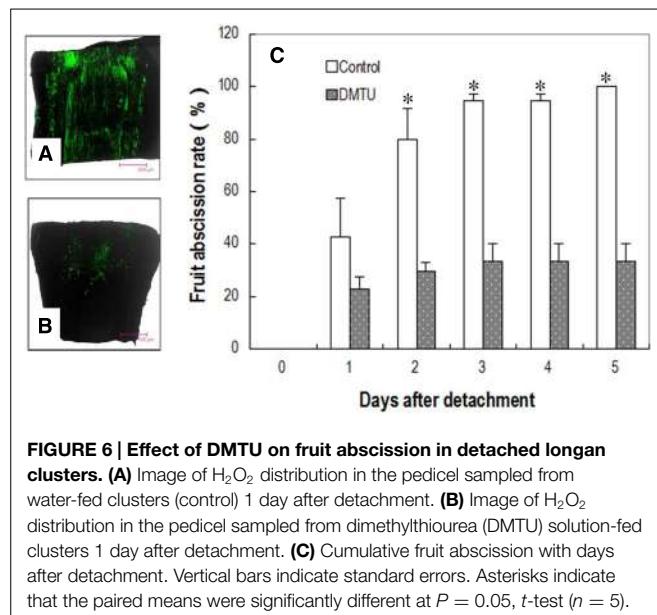
As a mechanism that maintains ROS homeostasis, increased ROS up-regulates its scavenging enzymes (Yang and Poovaiah, 2002). The sharp decrease in PM-bound NADPH oxidase together with significant increases in H₂O₂-scavenging catalase and POD (Figure 5) might have led to the disappearance of H₂O₂ from 2 DAT. The reappearance of H₂O₂ on day 4 (Figure 4E) was found in the mitochondria as well as on the cell walls (Figure 4P), indicating mitochondrion-involved H₂O₂ generation took place in a later stage of fruit abscission.

Roles of ROS in the Regulation of Fruit Abscission Under Carbohydrate Stress

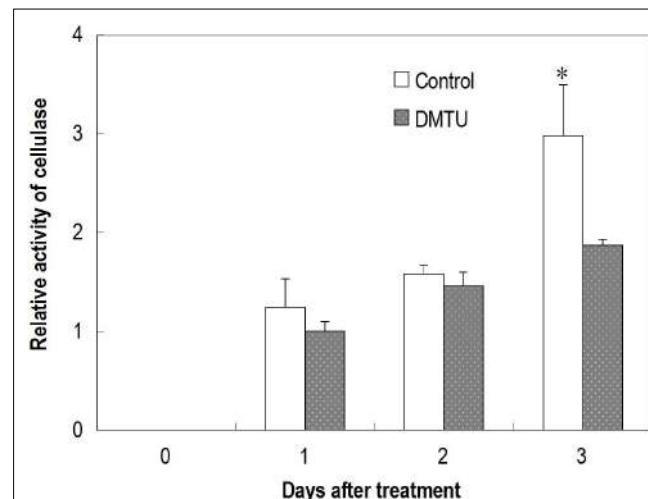
Limited evidence is available for a role of ROS in abscission. Lai et al. (2001) found that H₂O₂ reduced abscission in wax apple at low temperatures. A number of other studies showed that H₂O₂ accumulated prior to and promoted organ abscission (Ueda et al., 1991; Sakamoto et al., 2008; Zhou et al., 2008). Sakamoto et al. (2008) found the abscission of excised pepper leaves was



of longan. Vertical bars indicate standard errors. Asterisks indicate that the paired means were significantly different at $P = 0.05$, *t*-test ($n = 5$).



increased by exogenous H₂O₂ and decreased by H₂O₂ biosynthetic inhibitors or scavengers. Cohen et al. (2014) suggested that ROS was responsible for rapid root abscission in *Azolla*. In the present study, exogenous H₂O₂-scavenger DMTU significantly suppressed fruit abscission under carbohydrate stress.



These results suggest that ROS plays an essential role in organ abscission.

The location of H₂O₂ in the cells displays mechanisms of its generation and provides clues of its roles in abscission. H₂O₂

induced by carbohydrate stress was initially found along the plasma membrane (1 DAT; **Figure 4G**) revealing H₂O₂ signal sensing at plasma membrane as well as a membrane-bound ROS generation mechanism. Later, H₂O₂ was exclusively located in the cell walls (**Figure 4H**), suggesting its actions on the cell walls. Studies thus far have indicated at least three roles for H₂O₂ in wall modification. First, H₂O₂, as a substrate, directly participates in the peroxidase-catalyzed cross-linking reactions between lignin monomers and between phenolic residues in the structural macro-molecular components of the cell walls (Brett and Waldron, 1990; Brisson et al., 1994). Second, •OH, the product of Fenton reduction of H₂O₂, directly breaks the polysaccharide chains, loosening the cell walls during cell elongation (Liszkay et al., 2004; Dunand et al., 2007). Involvement of •OH-mediated oxidative bond cleavage in the cell walls of the abscission zone has also been reported during rapid root abscission in *Azolla* (Cohen et al., 2014). Our observation of increased generation of DCF* from DCFH in the abscission zone after carbohydrate stress (**Figure 4**) is indicative of the generation of a potent •OH-like oxidant (Kalyanaraman et al., 2012). As cell separation during abscission involves both the cleavage of structural polysaccharides and lignin accumulation (Poovaiah, 1974), there is a possibility that both of the above-mentioned roles of H₂O₂ in wall modification are involved in the abscission induced by carbohydrate stress. Third, H₂O₂ was found to up-regulate the gene encoding cellulase during leaf abscission (Sakamoto et al., 2008). Our study provided further evidence of the involvement of H₂O₂ in the up-regulation of cellulase activity during fruit abscission (**Figure 7**). In addition to cell wall modifications, ROS as a plant signal may trigger a

wide range of biochemical changes leading to abscission. Recently, researchers found programmed cell death (PCD) serves as a key mechanism in fruit abscission of tomato (Bar-Dror et al., 2011). There are sound evidences showing H₂O₂ generated by NADPH oxidase signals PCD in response to stresses (Gechev and Hille, 2005; Vannini et al., 2012; Huang et al., 2014). In the current study, accumulation of ROS in the mitochondria (**Figure 4P**) and signs of cell death (**Figures 4O,P**) in the abscission zone were observed, indicating H₂O₂-induced PCD in the abscission zone might be involved in fruit abscission under carbohydrate stress. Further studies are needed to clarify the signal pathways mediating ROS and abscission.

Author Contributions

ZY and XZ conducted the major part of the experiments, data processing and writing the draft of the paper. YF took part in field data collecting, sample preparing, data processing, and draft improvement. JL, HW, and XH contributed experiment design, final data analysis and editing the manuscript. ZY and XZ contributed equally to the work.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptome Profiling of Petal Abscission Zone and Functional Analysis of an Aux/IAA Family Gene *RhIAA16* Involved in Petal Shedding in Rose

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Roses are one of the most important cut flowers among ornamental plants. Rose flower longevity is largely dependent on the timing of petal shedding occurrence. To understand the molecular mechanism underlying petal abscission in rose, we performed transcriptome profiling of the petal abscission zone during petal shedding using Illumina technology. We identified a total of 2592 differentially transcribed genes (DTGs) during rose petal shedding. Gene ontology term enrichment and pathway analysis revealed that major biochemical pathways the DTGs were involved in included ethylene biosynthesis, starch degradation, superpathway of cytosolic glycolysis, pyruvate dehydrogenase and TCA cycle, photorespiration and the lactose degradation III pathway. This suggests that alterations in carbon metabolism are an important part of rose petal abscission. Among these DTGs, approximately 150 genes putatively encoding transcription factors were identified in rose abscission zone. These included zinc finger, WRKY, ERF, and Aux/IAA gene families, suggesting that petal abscission involves complex transcriptional reprogramming. Approximately 108 DTGs were related to hormone pathways, of which auxin and ethylene related DTGs were the largest groups including 52 and 41 genes, respectively. These also included 12 DTGs related to gibberellin and 6 DTGs in jasmonic acid pathway. Surprisingly, no DTGs involved in the biosynthesis/signaling of abscisic acid, cytokinin, brassinosteroid, and salicylic acid pathways were detected. Moreover, among DTGs related to auxin, we identified an Aux/IAA gene *RhIAA16* that was up-regulated in response to petal shedding. Down-regulation of *RhIAA16* by virus-induced gene silencing in rose promoted petal abscission, suggesting that *RhIAA16* plays an important role in rose petal abscission.

Keywords: *Rosa chinensis*, petal abscission, transcriptome, auxin signaling, *RhIAA16*

INTRODUCTION

Plant organ abscission is a crucial process that occurs throughout the life span of plants, and regulates the detachment of organs from main body (Roberts et al., 2002). This will benefit plants for recycling nutrients for continuous growth, propagating, facilitating reproduction, and preventing from disease infections (Addicott, 1982; Nakano et al., 2013). In particular, flower, fruit, and seed abscission is closely correlated with plant reproductive success, crop quality, and productivity (Addicott, 1982; Roberts et al., 2002; Estornell et al., 2013; Nakano and Ito, 2013).

Initiation of flower organ abscission is triggered by both internal and external cues (Taylor and Whitelaw, 2001). As internal cues, interaction of auxin and ethylene plays a critical role in abscission initiation (Meir et al., 2010). Depletion of the polar flow of auxin passing through the abscission zone (AZ) makes the AZ sensitive to ethylene, which triggers the separation process (Abeles and Rubinstein, 1964; Addicott, 1982; Taylor and Whitelaw, 2001). Ethylene biosynthesis and signal transduction pathways are involved in the regulation of abscission. In *Arabidopsis*, ethylene-insensitive mutants *etr1-1* and *ein2* inhibited flower organ shedding, suggesting the roles of ETR1 and EIN2 in abscission (Patterson and Bleecker, 2004). In tomato, repression of EIN3-like gene *LeEIL* expression retarded the flower pedicel abscission and fruit ripening processes (Tieman et al., 2001). Tomato *never ripe* (*nr*), *sletr1-1*, and *sletr1-2* mutants affected ethylene receptor function and ethylene sensitivity, thereby delayed fruit ripening and organ abscission (Lanahan et al., 1994; Okabe et al., 2011). On the contrary, auxin as a negative regulator of abscission inhibits the cell separation process (Addicott, 1982; Estornell et al., 2013). The change in auxin flow results in the changes of transcript abundance of many genes involved in auxin biosynthesis, signal transduction, and transport. Functional studies of auxin response factors (ARFs) 1, 2, 7, and 19 demonstrated that these transcriptional regulators have functions in floral organ abscission (Ellis et al., 2005; Okushima et al., 2005). However, the roles of other gene families in the auxin pathway in the regulation of the petal abscission process are still largely unknown.

The perception and transduction of auxin signaling involve the cooperative action of several components. Among them, auxin/indole-3-acetic acid (Aux/IAA) proteins act as transcription repressors by dimerizing with auxin response factors (ARFs; Leyser, 2002; Woodward and Bartel, 2005). In presence of auxin, Aux/IAA proteins binding to the transport inhibitor response one/auxin signaling F-box (TIR1/AFB) cause degradation of Aux/IAA proteins, which then releases ARFs to trigger the expression of auxin responsive genes (Kepinski and Leyser, 2002; Woodward and Bartel, 2005). In *Arabidopsis*, analyses of Aux/IAA gain-of-function mutants revealed functional redundancy among Aux/IAA members (Overvoorde et al., 2005). However, distinctive expression patterns in different organs and tissues among Aux/IAA genes are displayed in several plant species such as rice (Jain et al., 2006), tomato (Audran-Delalande et al., 2012), *Populus* (Kalluri et al., 2007). In addition, functional analyses of

Aux/IAA homologues in different plant species demonstrated the distinct and diverse roles of Aux/IAA proteins in plant development and growth (Audran-Delalande et al., 2012). In tomato, expression of an Aux/IAA family gene *Sl-IAA3* is auxin- and ethylene-dependent. The phenotypes resulting from *Sl-IAA3*-silenced transgenic tomato suggested that *Sl-IAA3* plays a role in interaction of auxin and ethylene in differential growth (Chaabouni et al., 2009a,b). However, the roles of Aux/IAAs in flower petal abscission are not well documented.

Roses are one of the most important cut flowers among ornamental plants. The opening and longevity of rose flower are major factors in determining the ornamental value of rose flower. Moreover, rose flower longevity is largely dependent on the timing of petal shedding occurrence. However, information on the molecular mechanism governing the rose petal abscission is scarce. To date, the regulatory genes in abscission signaling pathway, including *IDA* (Cho et al., 2008), *NEVERSCHED* (Liljegren et al., 2009), *EVERSHED* (Leslie et al., 2010), were identified and characterized in model plants by genetic mapping of mutants. This forward genetic technique is difficult and time-consuming to identify and characterize the genes in non-model plant systems including rose. Next generation sequencing technology has become an effective method to investigate the regulatory network of abscission. Transcriptome studies of the flower abscission process were previously performed in several plant species including tomato (Meir et al., 2010; Nakano et al., 2013; Wang et al., 2013; Ma et al., 2015a; Sundaresan et al., 2016), olive (Gil-Amado and Gomez-Jimenez, 2013), melon (Corbacho et al., 2013), apple (Botton et al., 2011; Zhu et al., 2011), and litchi (Li et al., 2013). Most of these studies have focused on dissecting the regulatory mechanism of pedicel abscission that is the last phase of fruit development and ripening (Giovannoni, 2004). Combined with reverse genetic techniques, roles of several genes obtained from those transcriptome data related to abscission have been confirmed, including *SIERF52* (Nakano et al., 2012, 2014) and *KD1* (Ma et al., 2015a). In *Arabidopsis*, HAE (HAE) and HAE-LIKE2 (HSL2)-dependent pathways were revealed to be involved in petal AZ by the comparison of the transcriptomes of wild-type and *hae hsl2* double mutant (Niederhuth et al., 2013). However, the mechanism regulating petal abscission in non-model plants is still not well understood.

Here we investigated the transcriptome dynamics of the petal AZ during petal shedding in rose by Illumina technology and dissected the transcriptional network governing the abscission process. Furthermore, we identified and characterized an Aux/IAA gene, *RhIAA16*, which we revealed to have an important role in controlling the timing of petal abscission in rose.

MATERIALS AND METHODS

Plant Materials

Rose flowers (*Rosa chinensis* Jacq. cv. Gold Medal) were grown at a greenhouse on the campus of China Agricultural University,

Beijing, China. Rose flower opening was divided into six stages: stage 1, partially opened bud; stage 2, completely opened bud; stages 3 and 4, partially opened flower; stage 5, fully opened flower with visible anthers; stage 6, fully opened flower with abscised petals (**Figure 1**). Rose flowers at different opening stages were harvested. The flower stems were placed immediately in water, and transported to the laboratory within 15 min. The flower stems were re-cut to 20 cm in length under water and placed in deionized water until further processing. The petal was shed at separation layer of abscission zone. Both distal and proximal sides of separation layer belong to abscission zone. Distal side attaches to petal organ, and proximal side attaches to receptacle (**Figure 1**). Therefore, we took sample of petal abscission zone by excising the base of petal (less than 1 mm in length) and the receptacle where petals locate (less than 1 mm in length). Since petals at stage 6 started to abscise, we only focused on the stages prior to petal shedding. Therefore, AZ samples at stages 1, 3, and 5 were collected and used for the transcriptome profiling. Three biological samples were collected for each stage.

Our preliminary tests demonstrated that *R. hybrida* cv. Samantha showed better responses to virus-induced gene silencing (VIGS) and much higher silencing efficiency than *R. chinensis* Jacq. cv. Gold Medal that was used for the transcriptome profiling (data not shown). In addition, the plantlets of *R. hybrida* cv. Samantha bloom as quickly as 40 days after rooting under our growth conditions. Therefore, rose plantlets of *R. hybrida* cv. Samantha were selected for VIGS. Rose plantlets were propagated by tissue culture. Rose shoots with at least 1 node and approximately 2 cm in length were used as explants and cultured on Murashige and Skoog (MS) medium supplemented with 1.0 mg/L 6-Benzyl aminopurine, 3 mg/L Gibberellic Acid, and 0.05 mg/L α -Naphthalene acetic

acid for 30 days, then transferred to 1/2-strength MS medium supplemented with 0.1 mg/L NAA for 30 days for rooting.

Total RNA Extraction and RNA-Seq Library Preparation

Total RNA was extracted using the hot borate method according to previously described (Wan and Wilkins, 1994), and treated with RNase-free DNase I (Promega) to remove any contaminating genomic DNA. Three biological replicates were performed for each developmental stage (stages 1, 3, and 5). Strand-specific RNA libraries were constructed using the protocol described previously (Zhong et al., 2011), and sequenced on a HiSeq 2500 system according to the manufacturer's instructions. The raw reads were deposited into NCBI SRA database under accession no. PRJNA325324.

RNA-Seq Data Processing, De novo Assembly and Annotation

RNA-Seq reads were first processed to remove low quality and adaptor sequences using Trimmomatic (Bolger et al., 2014). Reads shorter than 40 bp were removed. The resulting RNA-Seq reads were then aligned to the ribosomal RNA (rRNA) database (Quast et al., 2013) using Bowtie with default parameters (Li and Durbin, 2010). Reads mapped to the rRNA database were discarded. The high-quality cleaned reads were assembled *de novo* into contigs using the Trinity program (Grabherr et al., 2011). To remove the redundancy of Trinity-assembled contigs, the contigs were further assembled *de novo* using iAssembler (Zheng et al., 2011). The final assembled rose contigs were blasted against the UniProt (Swiss-Prot and TrEMBL; The UniProt Consortium, 2014) and *Arabidopsis* protein (version TAIR10) databases (Lamesch et al., 2012) with a cutoff e-value of $1e^{-5}$. Based on



FIGURE 1 | Flower opening stages in rose (**Left**), and petal abscission zone region used for RNA-Seq (**Right**). (**Left**) flower opening of rose was divided into six stages: stage 1, partially opened bud; stage 2, completely opened bud; stages 3 and 4, partially opened flower; stage 5, fully opened flower with visible anthers; stage 6, fully opened flower with abscised petals. (**Right**) abscission zone (AZ) sample used for RNA-Seq was the AZ at the base of petal (less than 1 mm in length) and AZ at the receptacle where petals locate (less than 1 mm in length).

the UniProt and *Arabidopsis* protein blast results, functional descriptions (human readable descriptions) were assigned to each unigene using AHRD¹. Gene ontology (GO) terms were assigned to the rose assembled transcripts based on the GO terms annotated to their corresponding homologues in the UniProt database. Biochemical pathways were predicted from the rose transcripts using the Pathway Tools (Karp et al., 2002).

To identify differentially expressed genes, high-quality cleaned reads were aligned to rose contigs using Bowtie allowing up to two mismatches. Only the best alignments for each read were retained. Following alignments, raw read count for each rose contig in each sample was derived and normalized to reads per kilo base exon model per million mapped reads (RPKM). The significance of differential gene expression between different samples was determined using DESeq (Anders and Huber, 2010), and raw *p*-values of multiple tests were corrected using false discovery rate (Benjamini and Hochberg, 1995).

Quantitative RT-PCR

To analyze the transcript abundance of selected genes for confirmation of RNA-Seq data, quantitative RT-PCR (qRT-PCR) reactions were performed, as previously described (Ma et al., 2015b). Briefly, total RNAs were isolated from AZ samples with three biological repeats. 1 μ l of the first strand cDNA was used as template with the Step One PlusTM real-time PCR system (Applied Biosystems) using KAPA™ SYBR® FAST quantitative PCR kits (Kapa Biosystems). *RhActin5* was used as a reference gene (Pei et al., 2013). The primers used for determining transcript abundance were listed in **Supplementary Table S1**.

Virus-Induced Gene Silencing

A 290 bp fragment in the 3'end of *RhIAA16* gene was amplified by PCR from rose cDNAs to specifically silence *RhIAA16*. The fragment was then inserted into pTRV2 vector to generate the pTRV2-*RhIAA16* construct. The primers used for amplifying *RhIAA16* are listed in **Supplementary Table S1**.

Constructs of pTRV1, pTRV2, and pTRV2-*RhIAA16* were transformed into *Agrobacterium tumefaciens* GV3101. *A. tumefaciens* were cultured in Luria-Bertani medium supplemented with 10 mM MES, 20 μ M acetosyringone, 50 μ g/ml gentamicin sulfate, and 50 μ g/ml kanamycin. The cultured bacterium was collected by centrifuge at 4,000 rpm for 10 min, and re-suspended in infiltration buffer (10 mM MgCl₂, 200 μ M acetosyringone, 10 mM MES, pH 5.6) to OD₆₀₀ of ~1.5 (Yin et al., 2015). Mixtures of cultures containing an equal ratio (v/v) of pTRV1 and pTRV2 or pTRV2-*RhIAA16* were used for inoculation. VIGS of rose plantlets was performed as previously described (Tian et al., 2014). Rose plantlets, as shown in **Supplementary Figure S1**, were immersed in bacterial suspension solution and infiltrated under a vacuum at 0.7 MPa for 2 min. After infiltration, plantlets were washed in deionized water, and transplanted into pots

containing a mixture of 1:1 (v/v) of peat and vermiculite. The plantlets were immediately placed in dark at 8°C for 3 days in a low temperature incubator (MIR-253, SANYO), and then grown in a culture room at 22 ± 1°C, 40% relative humidity. Three independent experiments were performed. 30 plantlets were used for each experiment. Prior to the petal abscission test, we PCR-screened the plants and determined the transcript abundance of *RhIAA16* in petals among the 30 plantlets. We found that the transcript levels of *RhIAA16* in more than 30% plantlets were reduced, compared to uninoculated and empty vector controls. These plants with down-regulated *RhIAA16* expression were used for petal abscission assay.

RESULTS

Sequencing and *De novo* Assembly of Petal Abscission Zone Transcriptome in Rose

To perform transcriptomic analysis, petal AZs of rose flowers (**Figure 1**) with three biological replicates at stage 1, 3, and 5 were collected to construct a total of nine RNA-Seq libraries. A total of 75,752,884 paired-end raw reads with length of 100 nucleotides (nt) were obtained. After further filtering and cleaning, a total of 57,312,389 clean read pairs were obtained. *De novo* assembly of these high-quality cleaned reads generated 80,226 unique transcripts with an average length of 743 bp (**Table 1**). The size distribution indicated that the lengths of the 19,414 transcripts were more than 1000 bp (**Supplementary Figure S2**). Correlation coefficients of transcriptome profiles among the nine libraries and between the biological replicates were calculated (**Supplementary Table S2**). High correlation coefficients were obtained, suggesting the robustness of our RNA-Seq dataset.

To further validate the expression profiles of RNA-Seq data, four selected transcripts were analyzed by qRT-PCR. The results from qRT-PCR analysis were generally in agreement with the expression profiles obtained by RNA-Seq data (**Figure 2**).

Dynamic Transcriptome Profiles during Petal Abscission in Rose

Differentially transcribed genes (DTGs) were determined using a cutoff ratio of >2 or <0.5 when comparing their transcript

TABLE 1 | Summary of rose petal abscission zone transcriptome sequencing dataset.

Items	Total
No. of reads	75,752,884
No. of cleaned reads	57,312,389
No. of mapped reads	44,769,490
No. of assembled transcripts	80,226
Average length of transcripts	743.1 bp
Total length of transcripts	59,617,563 bp

¹<https://github.com/asishallab/AHRD-1>

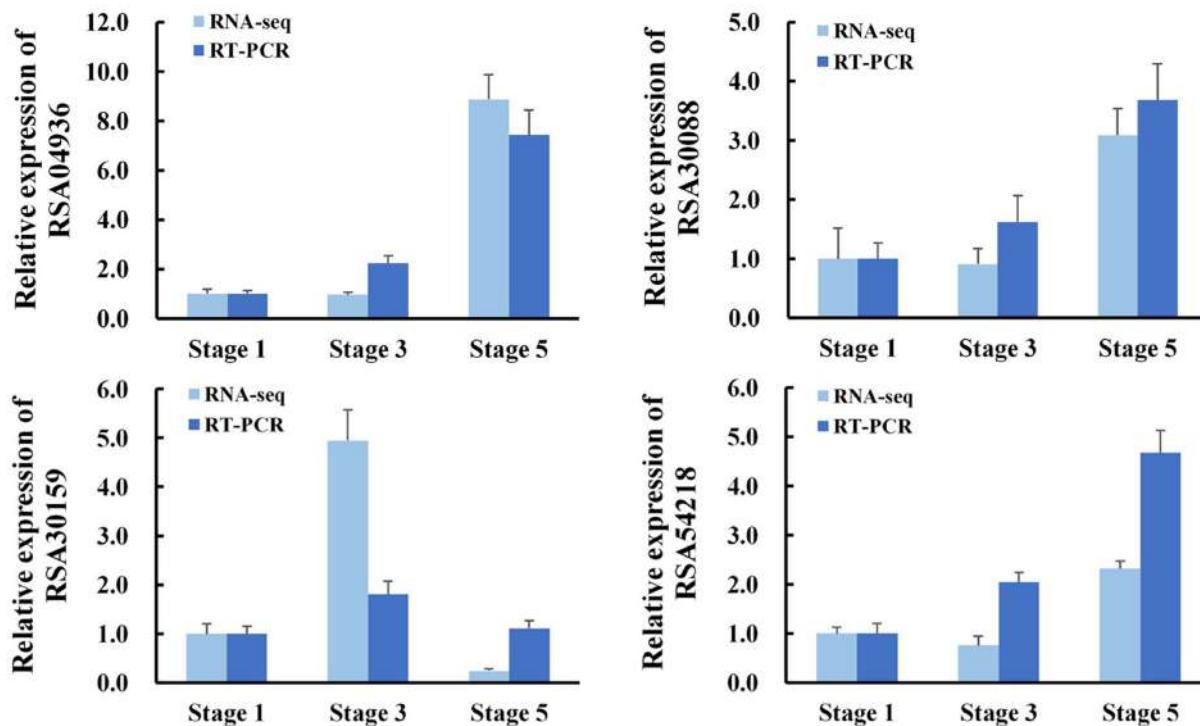


FIGURE 2 | Validation of RNA-Seq results by quantitative RT-PCR (qRT-PCR). RNA was extracted from petal AZ at the indicated flower opening stage. *RhActin5* was used as an internal control. The results were the means of three biological replicates \pm SD. RSA04936, GDSL esterase/lipase; RSA30088, Receptor-like protein kinase; RSA30159, Lachrymatory-factor synthase; RSA54218, Zinc transporter.

abundance in stage 3 to that in stage 1 (S3 vs. S1), and/or in stage 5 to that in stage 3 (S5 vs. S3). A total of 2592 DTGs were obtained (**Supplementary Table S3**). Based on the change in ratio of DTG transcript abundance, the number of DTGs at stage 3/1 and stage 5/3 was counted (**Figure 3**). Compared with stage 1, 782 DTGs were up-regulated and 300 DTGs were down-regulated in stage 3. Compared with stage 3, 1179 transcripts were increased and 1408 transcripts were decreased in stage 5 (**Figure 3**), suggesting that major transcriptional dynamic for petal abscission occurs just prior to petal shedding (stage 5).

To evaluate the potential functions of genes that showed transcriptional changes during petal abscission, we identified the GO terms of the DTGs in the biological process category (**Figure 4**). At stages 3 and 5, the biological processes were enriched in the metabolic process and defense responses including response to abiotic stimulus, external stimulus, organic substance (**Figures 4A,B**).

To identify the biochemical pathways involved in petal abscission, we analyzed DTGs using the Pathway Tools (Karp et al., 2002). The 108 DTGs at stage 3 and 261 DTGs at stage 5 were classified into 42 and 92 biochemical pathways, respectively (**Supplementary Table S4**). The major pathways both at stage 3 and 5 included ethylene biosynthesis, starch degradation, superpathway of cytosolic glycolysis, pyruvate dehydrogenase and TCA cycle, and photorespiration (**Supplementary Table S4**). In addition, one of the major pathways at stage 5 is related to

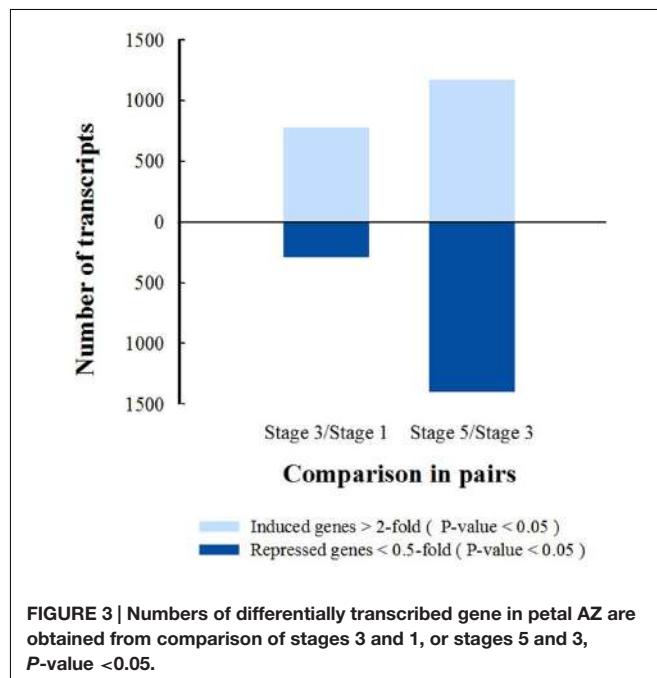


FIGURE 3 | Numbers of differentially transcribed gene in petal AZ are obtained from comparison of stages 3 and 1, or stages 5 and 3, *P*-value < 0.05.

the lactose degradation III pathway (**Supplementary Table S4**). These results suggested that alterations in carbon metabolism play an important part in rose petal abscission.

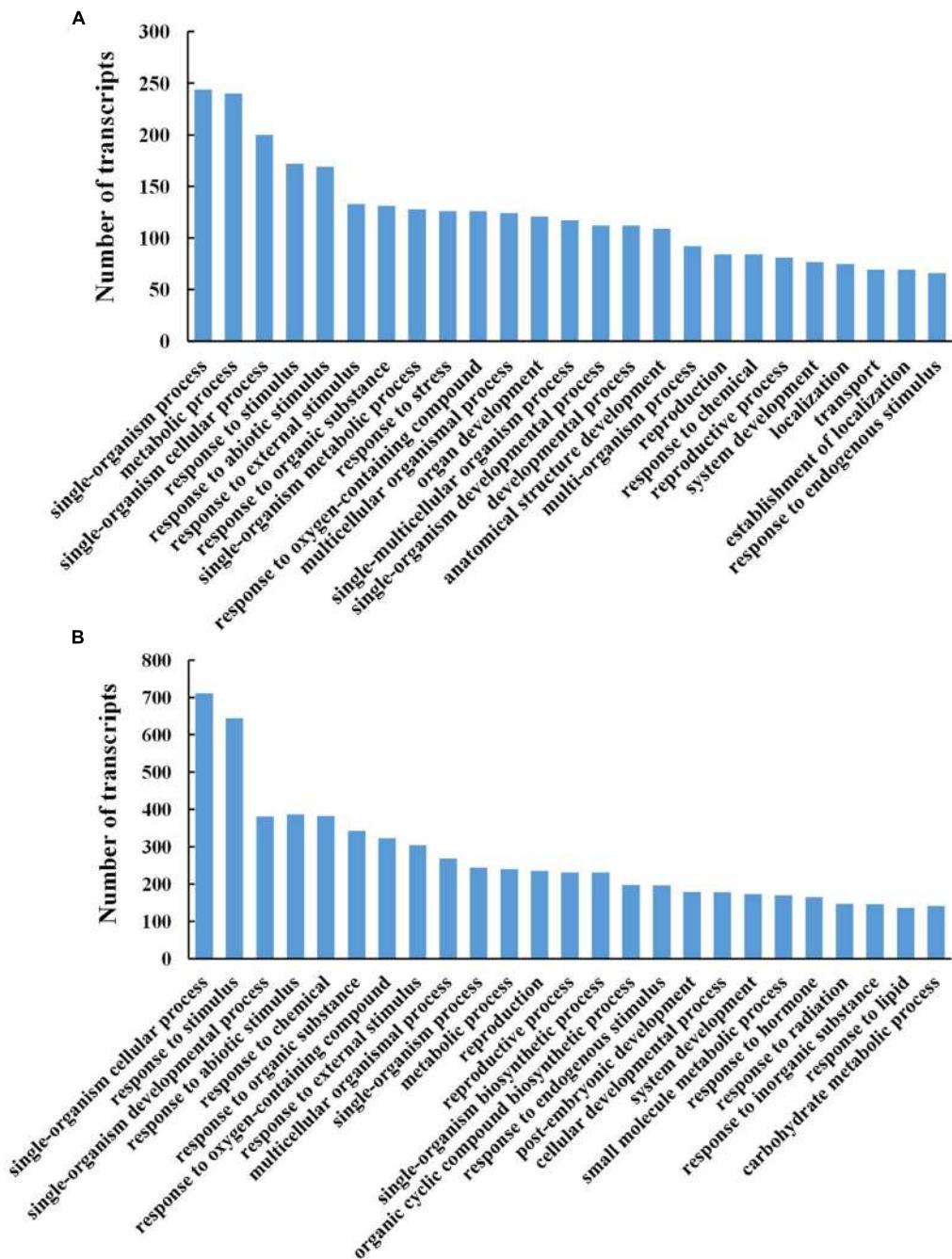
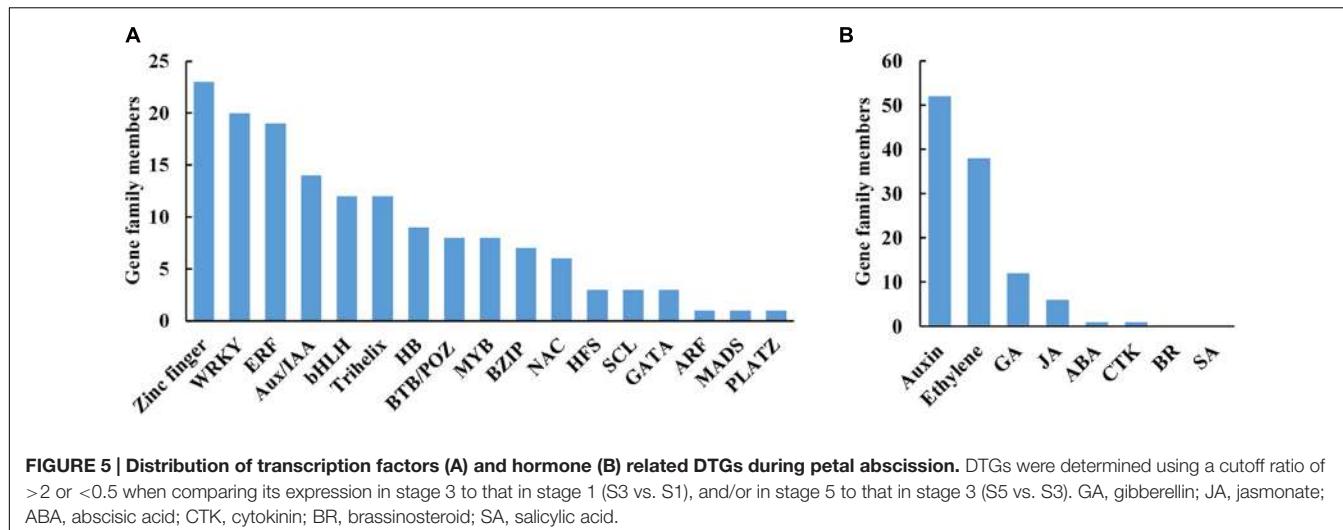


FIGURE 4 | Gene ontology functional classification analysis of differentially transcribed genes (DTGs) during petal abscission. Histograms representing functional distributions (GO biological process class) of DTGs obtained from stage 3 compared to stage 1 (**A**), and from stage 5 compared to stage 3 (**B**). DTGs were determined using a cutoff ratio of >2 or <0.5 when comparing its expression in stage 3 to that in stage 1 (S3 vs. S1), and/or in stage 5 to that in stage 3 (S5 vs. S3).

Abscission-Responsive Transcriptional Regulators in Rose Petal Abscission Zone

Of 2592 DTGs, 150 encoded putative transcription factors (TFs; **Figure 5A**). More specifically, 15.3% (23/150) belonged to zinc finger family, 13.3% (20/150) to WRKYs, 12.7% (19/150)

to ERFs (ethylene responsive factors), and 9.3% (14/150) to Aux/IAAs (**Figure 5A**). As the largest group of abscission-responsive TFs, the transcript abundance of most of the zinc finger family members (18/23) was increased in stage 3 or stage 5 (**Supplementary Table S5**). Furthermore, the transcript abundance of all the WRKY family members was induced during



rose flower opening (**Supplementary Table S5**). The results suggested a complex transcriptional reprogramming of petal abscission.

Abscission-Induced Hormone Pathway Changes in Rose Petal Abscission Zone

Hormones act as internal cues to initiate abscission process (Addicott, 1982; Estornell et al., 2013). We identified 108 DTGs related to hormone pathways. Among them, DTGs related to auxin and ethylene pathways including 52 DTGs and 38 DTGs, respectively, were the largest group (**Figure 5B; Table 2; Supplementary Table S6**). In addition, 12 DTGs related to gibberellin were obtained, and 6 DTGs in jasmonic acid pathway (**Figure 5B; Supplementary Table S6**). No DTGs involved in the biosynthesis/signaling of abscisic acid, cytokinin, brassinosteroid, salicylic acid pathways were detected (**Figure 5B**).

Among DTGs related to auxin pathway, six auxin transporter genes were identified including five auxin efflux carrier genes (**Table 2**). In addition, 14 Aux/IAA family members were obtained, of which six members were up-regulated in stage 5, and eight members were down-regulated in stage 5 compared to stage 3 (**Table 2**). Among DTGs related to ethylene pathway, 15 DTGs encoded ethylene biosynthesis related 1-Aminocyclopropane-1-carboxylic acid oxidase (ACO). Transcript abundance of 10 ACO genes was accumulated in stage 5 compared to stage 3. Furthermore, 19 DTGs encoded ERFs were detected (**Supplementary Table S6**). The transcript abundance of 16 ERFs was increased in stage 5 compared to stage 3 (**Supplementary Table S6**). Overall, our results suggested that auxin and ethylene may play central roles in petal abscission of rose.

RhIAA16 Transcript Abundance Is Induced during Petal Abscission

To identify key regulators governing petal abscission, we initiated functional analysis of DTGs using VIGS. We primarily focused on the up-regulated DTGs, hypothesizing that VIGS-down regulation of these genes might lead to changes in the petal

abscission processes. Given the potential important roles that auxin plays in the regulation of abscission, we first examined the functions of several Aux/IAA genes (RSA33069, RSA04500, RSA45030) that were up-regulated in the rose abscission zone using rose cut flowers. We found that VIGS-silencing of the contig of RSA33069 exhibited accelerated petal abscission phenotype. Analysis of the RSA33069 cDNA sequence revealed that it encoded a deduced protein of 253 amino acids with a 762 bp predicted open reading frame (**Figure 6A**). The predicted amino acid sequence of RSA33069 showed that it belongs to the Aux/IAA family, and has the four canonical conserved domains known for this family (**Figure 6A**) (Overvoorde et al., 2005). In addition, phylogenetic tree analysis suggested that the protein has high degree of sequence homology to FvIAA16 from *Fragaria vesca*, therefore was designated as RhIAA16 (**Figure 6B**). RT-PCR analysis demonstrated that the transcript abundance of RhIAA16 in petal AZ was significantly induced during flower development to peak at stage 5 (**Figure 7A**). These results were consistent with the transcript abundance changes of RhIAA16 in the RNA-Seq data.

Reduction of RhIAA16 Expression Promotes Petal Abscission in Rose

To further confirm the potential role of RhIAA16 in petal abscission, we chose a fragment from RhIAA16-specific 3' untranslated region (UTR) to silence RhIAA16 in rose plantlets. qRT-PCR results showed that transcript abundance of RhIAA16 in RhIAA16-silenced (TRV2-RhIAA16) petal was significantly reduced compared to TRV2 control (**Figure 7B**). Petal abscission was detected at 5 days after full opening in the RhIAA16-silenced plantlets in contrast to 9 days after full opening in TRV2 control plantlets (**Figures 7C,D**), suggesting that silencing of RhIAA16 accelerated the timing of initial petal abscission. At 9 days and 11 days after full opening, petals in ~40.5 and 69.0% of flowers in RhIAA16-silenced plantlets had abscised whereas petals in only 17.8 and 37.9% of TRV2 control flowers were abscised, respectively (**Figure 7C**).

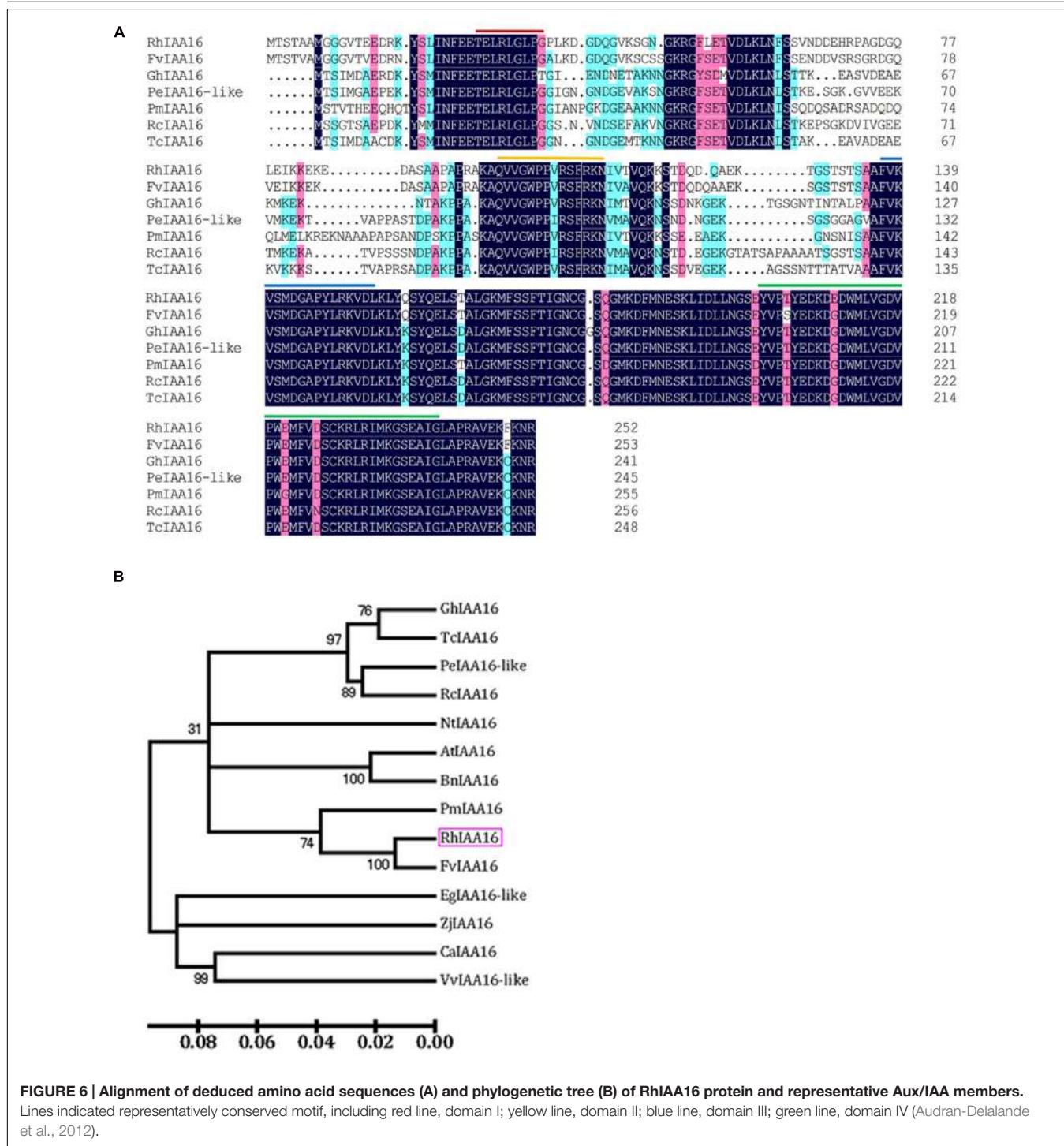
TABLE 2 | Expression changes of transcripts related auxin pathway in response to abscission.

GeneID	Annotation	Transcript abundance (RPKM)			Ratio Stage 3/1	Ratio Stage 5/3
		Stage 1	Stage 3	Stage 5		
Auxin transporter						
RSA03469	Auxin efflux carrier family protein	17.66	32.95	76.18	1.87	2.31
RSA57045	Auxin transporter-like protein	11.79	15.09	33.64	1.28	2.23
RSA40292	Auxin efflux carrier family protein	23.29	32.14	69.9	1.38	2.17
RSA48611	Auxin efflux carrier	106.54	56.24	26.35	0.53	0.47
RSA02921	Auxin efflux carrier family protein	30.53	13.13	2.53	0.43	0.19
RSA22467	Auxin efflux carrier family protein	25.07	10.37	0.27	0.41	0.03
Aux/IAA family						
RSA45030	Aux/IAA27-like	42.87	20.59	81.81	0.48	3.97
RSA47413	Aux/IAA26-like	8.76	5.37	19.73	0.61	3.67
RSA38191	Aux/IAA27-like	58.7	28.52	101.47	0.49	3.56
RSA04500	Aux/IAA27	33.66	33.19	88.58	0.99	2.67
RSA33069	Aux/IAA16	73.94	80.94	165.03	1.09	2.04
RSA52969	Aux/IAA8-like	55.95	62.56	27.42	1.12	0.44
RSA45028	Aux/IAA28-like	79.15	394.48	169.38	4.98	0.43
RSA45029	Aux/IAA28-like	85.33	416.25	177.08	4.88	0.43
RSA45026	Aux/IAA28-like	78.92	394.68	168.36	5	0.43
RSA45027	Aux/IAA28-like	83.91	411.14	173.63	4.9	0.42
RSA05184	Aux/IAA1-like	1.79	5.96	0.67	3.32	0.11
RSA37816	Aux/IAA13 isoform X1	33.29	13.14	6.55	0.39	0.5
RSA37817	Aux/IAA13 isoform X1	31.83	13.3	6.68	0.42	0.5
RSA63979	Aux/IAA13 isoform X1	9.72	7.96	16.83	0.82	2.11
Others						
RSA29268	Auxin-regulated protein	6.83	4.76	19.07	0.7	4
RSA55555	Auxin-induced in root cultures protein 12-like	18.79	22.87	89.63	1.22	3.92
RSA40153	Auxin-induced protein 5NG4, putative	4.31	3	11.37	0.7	3.79
RSA55554	Auxin-induced in root cultures protein 12-like	13.27	14.59	53.88	1.1	3.69
RSA47053	Auxin response factor three family protein	30.04	14.91	35.9	0.5	2.41
RSA46802	Auxin-responsive protein SAUR36	2.36	11.11	26.6	4.7	2.4
RSA46801	Auxin-responsive protein SAUR36	2.16	7.63	18.02	3.53	2.36
RSA01454	Auxin-induced protein 5NG4, putative	25.62	11.88	3.61	0.46	0.3
RSA01453	Auxin-induced protein 5NG4, putative	28.64	14.67	3.93	0.51	0.27
RSA64224	Auxin-induced protein 15A-like	4.11	9.56	2.56	2.32	0.27
RSA64234	Auxin-induced protein-like protein	9.62	13.79	3.57	1.43	0.26
RSA06545	Auxin-induced protein-like protein	4.69	6.24	1.5	1.33	0.24
RSA64231	Auxin-induced protein 15A-like	8.15	12.1	2.9	1.48	0.24
RSA37530	Auxin-induced protein 15A-like	10.56	7.87	1.72	0.74	0.22
RSA64235	Auxin-induced protein 15A-like	7.93	8.89	1.87	1.12	0.21
RSA64222	Auxin-induced protein 15A-like	5.06	9.98	1.78	1.97	0.18
RSA58610	Auxin-induced protein 22D-like	1.13	37.04	6.43	32.69	0.17
RSA06546	Auxin-induced protein 15A-like	4.57	8.65	1.36	1.89	0.16
RSA64232	Auxin-induced protein 15A-like	5.75	9.72	1.42	1.69	0.15
RSA64228	Auxin-induced protein 15A-like	5.98	10.76	1.66	1.8	0.15
RSA10174	Auxin-responsive protein	2.33	7.69	1.17	3.3	0.15
RSA64229	Auxin-induced protein 15A-like	4.31	6.99	0.92	1.62	0.13
RSA64230	Auxin-induced protein 15A-like	5.42	8.03	0.87	1.48	0.11
RSA64233	Auxin-induced protein 15A-like	6.44	10.1	1.15	1.57	0.11
RSA64225	Auxin-induced protein 15A-like	6.7	9.89	1.11	1.48	0.11
RSA54814	Auxin-induced protein 15A-like	3.75	6.04	0.69	1.61	0.11
RSA54813	Auxin-induced protein 15A-like	3.5	5.26	0.54	1.51	0.1
RSA64226	Auxin-induced protein 15A-like	5.57	8.26	0.86	1.48	0.1

(Continued)

TABLE 2 | Continued

GeneID	Annotation	Transcript abundance (RPKM)			Ratio Stage 3/1	Ratio Stage 5/3
		Stage 1	Stage 3	Stage 5		
RSA54803	Auxin-induced protein 15A-like	2.74	6.44	0.55	2.35	0.09
RSA54811	Auxin-induced protein ARG7-like	1.87	5.13	0.46	2.74	0.09
RSA39307	Early auxin response protein	2.17	12.55	1.19	5.79	0.09
RSA31518	Auxin-responsive protein SAUR71-like	1.09	183.12	0.85	168.52	0

**FIGURE 6 | Alignment of deduced amino acid sequences (A) and phylogenetic tree (B) of RhIAA16 protein and representative Aux/IAA members.**

Lines indicated representatively conserved motif, including red line, domain I; yellow line, domain II; blue line, domain III; green line, domain IV (Audran-Delalande et al., 2012).

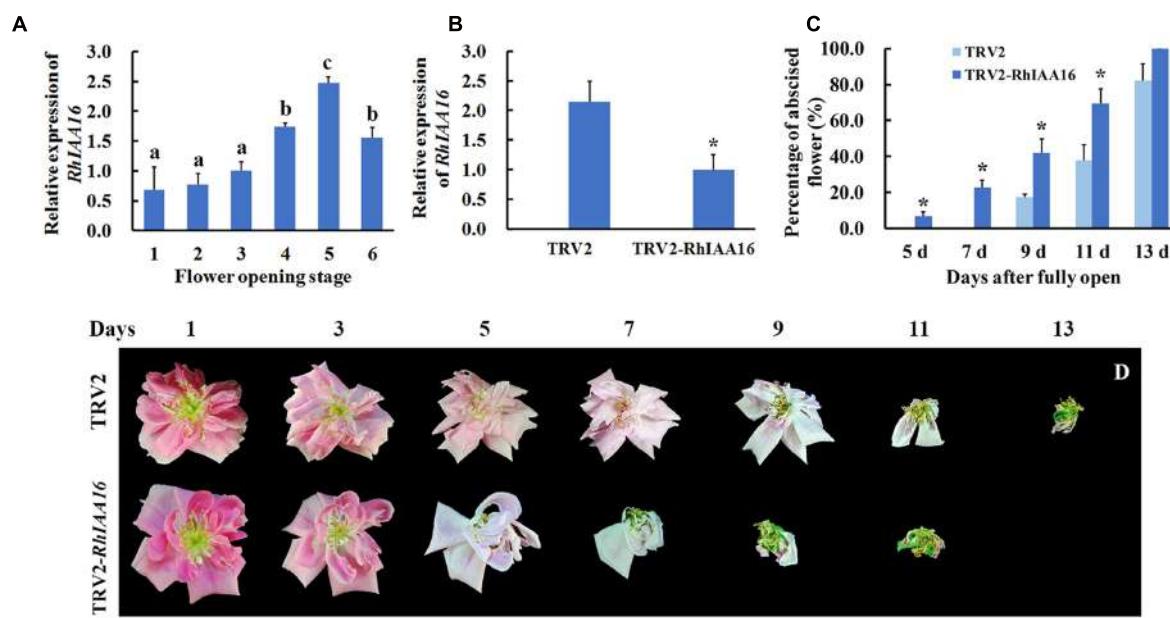


FIGURE 7 | Silencing of *RhIAA16* promotes petal abscission. **(A)** Expression of *RhIAA16* during flower opening was analyzed by qRT-PCR. **(B)** Expression of *RhIAA16* was analyzed by qRT-PCR in *RhIAA16*-silenced (TRV2-RhIAA16) and control (TRV2) plants. **(C)** The percentage of abscised flowers were determined at intervals after fully open in *RhIAA16*-silenced (TRV2-RhIAA16) and control (TRV2) plants. Abscised flower was defined as the flower with all the petals shed. **(D)** The phenotypes of flowers were recorded and photographed every 2 days. The results were the means of three biological replicates with standard deviation. Letters indicated significant differences according to Duncan's multiple range test ($P < 0.05$), and asterisks indicated statistically significant differences (Student's *t*-test, $P < 0.05$).

DISCUSSION

In this study, high-throughput sequencing and *de novo* assembly strategies permitted us to dissect the transcriptome of rose petal AZ during petal shedding. Our results demonstrated that among DTGs related to hormones during petal abscission, most of them were associated with auxin and ethylene pathways, suggesting that auxin and ethylene play important roles in petal abscission in rose flowers (Figure 5B). This conclusion is in good agreement with tomato abscission studies (Meir et al., 2010). Furthermore, functional characterization of *RhIAA16* partially supports this notion (Figure 7).

Auxin and ethylene as key hormones in the initiation of abscission have been demonstrated not only by physiological experiments, but also by transcriptome studies from different organ AZ. In tomato pedicel AZ, auxin depletion by auxin transport inhibitor, or flower removal stimulated pedicel abscission while ethylene action inhibitor treatment prevented the abscission induced by auxin depletion (Meir et al., 2010). In addition, the transcriptome of pedicel AZ demonstrated that acquisition of ethylene sensitivity in the AZ is associated with altered expression of auxin-regulated genes (Meir et al., 2010). Auxin homeostasis and signaling are usually modulated by the Aux/IAA genes (Song et al., 2009). Canonical Aux/IAA proteins function as transcriptional repressors of auxin-regulated genes (Tiwari et al., 2001, 2004). In our study, the DTGs included 11 Aux/IAA family members (Table 2). Among them, the transcript abundance of six

members of Aux/IAA family was up-regulated during petal abscission (Table 2). Intriguingly, VIGS-silencing *RhIAA16*, one of the up-regulated Aux/IAA genes, accelerated the petal abscission process (Figure 7), suggesting that *RhIAA16* might be required for preventing premature abscission. In soybean, *IAA16* has been reported as abscission-specific transcription factor by a transcriptome analysis of soybean leaf abscission, although the expression of *IAA16* was down-regulated during soybean leaf abscission (Kim et al., 2016). In *Arabidopsis*, genetic evidences suggest that ARFs, which interact with Aux/IAA proteins, play regulatory roles in the petal abscission process. ARF1, ARF2, ARF7, and ARF19 were identified as regulators of abscission (Ellis et al., 2005). Given that ARF proteins interact with Aux/IAA in the auxin signal pathway (Leyser, 2002), further characterization of interactions between *RhIAA16* and ARFs in rose may shed light on petal abscission activation.

It is worth pointing out that the transcript levels of *RhIAA16* were not significantly changed in response to ethylene and ethylene action inhibitor 1-Methylcyclopropene (1-MCP) treatments (data not shown), suggesting that *RhIAA16* is involved in either an ethylene independent pathway or up-stream of the ethylene pathway during abscission initiation.

In tomato, microarray assay showed that the expression of genes related to different steps of ethylene biosynthetic pathway, including *S-adenosylmethionine (SAM) synthase*, *ACC synthase*, and *ACO* genes, were altered during the pedicel abscission process (Meir et al., 2010). However, our transcriptome study

revealed that multiple *ACO* genes in the final step of ethylene biosynthesis (Yang and Hoffman, 1984) were increased in AZ of stage 5 flower (**Supplementary Table S6**), indicating the critical role of *ACO* in controlling the rose petal abscission. Furthermore, the transcript abundance of many *ERFs* was induced in stage 5 compared with stage 3 (**Supplementary Table S6**). The importance of *ERFs* in flower abscission was recently demonstrated in tomato (Nakano et al., 2014). These researchers found that silencing of *SIERF52*, which is specifically expressed in the pedicel AZ, delayed tomato flower pedicel abscission.

In *Arabidopsis*, the transcriptomic analysis of petal abscission indicated that ethylene and ABA pathways were enriched during petal abscission (Niederhuth et al., 2013). In addition, JA signaling and biosynthesis genes were also involved in petal abscission (Niederhuth et al., 2013). Functional analysis showed that mutants of JA biosynthesis gene *allene oxide synthase* (*AOS*) retarded the petal abscission (Kim et al., 2013). This delayed abscission phenotype can be enhanced by ethylene insensitive mutant *ein2* and ABA deficient mutant *aba2*, suggesting that ethylene, ABA and JA might synergistically regulate the petal abscission in *Arabidopsis* (Ogawa et al., 2009; Kim et al., 2013). In our study, the transcriptomic analysis suggested that among these three hormones, ethylene might play a major role, and JA might also be recruited in rose petal abscission, but not ABA (**Figure 5**). In GO analysis of our transcriptome showed that defense responses such as response to abiotic stimulus and external stimulus were enriched during petal abscission process (**Figure 4**), suggesting that the genes responsive to stress are involved in the activation of abscission. Indeed, abscission is considered to be a physiological process related to hormone-mediated stress responses (Addicott, 1982; Estornell et al., 2013). In *Arabidopsis*, GO enrichment analysis of petal AZ transcriptome also demonstrated that biological processes that significantly enriched include defense response to abiotic and biotic stresses (Niederhuth et al., 2013). Similarly, in soybean, the biological processes of leaf AZ transcriptome significantly enriched include responses to endogenous and external stimuli (Kim et al., 2016).

Zinc finger transcription factors are a large and diverse family involved in many aspects of plant growth and development and play critical roles in cellular functions such as transcriptional regulation, RNA binding, and protein-protein interactions (Ciftci-Yilmaz and Mittler, 2008). Our results showed that zinc finger genes are the largest group among differentially transcribed transcription factors in rose petal AZ (**Figure 5A**). In *Arabidopsis*, *ZINC FINGER PROTEIN2* (*ZFP2*) encoding a ZFP, has been revealed in stamen AZ transcriptome profiling, which was increased during floral organ abscission process. Overexpression of *ZFP2* exhibited delayed floral organ abscission phenotype (Cai and Lashbrook, 2008). Our data showed that 18 members of zinc finger gene were elevated in stage 5 (**Supplementary Table S5**), indicated the important roles of zinc finger gene in rose petal

abscission. Functional analysis of these regulatory genes would be an important step toward elucidating their roles in petal abscission in the future.

CONCLUSION

Our RNA-Seq analysis has permitted us to dissect the rose AZ transcriptome during petal abscission, and reveal that auxin and ethylene are important hormones in the regulation of the abscission process. Furthermore, our data demonstrated that an *Aux/IAA* gene, *RhIAA16*, played an important role in rose petal abscission.

AUTHOR CONTRIBUTIONS

CM and C-ZJ conceived and supervised the study. YG, CL, XL, HX, and YL performed the experiments. YG and CL analyzed the data and drafted the manuscript. CM, NM, ZF, C-ZJ, and JG provided technical support, conceptual advice, analyzed the data, and participated in writing the manuscript. C-ZJ extensively revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.01375>

FIGURE S1 | Rose seedling for virus-induced gene silencing.

FIGURE S2 | Length distribution of rose petal abscission zone unique transcripts.

TABLE S1 | The primer list.

TABLE S2 | Correlation coefficients of transcriptome profiles among RNA-Seq samples.

TABLE S3 | Differentially transcribed genes.

TABLE S4 | Pathways of differentially transcribed genes.

TABLE S5 | Differentially transcribed transcription factor.

TABLE S6 | Differentially transcribed genes related to hormones.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Transcriptome Analysis of Soybean Leaf Abscission Identifies Transcriptional Regulators of Organ Polarity and Cell Fate

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Abscission, organ separation, is a developmental process that is modulated by endogenous and environmental factors. To better understand the molecular events underlying the progression of abscission in soybean, an agriculturally important legume, we performed RNA sequencing (RNA-seq) of RNA isolated from the leaf abscission zones (LAZ) and petioles (Non-AZ, NAZ) after treating stem/petiole explants with ethylene for 0, 12, 24, 48, and 72 h. As expected, expression of several families of cell wall modifying enzymes and many pathogenesis-related (PR) genes specifically increased in the LAZ as abscission progressed. Here, we focus on the 5,206 soybean genes we identified as encoding transcription factors (TFs). Of the 5,206 TFs, 1,088 were differentially up- or down-regulated more than eight-fold in the LAZ over time, and, within this group, 188 of the TFs were differentially regulated more than eight-fold in the LAZ relative to the NAZ. These 188 abscission-specific TFs include several TFs containing domains for homeobox, MYB, Zinc finger, bHLH, AP2, NAC, WRKY, YABBY, and auxin-related motifs. To discover the connectivity among the TFs and highlight developmental processes that support organ separation, the 188 abscission-specific TFs were then clustered based on a >four-fold up- or down-regulation in two consecutive time points (i.e., 0 and 12 h, 12 and 24 h, 24 and 48 h, or 48 and 72 h). By requiring a sustained change in expression over two consecutive time intervals and not just one or several time intervals, we could better tie changes in TFs to a particular process or phase of abscission. The greatest number of TFs clustered into the 0 and 12 h group. Transcriptional network analysis for these abscission-specific TFs indicated that most of these TFs are known as key determinants in the maintenance of organ polarity, lateral organ growth, and cell fate. The abscission-specific expression of these TFs prior to the onset of abscission and their functional properties as defined by studies in *Arabidopsis* indicate that these TFs are involved in defining the separation cells and initiation of separation within the AZ by balancing organ polarity, roles of plant hormones, and cell differentiation.

Keywords: transcription factors, network, abscission, soybean, *Glycine max*, organ polarity, cell fate

INTRODUCTION

Cell separation processes take place throughout the life cycle of a plant including root emergence during seed germination, dehiscence of anthers and seedpods, and shedding of organs (abscission; Roberts et al., 2002). Abscission is an active biological process critical to the survival and reproduction of plants (Bleecker and Patterson, 1997; Patterson, 2001; Taylor and Whitelaw, 2001; Lewis et al., 2006; Meir et al., 2010; Basu et al., 2013). Plant hormones and abiotic/biotic stresses activate the process when the organ (e.g., leaf, flower, fruit) has completed its purpose on the main body of a plant or as part of a defense mechanism. Regulated by various endogenous and exogenous signals, abscission occurs within a specialized tissue called the abscission zone (AZ) consisting of small, less vacuolated cells (Addicott, 1982). Recent transcriptome studies of abscission in a diverse set of plants have provided many insights into the regulation and cellular mechanisms used for organ separation (Cai and Lashbrook, 2008; Meir et al., 2010; Nakano et al., 2013; Wang et al., 2013; Zhang et al., 2015). These studies revealed that there are functional categories of genes such as cell wall hydrolytic enzymes (e.g., polygalacturonases and cellulases) that are commonly up-regulated in abscission of different organs in multiple species. However, although some functional categories of genes may be common to many forms of abscission, the signals that initiate their expression may vary. For instance, more than 100 years ago the plant hormone ethylene was discovered to play an important role in abscission; however, although ethylene has been demonstrated to be essential for abscission in tomato (Lanahan et al., 1994; Meir et al., 2010) and soybean (Tucker and Yang, 2012), ethylene is not essential for floral organ abscission in *Arabidopsis* (Patterson, 2001). Moreover, genetic and biochemical studies of *Arabidopsis* floral organ abscission identified additional key signaling components in the regulation of organ separation that are independent of ethylene (Butenko et al., 2003; Patterson and Bleecker, 2004; Liljegren et al., 2009; Leslie et al., 2010; Lewis et al., 2010; Burr et al., 2011; Kim et al., 2013; Gubert et al., 2014; Tucker and Kim, 2015).

Based on many years of abscission research, a working model for abscission has been established. The model differentiates abscission into four developmental phases: Phase (1) establishment of the AZ; Phase (2) acquisition of competence to respond to abscission signals; Phase (3) activation of abscission/cell separation; and Phase (4) trans-differentiation between the separating sides of the AZ cells and formation of a protective scar (Bleecker and Patterson, 1997; Patterson, 2001; Lewis et al., 2006; Liljegren, 2012; Kim, 2014; Tucker and Kim, 2015). Although we have learned much from genetic, gene expression and proteomic studies of abscission, the regulation of gene expression in the AZ by transcription factors (TFs) and their regulatory networks are only beginning to be deciphered (Nath et al., 2007; Nakano et al., 2013; Niederhuth et al., 2013; Wang et al., 2013; Ito and Nakano, 2015). In the current study, we conducted RNA-seq using RNA isolated from soybean leaf AZ (LAZ) and non-abscission zone (NAZ) petiole tissues exposed to ethylene for 0, 12, 24, 48, and 72 h. After removal of the leaf blade, which is a source of auxin that inhibits abscission, exposure

to ethylene induces and synchronizes abscission in the LAZ culminating in nearly 100% organ separation by 72 h. Moreover, by exposing both LAZ and petioles to a high concentration of ethylene this reduces differential gene expression between the LAZ and NAZ that might otherwise occur due to unequal synthesis of ethylene in the AZ compared to the petiole, which would cause unequal ethylene-induction of senescence and ethylene-associated defense responses (Tucker and Yang, 2012). We focus herein on the regulatory networks that underlie soybean leaf abscission. The objective of our study was to identify transcription factors that potentially regulate genes affecting the second and third phases of abscission, competence to respond to abscission signals and activation of abscission. We identified transcriptional networks in which transcription factors themselves regulate other transcriptional co-regulators that mediate the separation process.

MATERIALS AND METHODS

Plant Material

Soybean (*Glycine max*, cv. Williams82) seeds were germinated and plants grown in the greenhouse until the primary leaves were fully expanded (19–24 days) and the stem, petioles, and leaves were harvested together by cutting the stem immediately above the soil. Explants were prepared as previously described (Kim et al., 2015) by cutting off the leaf blade leaving about a 5-mm triangular portion of the leaf, and then cutting the stem ~4 cm below the node (Figure 1A). Removing the leaf blade is essential because it removes a source of auxin that inhibits abscission (Addicott, 1982; Meir et al., 2010). Explants were put into Erlenmeyer flasks with water and placed in a dark chamber wherein 25 $\mu\text{L L}^{-1}$ ethylene in air saturated with water was passed through at a flow rate of 2 L min^{-1} (Figure 1B). Treatment with ethylene both accelerates and synchronizes abscission, which is important because AZs and petioles were collected from 20 explants per time point (Addicott, 1982; Tucker et al., 1988). Explants were removed from the chamber after 12, 24, 48, and 72 h, and a 2 mm section of the upper pulvinar AZ (LAZ) at the distal end of the petiole was harvested and flash frozen in liquid nitrogen. In addition, the petiole, non-AZ (NAZ), between the upper and lower AZ was collected and flash frozen (Figure 1B). The upper AZ and petiole were also collected from explants immediately prior to the ethylene treatment, 0 h. To assess the extent of separation at the LAZ, the distal part of the LAZ was gently touched with forceps and, if it fell off the petiole, the LAZ was recorded as having fully abscised (Figure 1C). The AZs at the base of the petioles at the stem interface (lower AZ) were not collected, because the petioles for primary leaves of soybean are rather small and we wanted to avoid incidental collection of a portion of the lateral bud, which would compromise the interpretation of abscission-associated gene expression. LAZ and petioles (NAZ) were collected from 20 explants (two AZs and petioles per explant or 40 LAZ and NAZ total), and the entire process of plant growth, treatment, and collection of LAZ and NAZ was repeated three times.

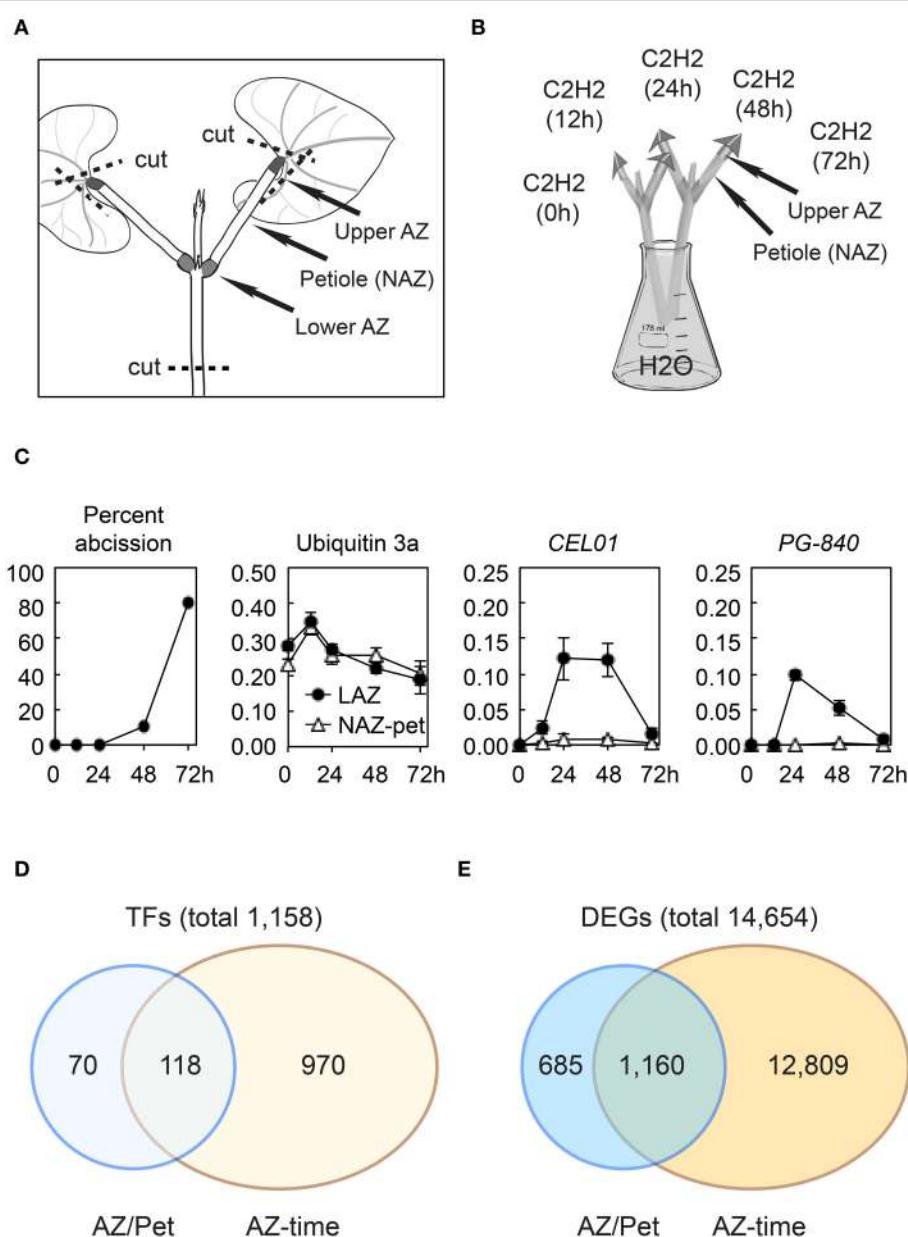


FIGURE 1 | Soybean leaf abscission system and the overview of transcriptome changes in abscission. (A) Stem-petiole explants were prepared by cutting the leaf blade off leaving an ~5-mm triangular portion of the leaf blade still attached to the petiole and cutting the stem at ~4 cm below the node (dashed line). **(B)** Explants were treated with 25 $\mu\text{L L}^{-1}$ ethylene in air saturated with water. The upper AZ (LAZ) and Non-AZ (NAZ, petiole) were collected at 0, 12, 24, 48, and 72 h. **(C)** Percentile of soybean leaf abscission and plots of RNA-seq results for a constitutive ubiquitin gene *UBI3a* (*Glyma20g27950*) and two abscission specific marker genes, *CEL01* (*Glyma11g02350*) and *PG-840* (*Glyma20g02840*). **(D)** Summary of soybean transcription factors (TFs) differentially regulated more than eight-fold ($\log_2 > 3$ or < -3 , $p < 0.015$). **(E)** Summary of differentially expressed genes (DEGs) in the entire transcriptome that were differentially expressed more than eight-fold ($\log_2 > 3$ or < -3 , $p < 0.015$).

RNA Sequencing

RNA was isolated from LAZ and NAZ tissue collected after 0, 12, 24, 48, and 72 h after ethylene exposure using a Qiagen RNeasy Mini Kit following the standard protocol (Qiagen, Germantown, MD, USA). Three experimental replications resulted in 30 RNA samples. Further RNA purification, cDNA synthesis and sequencing on an Illumina GAII sequencer were performed at

Cornell University, Ithaca, NY, USA as previously described (Zhong et al., 2011; Grassi et al., 2013). The 30 RNA samples were processed, barcoded and run together in a single lane on the GAII sequencer. The raw sequence files have been submitted to the NCBI SRA databases with the study accession SRP050050. On average, each RNA sample produced ~4 million reads. Raw sequences were trimmed to remove ambiguous ends.

Using Bowtie (Langmead et al., 2009), ~40,000 (1%) of the reads mapped to ribosomal RNA (rRNA), which were removed from the dataset. Using TopHat (Trapnell et al., 2009), ~90% of the remaining RNA mapped to a predicted data set of soybean transcripts (cds, *G. max* 189 genome assembly) for 54,175 soybean genes. Multiple versions (splice variants) were not taken into account. A single version (usually the last version) was used for alignment. The trimmed sequences (reads) were aligned to the genome assembly and the number of reads aligning with each transcript was normalized to Reads Per Kilobase per Million mapped reads (RPKM) (Mortazavi et al., 2008). For a gene to be counted as expressed in the LAZ or NAZ, we required that the mean RPKM for the three replicates be at least 1.0 or greater in at least one of the treatments. To avoid ratios with a zero in the numerator or denominator, any RPKM of <0.1 was given the minimal value of 0.1. qPCR was performed as previously described (Tucker et al., 2007) on a few selected genes to confirm that the RNA-seq and RPKM normalization produced the expected expression profile (results not shown).

Cluster Analysis

Ratios for the change in expression over time were obtained by dividing the ethylene-treated time values (12, 24, 48, and 72 h) with corresponding values at 0 h, and ratios for abscission-specificity were obtained by dividing the expression in the LAZ relative to the expression in the petioles (NAZ) at the same time interval. Genes were selected for further analysis based on differential expression greater than eight-fold ($\log_2 > 3$ or < -3 , $p < 0.015$) in the LAZ relative to the NAZ (LAZ/NAZ) and also those that changed >eight-fold over time in the LAZ (LAZ at 12/0, 24/0, 48/0, or 72/0 h). These genes and subsets of these genes were then clustered and the uncertainty of the clustering determined using pvclust in the R statistical package (Suzuki and Shimodaira, 2006).

Gene Ontology Analyses and Generation of Transcriptional Networks

The Arabidopsis orthologs that best matched the selected soybean TFs and differentially expressed genes (DEGs) were analyzed for Gene Ontology (GO) enrichment and process interactions using BiNGO_Biological_Process (Maere et al., 2005) on Cytoscape v. 3.2.1 using a Benjamini and Hochberg False Discovery Rate (FDR) < 0.05 (5%).

To predict transcriptional networks underlying soybean leaf abscission, we used the Arabidopsis Transcriptional Regulatory Map (ATRM) data set as described by Jin et al. (2015). Details of transcriptional interactions (either activation or repression) are as described in their **Figure S1B** and Materials and Methods Section (Jin et al., 2015) (<http://atrm.cbi.pku.edu.cn/download.php>). Using the Arabidopsis TFs that were most similar to the soybean TFs and were also in the ATRM data set (**Table 1**), transcription networks were generated that reflect interactions that presumably also occur in soybean leaf abscission. Visualizations of the transcriptional networks were generated using Cytoscape v. 3.2.1.

RESULTS AND DISCUSSION

Overview of Transcriptome Changes in Soybean Leaf Abscission

We conducted RNA-seq using RNA isolated from soybean leaf AZ (LAZ) and non-abscission zone (NAZ) petiole tissues exposed to ethylene for 0, 12, 24, 48, and 72 h. The RNA-seq expression results for all 54,175 soybean genes can be found at <http://sgil.ba.ars.usda.gov/mtucker/Public/Tucker.html>. To validate our RNA-seq data, the isolated RNA was used to perform qPCR for several marker genes known to be specifically expressed in the AZ (e.g., cellulase, polygalacturonase, etc; Tucker et al., 1988; Kalaitzis et al., 1999; Kim and Patterson, 2006; Kim et al., 2006; results not shown). Additionally, when the RNA-seq data was plotted for a constitutively expressed ubiquitin and differentially expressed cellulase and polygalacturonase that correlate with cell wall loosening and cell separation, the expression patterns were as previously reported (Tucker et al., 2007; **Figure 1C** and **Table S1**).

For a gene to be scored as expressed, we required that a gene have a minimum RPKM value > 1 in either the LAZ or petiole at any time interval between 0 and 72 h of ethylene treatment. Using this criterion, we selected 37,572 of the 54,175 soybean genes in the *G. max* 189 genome assembly as being expressed in our tissue collections. Based on the predicted Gene Ontology (GO) biological process and cellular component for the most similar gene identified in Arabidopsis, we selected 5,206 genes in the soybean genome as having transcriptional activity, i.e., transcription factors (TFs) (<http://sgil.ba.ars.usda.gov/mtucker/Public/Tucker.html>). Within these 5,206 TFs, 3,593 TFs were expressed in the LAZ and/or petiole. To identify transcriptional dynamics associated with organ separation, we narrowed down the TFs for further analysis by selecting only genes that were differentially expressed more than eight-fold ($\log_2 > 3$ or < -3 , $p < 0.015$) in the LAZ relative to the NAZ (LAZ/NAZ) and also those that changed >eight-fold over time in the LAZ (LAZ at 12/0, 24/0, 48/0, or 72/0 h; **Figure 1D** and **Table S2**). In addition, we then limited the selection of TFs by eliminating genes having transcriptional activities that are associated with DNA methylation or demethylation, e.g., Glyma20g32960 (At2g36490, Demeter-like 1) and genes with DNA-directed RNA polymerase activity, e.g., Glyma18g17166 (At1g60620, RNA-polymerase I subunit 43). After excluding these genes, there were 1,158 TFs with a more than eight-fold differential expression pattern (**Figure 1D**). This selection includes genes that are more highly expressed in the petiole than the LAZ ($\log_2 < -3$). Although it is reasonable to conclude that TF gene expression that is higher in the petiole than the LAZ is more closely tied to processes specific to the petiole, it is also possible that the lack of a TF (e.g., transcriptional repressor) in the AZ could be important to LAZ gene expression and abscission. Therefore, TFs whose expression was lower in the LAZ than the NAZ ($\log_2 < -3$) were included in the gene clustering and subsequent analyses. However, rather than unnecessarily complicate the discussion of gene expression, both significantly higher and lower expression in the LAZ will be referred to as simply abscission-specific unless it is relevant to the discussion. Thus, within this eight-fold subgrouping, 188

TABLE 1 | List of soybean abscission-specific transcription factors used to generate transcriptional network.

Soybean TF (58)	Cluster	AZ/NAZ		TIME/Oh				Arabidopsis				
		Figure 3	O h	12 h	24 h	48 h	72 h	12 h	24 h	48 h	72 h	TF (40)
Glyma02g02630			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G01470 Homeobox 1, ATHB-1
Glyma17g35951			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G36930 SPATULA, SPT AT1G67260 TCP1
Glyma18g51581			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G67260 TCP1
Glyma06g45554			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G23250 Myb domain protein 15
Glyma02g08241			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G54220 SCARECROW, SCR, SGR1, SHOOT GRAVITROPISM 1
Glyma03g34960			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G03680 PETAL LOSS, PTL
Glyma03g31530	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G14550 Indole-3-acetic acid inducible 14, SOLITARY ROOT
Glyma19g34380			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G14550 Indole-3-acetic acid inducible 14, SOLITARY ROOT
Glyma02g16071	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G04730 Indoleacetic acid-induced protein 16
Glyma13g22620			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G26580 YAB5, YABBY5
Glyma17g12200	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G26580 YAB5, YABBY5
Glyma08g28691	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G67260 TCP1
Glyma18g16390	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G01470 Homeobox 1, ATHB-1
Glyma03g34710	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G03790 HOMEOBOX 51, LATE MERISTEM IDENTITY1, LMI1
Glyma05g04260	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G45190 ABNORMAL FLORAL ORGANS, AFO, FIL, YAB1, YABBY1
Glyma17g14710	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G45190 ABNORMAL FLORAL ORGANS, AFO, FIL, YAB1, YABBY1
Glyma02g16080	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G23050 Indole-3-acetic acid 7
Glyma04g10125	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G23420 INNER NO OUTER, INO
Glyma06g10110	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G23420 INNER NO OUTER, INO
Glyma19g36100	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G37260 TRANSPARENT TESTA GLABRA 2, TTG2, WRKY44
Glyma08g39951	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G43060 IL11 binding bHLH 1
Glyma07g03840	TF C1		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G15540 Indole-3-acetic acid inducible 19
Glyma1Og03720			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G23050 Indole-3-acetic acid 7
Glyma1Og27881			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G62100 Indole-3-acetic acid inducible 30
Glyma08g40705			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G01470 Homeobox 1, ATHB-1
Glyma05g03020			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G66350 RGA-like 1
Glyma18g45220			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G54220 SCARECROW, SCR, SGR1, SHOOT GRAVITROPISM 1
Glyma19g05921			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G67260 TCP1
Glyma03g33376			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G37260 TRANSPARENT TESTA GLABRA 2, TTG2, WRKY44
Glyma1Og07730			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G03680 PETAL LOSS, PTL
Glyma09g33241			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G10510 AINTEGUMENTA-like 6
Glyma03g19030			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G37630 ASYMMETRIC LEAVES 1, MYB91, PHANTASTICA-LIKE 1
Glyma02g40650			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G37020 Auxin response factor 8
Glyma15g01960			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G79840 GL2, GLABRA 2
Glyma13g37111			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1Q03790 SOMNUS (SOM)
Glyma03g06225			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G05100 Myb domain protein 74
Glyma04g03801	TF C3		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G42630 ABERRANT TESTA SHAPE, ATS, KAN4, KANADI 4
Glyma06g03901			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G42630 ABERRANT TESTA SHAPE, ATS, KAN4, KANADI 4
Glyma16g26291	TF C4		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT3G26744 ICE1, INDUCER OF CBF EXPRESSION 1, SCREAM
Glyma09g29940			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT1G17950 Myb domain protein 52
Glyma03g26520			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G44840 Ethylene-responsive element binding factor 13
Glyma14g10830			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G26150 CGA1, CYTOKININ-RESPONSIVE GATA1, GATA22, GNL
Glyma12g13710			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G28500 NAC PROTEIN 73, SECONDARY WALL-ASSOCIATED
Glyma17g06290			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G56860 GATA TRANSCRIPTION FACTOR 21, GNC
Glyma06g21495			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G61270 Phytochrome-interacting factor 7
Glyma08g02020			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT5G41410 BEL1, BELL 1
Glyma05g21726			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G32880 Homeobox gene 8, ATHB8
Glyma18g44030			Y	Y	Y	Y	Y	Y	Y	Y	Y	AT2G38470 WRKY33
Glyma06g44250	TF C5		Y	Y	Y	Y	Y	Y	Y	Y	Y	AT4G28500 NAC PROTEIN 73, SECONDARY WALL-ASSOCIATED

(Continued)

TABLE 1 | Continued

Soybean TF (58)	Cluster	AZ/NAZ		TIME/Oh				Arabidopsis		
		Figure 3	O h	12 h	24 h	48 h	72 h	12 h	24 h	48 h
Glyma12g33460	TF C5							AT4G28500	NAC PROTEIN 73, SECONDARY WALL-ASSOCIATED	
Glyma1Og28820								AT1G23380	KNOTTED1-like homeobox gene 6	
Glyma11g04910								AT4G37750	AINTEGUMENTA	
Glyma18g04580								AT5G16600	Myb domain protein 43	
Glyma10g27860	TF C6							AT4G04450	WRKY42	
Glyma18g48730								AT2G44840	Ethylene-responsive element binding factor 13	
Glyma05g38530								AT3G26744	ICE1, INDUCER OF CBF EXPRESSION 1, SCREAM	
Glyma03g39041								AT1G23380	KNOTTED1-like homeobox gene 6	
Glyma17g00650								AT2G02450	ANAC034, NAC 35, LONG VEGETATIVE PHASE 1	

Of the 188 abscission-specific soybean transcription factors, 58 soybean TFs matched 40 different Arabidopsis homologs found in the high-confidence ATRM data set. More information on these 40 TFs can be found in **Table S5**. Expression patterns (Up or Down) and the TF Cluster numbers are as shown in **Figure 3**. AZ/NAZ, represents the difference in expression in the two tissues at the indicated time; Time/Oh, represents the change in expression at the indicated time relative to 0 h; yellow, up-regulation; black, neutral; blue, down-regulation.

TFs were found to be abscission-specific, and, among these, 118 TFs changed more than eight-fold over time and were also abscission-specific (**Figure 1D** and **Table S2**).

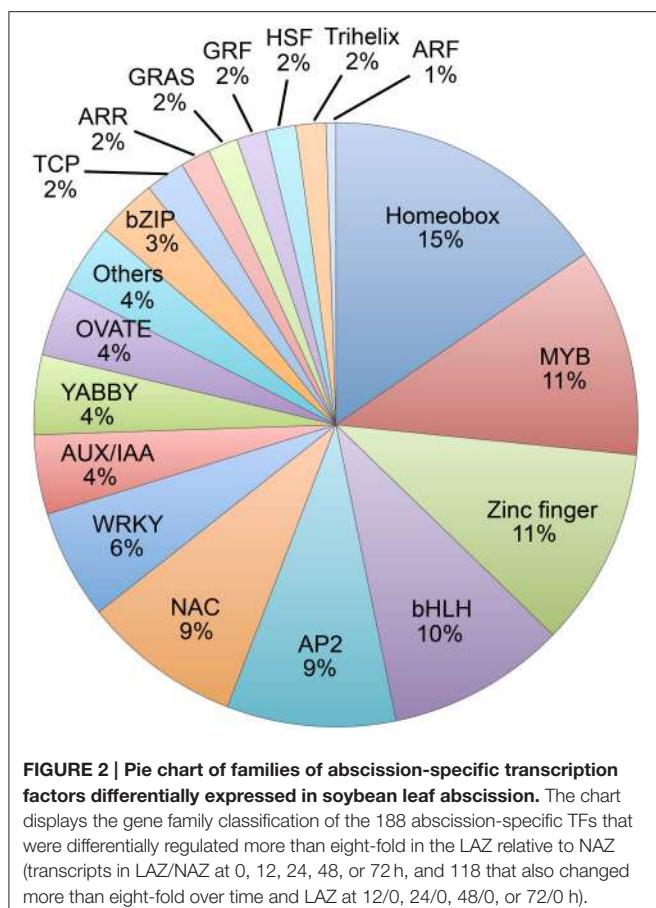
Because these differentially expressed TFs presumably regulate downstream events in organ separation and these target processes are also of interest, we selected genes of all types in the entire transcriptome that displayed a more than eight-fold change in expression, i.e., differentially expressed genes (DEGs). When the same criterion ($\log_2 > 3$ or < -3 , $p < 0.015$) was applied, we found that 14,654 genes were differentially expressed in the AZ relative to the NAZ or changed more than eight-fold in LAZ over time (**Figure 1E** and **Table S1**). To be consistent, we have included both higher ($\log_2 > 3$) and lower ($\log_2 < -3$) LAZ/NAZ gene expression; nonetheless, we recognize that there is a difference between the absence of a TF in the AZ, which might be a repressor of gene expression, and the absence of an enzyme or structural protein in the AZ. There were 13,969 genes whose expression changed more than eight-fold at any time collection between the 0 and 72 h treatment and 1,845 genes whose expression was abscission-specific (higher or lower in the LAZ relative to the NAZ; **Figure 1E**). Within this grouping were 1,160 genes whose expression was both abscission-specific and changed more than eight-fold over time (**Table S1**). These 1,160 DEGs may be regulated or co-regulated by the 118 TFs that were similarly expressed. Below we focus our analysis on the 188 abscission-specific TFs and the 1,845 potential targets for these TFs that we predict are specifically involved in the abscission process. We have included in our analysis the 70 TFs (**Figure 1D**) and 685 DEGs (**Figure 1E**) that were abscission-specific but did not change more than eight-fold over time because by simply being abscission-specific may indicate a special role in abscission.

Characterization of Abscission-Specific Transcription Factors in Soybean

Among the 188 abscission-specific TFs, there were 18 different families of TFs including homeobox, MYB, various types of Zinc finger, bHLH, AP2, NAC, WRKY, YABBY (YAB), IAA, and others (**Figure 2** and **Table S2**), suggesting a complex regulation

of organ separation. In particular, over 15% (29 of 188) of the abscission-specific TFs contained a homeobox domain and this was followed by MYB (21 of 188, 11%), Zinc finger (20 of 188, 11%), bHLH (18 of 188, 10%), and AP2 domain TFs (17 of 188, 9%). In addition, there were several hormone related TFs represented by AUX/IAA (8 of 188, 4%), ARR (3 of 188, 2%), and ARF (1 of 188, 1%). Of particular interest in this group was the plant-specific YABBY family of TFs, which accounted for more than 4% (8 of 188) of the TFs. The YABBY gene family is a relatively small family of genes found only in the plant kingdom. Moreover, YABBY gene expression was most abundant in the group with higher expression in the LAZ and not highly expressed in the NAZ (**Table 1**, **Table S2**).

As mentioned above, a working model for abscission predicts four developmental phases that culminate in organ separation and synthesis of a protective scar: Phase (1) establishment of the AZ; Phase (2) acquisition of competence to respond to abscission signals; Phase (3) activation of abscission/cell separation; and Phase (4) trans-differentiation between the separating sides of the AZ cells and deposition of a protective layer (Bleecker and Patterson, 1997; Lewis et al., 2006; Liljegren, 2012; Kim, 2014; Tucker and Kim, 2015). Because abscission is a nexus of multiple developmental and environmental signals, abscission should be thought of as a mutually interconnected process that occurs within a developmental time frame (Tucker and Kim, 2015). To identify transcriptional regulation that underlies the developmental processes coordinating organ separation, we performed a cluster analysis of the 188 abscission-specific TFs to group TFs that presumably share a common role in the regulation of the different phases of soybean leaf abscission. The 188 TFs were clustered based on a minimum expression of $>$ four-fold up- or down-regulation in two consecutive time points ($\log_2 > 2$ or < -2 , $p < 0.015$; i.e., LAZ/NAZ at 0 and 12 h, 12 and 24 h, 24 and 48 h, 48 and 72 h; **Figures 3A,B**). We chose to assess a sustained differential expression over two consecutive time points and not just one or more than two so that we could be more certain that the differential expression of a TF could be linked to a particular process or phase of abscission.



A total of seven clusters were identified, which included 48 of the original 188 genes (Figure 3 and Tables S2, S3). Four clusters included genes that were more highly expressed in the AZ than the petiole (34 genes) and three clusters where the genes were more highly expressed in the petiole (14 genes). TF Cluster 1, which included genes that were higher in the LAZ than NAZ ($\log_2 > 2$) at 0 and 12 h, contained the greatest numbers of TFs (17 genes; Figure 3A). All but 1 of the genes in Cluster 1 declined with time. Because organ separation at the LAZ was not detected until 48 h after ethylene treatment began, we presume that the Cluster 1 TFs are associated primarily with phase 2 of abscission, i.e., acquisition of competence to respond to abscission signals. Gene Ontology (GO) analysis of TF Cluster 1 was enriched for biological processes associated with shoot system development, polarity specification of adaxial/abaxial axis, cell fate commitment/specification, reproductive structure development, response to endogenous/external stimuli, response to auxin, gibberellin metabolic process, regulation of the timing of meristematic phase transition, and positive regulation of transcription and metabolic processes (Figure 3C).

Of special interest in TF Cluster 1 were TFs encoding plant specific YABBY family proteins, which included INNER NO OUTER (INO), ABNORMAL FLORAL ORGANS/FILAMENTOUS FLOWER (AFO/FIL), YAB2, and YAB5. Current annotation for the YABBY TF family proteins

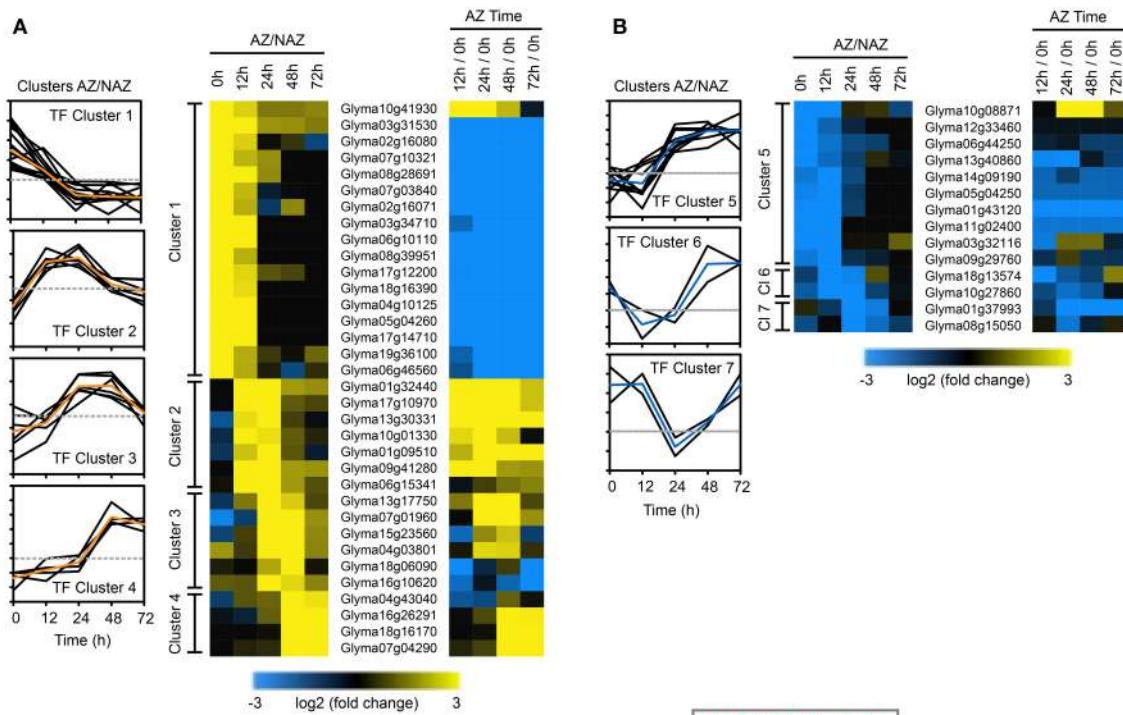
predicts six members in Arabidopsis (Siegfried et al., 1999; Plant TF Database <http://plntfdb.bio.uni-potsdam.de/v3.0/>) and 17 members in soybean (PlantTFDB at <http://planttfdb.cbi.pku.edu.cn>). There are five subfamilies of YABBY in angiosperms represented by AFO/YAB3, YAB2, YAB5, CRAB CLAW (CRC), and INO clades (Bonaccorso et al., 2012; de Almeida et al., 2014). It is therefore noteworthy that 6 out of the 17 TFs in TF Cluster 1 were YABs, which declined rapidly after induction of abscission (Tables S2, S3).

Also included in TF Cluster 1, were soybean TFs orthologous to HOMEobox 1 (ATHB-1) and LATE MERISTEM-IDENTITY-1 (LMI1/HOMEobox 51/ATHB-51). Both ATHB-1 and LMI1 have been demonstrated to affect cell fate and development (Aoyama et al., 1995; Saddic et al., 2006). Overexpression of ATHB-1 in tobacco resulted in de-etiolated seedlings in the dark and leaves in the light with light green sectors of spongy parenchyma cells rather than dark green palisade cells (Aoyama et al., 1995). It was concluded that ATHB-1 was a positive transcriptional activator that altered cell fate (Aoyama et al., 1995). LMI1 (ATHB-51) is a positive regulator of genes that regulate organ identity in the meristem (Saddic et al., 2006; Grandi et al., 2012).

Although TF Cluster 2 (high in both 12 and 24 h) did not predict any enriched biological processes, this cluster contained transcriptional co-regulators most similar to Arabidopsis OVATE 2 and OVATE 4. Also in TF Cluster 2 is a NAC domain protein that was both higher in the LAZ than NAZ and strongly up-regulated over time. Also in Cluster 2 was an AP2/ERF TF, which suggests that an ethylene response had begun in the AZ by 12 h. TF Cluster 3, which spans the 24 and 48 h time points, is enriched in GO terms for response to endogenous and hormone stimulus, ethylene-mediated hormone signaling (Figure S1A), and reproductive development, which included ETHYLENE AND SALT INDUCIBLE 3 (ESE3), GATA 9 and ABERRANT TESTA SHAPE/KANADI 4 (ATS/KAN4) (Tables S2, S3). In our explant system, ~10% of the upper AZ had separated by 48 h (Figure 1C). Presumably, changes in TFs associated with phase 3 of abscission, activation of abscission, began at 12 h when an increase in the ETHYLENE RESPONSE FACTOR (ERF) was observed and continued at least until 48 h when organ separation was detected. Thus, gene expression of TFs in TF Clusters 2 and 3 are most likely linked to activation of abscission.

TF Cluster 4, which spans the 48 and 72 h time collections, and most likely associated with phase 4 of abscission, trans-differentiation of proximal and distal cells, includes WRKY 72, DEHYDRATION RESPONSE ELEMENT-BINDING PROTEIN 26 (DREB26), INDUCER OF CBF EXPRESSION 1 (ICE1). GO term analysis of this cluster indicates that these TFs are best linked to responses to stress and, curiously, stomatal development (Figure S1B).

The second largest grouping in our TF cluster analysis is TF Cluster 5, which included 10 genes whose expression was lower in the AZ and higher in the petiole at 0 and 12 h. As we concluded for TF Cluster 1 genes, we presume that at least some of the genes in TF Cluster 5 are linked to phase 2 of abscission, acquisition of competence to respond to abscission signals. Because gene expression for these TFs



c GO analysis for TF Cluster 1

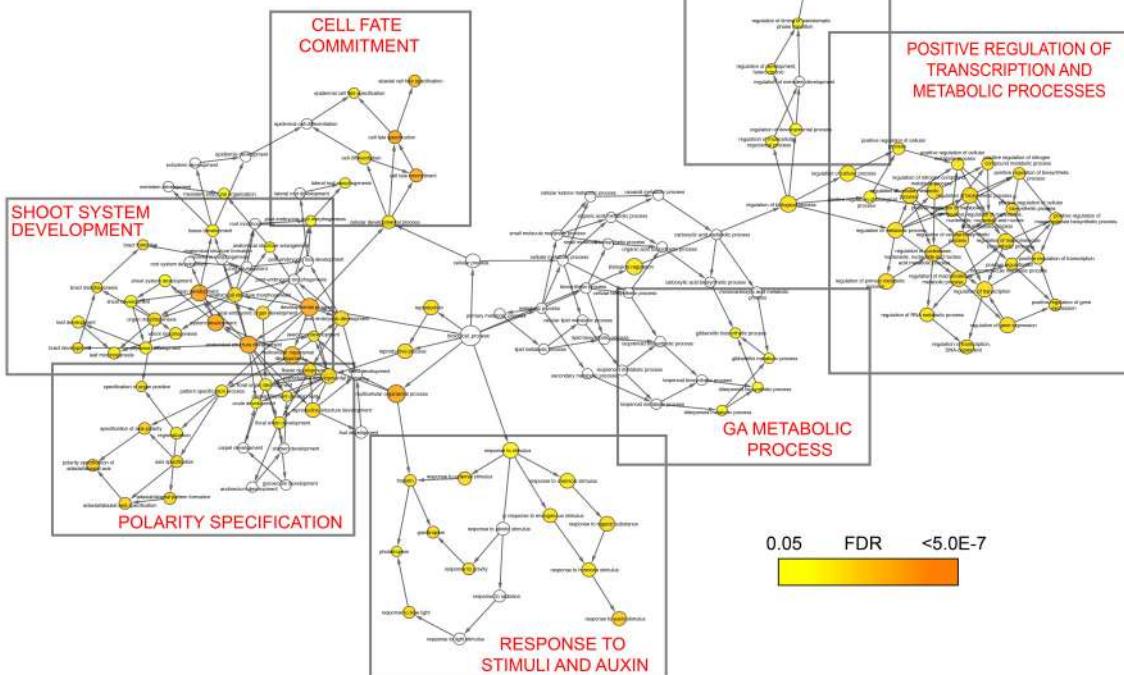


FIGURE 3 | Cluster analysis of abscission-specific transcription factors (188 TFs) more or less abundant in the AZ at two consecutive time points in soybean leaf abscission (48 TFs). (A) Heat map display of the 34 abscission-specific TFs that clustered based on expression greater than four-fold in the LAZ relative to the petiole (NAZ) ($\log_2 > 2, p < 0.015$) in two consecutive time points (i.e., LAZ/NAZ at 0 and 12 h, 12 and 24 h, 24 and 48 h, and 48 and 72 h). Change in expression for the same TFs in the LAZ over time (i.e., expression in LAZ at 12/0, 24/0, 48/0, and 72/0 h). **(B)** Similar heat map display of the 14 abscission-specific TFs that clustered based on expression of four-fold less in the LAZ relative to the NAZ ($\log_2 < -2, p < 0.015$) in two consecutive time points. **(C)** Gene Ontology (GO) term network analysis (BINGO) for TF Cluster 1 having four-fold higher expression in the LAZ/NAZ at 0 and 12 h. Enrichment clusters with similar biological processes

(Continued)

FIGURE 3 | Continued

are boxed and a summary of the biological process is printed in red inside the box. The range of colors from yellow to orange inside the circles for each identified biological process indicates the statistical significance from 0.05 to $< 5 \times 10^{-7}$, respectively, for the enrichment of the GO term in the test set, Cluster 1 TFs, (Maere et al., 2005). The color bar at the bottom right reflects the range of statistical significance where the *p*-value was adjusted using a Benjamini and Hochberg False Discovery Rate (FDR) correction.

is higher in the petiole than the LAZ, if these TFs are important to abscission, their absence from the LAZ would make possible the activation or binding of other TFs that evoke a differential gene expression linked to phase 3, activation of abscission. TF Cluster 5 was enriched in GO terms for asymmetric division and multicellular organismal development (Figure S1C and Tables S2, S3). The GO terms associated with TF Cluster 1 and 5 suggest that these 27 genes play critical roles in the control of cell fate and response to plant hormones, which includes auxin, cytokinin, gibberellic acid (GA), and ethylene. As expected, because the source of auxin was removed, TFs associated with auxin signaling (AUX/IAA) or co-regulators of auxin were rapidly down-regulated over time (Table S2).

Also in TF Cluster 5 were five NAC domain containing TFs. These NAC TFs, which were less abundant in the LAZ than the petiole are in contrast to the strongly AZ-specific NAC TF in Cluster 2 (Tables S2, S3). Moreover, there were several more NAC domain TFs that were either more abundant in the LAZ than the petiole or the reverse but did not fall into our clustering restriction of only two consecutive times (Table S2). NAC TFs are one of the largest families of TFs in the plant kingdom that regulate a variety of developmental and environmental responses (Olsen et al., 2005). Interestingly in regard to NAC domain TFs, when an 18 bp element (Z-BAC) from the promoter of the *BEAN ABSCISSION CELLULASE 1* (*BAC1*) (Tucker et al., 2002) was used in a yeast one-hybrid screen to identify putative DNA-binding proteins in a bean (*Phaseolus vulgaris*) leaf abscission zone (LAZ) cDNA library, the most common clones identified were for a NAC domain protein (*PvNAC1*) (results not shown). When the *PvNAC1* TF was co-expressed with a BAC1:luciferase promoter construct in a transient bean expression assay (Tucker et al., 2002), expression of the BAC promoter was suppressed by 84%. However, we were unable to demonstrate direct binding of the NAC protein to the *BAC1* promoter using an electrophoretic mobility shift assay (results not shown). Nonetheless, the dissimilar expression patterns of the NAC TFs accentuate the potential for both positive and negative regulation of gene expression by the same family of TFs (Olsen et al., 2005; Jin et al., 2015).

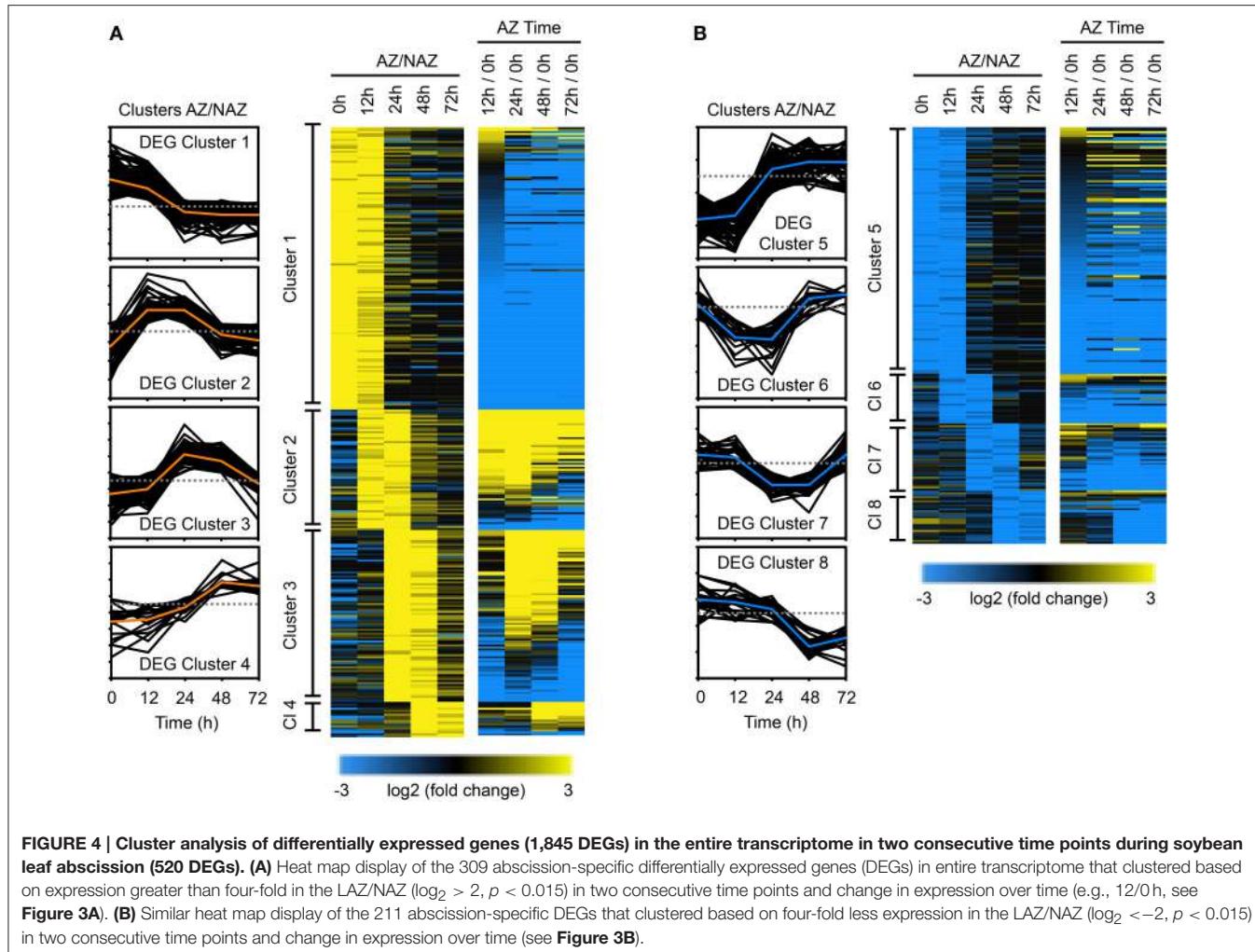
Clustering of Differentially Expressed Genes (DEGs) in the Entire Transcriptome

To gain insights into potential downstream events associated with the 188 abscission-specific TFs identified above, we examined changes in the soybean transcriptome having a similar pattern of up or down regulation in two consecutive times. When the same cutoff for expression levels ($\log_2 > 2$ or < -2 , $p < 0.015$) was applied to cluster the abscission-specific

genes (1,845 genes), we identified eight clusters that spanned two consecutive time points in an abscission-specific manner (Figure 4). The first four clusters were comprised of genes that were more abundantly expressed in the AZ (Figure 4A) and the latter four clusters contained genes that had lower abundance in the AZ relative to the petiole (Figure 4B). As was observed for the TF clusters, the first cluster (higher in the AZ than NAZ at 0 and 12 h) and the fifth cluster (lower in the AZ than NAZ at 0 and 12 h) contained the greatest numbers of genes. DEG Cluster 1 included 143 genes (Figure 4A and Table S4). GO term analysis of DEG Cluster 1 predicted similar but distinct results compared to that of TF Cluster 1 (Figure S2A). In addition to biological processes associated with response to endogenous/external stimuli, response to auxin, and reproductive structure development, the GO analysis of DEG Cluster 1 included cellular response to phosphate starvation, cytokinin transport, cutin transport, myo-insitol transport, flavonoid biosynthetic processes, cellular amino acid derivative, and metabolic processes. GO analysis of DEG Cluster 1 indicates that gene expression at 0 and 12 h is associated with inter-cellular communication and metabolic processes associated with an early abscission response. Moreover, GO analysis of DEG Cluster 1 suggests that the AZ cells have begun to respond to changes in cellular nutrition, which may be linked to the removal of the leaf blade. Also, as expected, expression of SAUR genes (SMALL AUXIN UPREGULATED RNA) declined rapidly from 0 to 12 h (Figure 4A and Table S4).

DEG Cluster 2 (61 genes), spanning the 12 and 24 h collections (Figure 4A and Table S4) includes homologs of ACC SYNTHASE 10, INFLORESCENCE DEFICIENT IN ABSCISSION (IDA)-like 1 and basic chitinase (PR-3), which have been previously linked to early stages of abscission in several species (Bleecker and Patterson, 1997; Roberts et al., 2002; Butenko et al., 2003; Meir et al., 2010; Tucker and Yang, 2012). GO term analysis of DEG Cluster 2 confirmed the abscission-specificity of this cluster (Figure S3A). DEG Cluster 3 at 24 and 48 h included 87 soybean genes enriched in GO terms for carbohydrate metabolic process and regulation of cell size (Figure S3B). Many of the genes in this cluster are annotated as cell wall hydrolytic and cell wall modifying enzymes that reflect the actual cell separation process (Figure 4A and Table S4). Although 18 genes grouped into DEG Cluster 4 spanning 48 and 72 h, there were no GO term predictions associated with these genes; nonetheless, this cluster included an arabinogalactan protein, which may reflect changes in the cell wall structure.

DEG Cluster 5, the second largest DEG cluster, included 126 genes that were more highly expressed in the petiole than the LAZ (Figure 4B and Table S4). GO term analysis of DEG Cluster



5 highlighted processes associated with cellulose biosynthesis, secondary cell wall biogenesis, rhamnogalacturonan I side chain metabolism, and proteolysis (Figure S2B). In addition, DEG Cluster 5 was also associated with biological processes for positive regulation of abscisic acid (ABA) and lipid biosynthesis (Figure S2B). It should be noted here that to be consistent with the clustering of TFs, we included DEGs that were more abundant in the petiole (higher in the NAZ than LAZ); however, as noted above, the lack of a TF in the AZ, which might be a repressor, is different than the lack of an enzyme in the AZ. A higher concentration of a DEG in the petiole at 0 and 12 h may reflect differences in processes that were occurring in the petiole at the time of harvest and may not be directly linked to abscission, e.g., cellulose biosynthesis.

GO term analysis for DEG Cluster 6 and 7 (less abundant in the LAZ at 12 and 24 h, and 24 and 48 h, respectively) did not highlight any special process; however analysis of DEG Cluster 8 (48 and 72 h) highlighted gene expression associated with response to stress and heat (Figure S3C), which might reflect the induction of programmed cell death and senescence at this time interval.

Regulatory Networks of Transcription Factors in Soybean Leaf Abscission

Although genomic-scale studies using microarray and RNA-seq have provided considerable data on TF gene expression in a variety of plant tissues in many plant species, information on the regulatory networks governed by TFs in species other than Arabidopsis is still lacking (Rhee and Mutwil, 2014). It is generally accepted that almost all eukaryotic genes are regulated by more than one TF and their target genes are also dependent on several TFs and/or co-regulators (Sorrells and Johnson, 2015), and a study of the transcriptional networks in Arabidopsis confirmed the importance of regulatory pairs of TFs (Jin et al., 2015). Moreover, the interactions of TFs in a regulatory network appear to be conserved during the evolution of multicellular organisms (Jin et al., 2015) in highly diverse species (Sorrells and Johnson, 2015). An interesting finding in the Arabidopsis study was that regulatory networks associated with development had more TFs and co-regulators per target gene than environmental or stress response networks (Jin et al., 2015). This would suggest that gene expression associated with developmental responses is more finely and discretely regulated than environmental

responses. Thus, abscission, which integrates both environmental and developmental cues, may be a complex interplay of several regulatory networks.

Above we focused on GO term analysis to identify developmental and environmental processes linked to abscission. To further substantiate and investigate transcriptional regulation of soybean leaf abscission, we used the publicly available data from the Arabidopsis Transcriptional Regulatory Map (ATRM) (Jin et al., 2015). In the 188 soybean abscission-specific TFs discussed above (**Figure 1D**), there were 133 different Arabidopsis TFs represented (**Table S2**). Among the 133 TFs, 40 TFs were found in the high-confidence regulatory ATRM data set (**Table 1**). These 40 were used to predict several interactive transcriptional networks for soybean leaf abscission (**Figure 5** and **Figure S4**). The largest network (**Figure 5**), which is discussed in the sub-sections below, consisted of TFs with domains for: AP2 [e.g., AINTEGUMENTA (ANT) and AINTEGUMENTA-like 6 (AIL6)], ASYMMETRIC LEAVES 1 (AS1), homeobox [e.g., KNOTTED-like 6 (KNAT6), ATS/KAN4, LMI1/HOMEOBOX 51, and BEL1], YABBY (e.g., INO, AFO, and YAB5), Zinc finger [e.g., GATA, NITRATE-INDUCIBLE, CARBON-METABOLISM INVOLVED (GNC) and CYTOKININ-INDUCED GATA1/GNC-like (GNL)], and Trihelix [e.g. PETAL LOSS (PTL)].

TFs Associated with Stem Cell Maintenance

ANT and AIL6 encode Arabidopsis TFs that belong to an eight-member subfamily of AIL/PLETHORA (PLT) in the larger AP2/ERF TF family (Nole-Wilson et al., 2005; Prasad et al., 2011). In Arabidopsis, it was demonstrated that ANT and AIL6 are associated with various developmental processes including the maintenance of shoot and flower meristems, organ size, flower initiation, and floral organ identity. Mutational studies of the *AIL* genes support their regulatory role in meristems as shown by loss or reduced growth of organs (loss-of-function) and ectopic formation or increased growth of organs (gain-of-function; Krizek, 2009; Horstman et al., 2014). The defects of *ant* and *ail6* mutants correlate with expression of the stem cell and floral organ regulatory genes, and changes in the expression of auxin responsive and transport genes (Krizek, 2009). Based on expression of the *ANT* gene in the *arf2* mutant, it was suggested that *ANT* might be regulated by ARF2 (Schruff et al., 2006). The results collectively suggest that ANT and other AIL TFs control the balance between cell proliferation and differentiation in response to auxin gradients that maintain growth and patterning in different developmental processes (Krizek, 2009).

The expression patterns of the soybean *ANT* and *AIL6* genes suggest a complex role in leaf abscission. Expression of *ANT* is higher in the petiole than the LAZ at 0, 12, and 24 h, but *AIL6* is significantly higher in the LAZ at 48 h than the petiole (**Figure 5** and **Table 1**), indicating that soybean AIL TFs may have distinct roles in leaf abscission. In fact, genetic studies in Arabidopsis and expression studies in other species demonstrated that AIL TFs have distinct and differing roles depending on the systems studied (Rieu et al., 2005; Mudunkothge and Krizek, 2012; Horstman et al., 2014). In regard to *ANT* and *AIL6* expression in response to auxin, it is worth noting that the ARF7/19 and

ARF1/2 are involved in the regulation of floral organ abscission in Arabidopsis (Ellis et al., 2005). Working forward from the Arabidopsis results, it would be interesting to know if an auxin-ANT/AIL module regulates the balance between cell proliferation and differentiation through translating an auxin gradient that might form during soybean leaf abscission (Louie and Addicott, 1970; Tucker and Kim, 2015).

TFs Associated with Hormone Signaling Pathways

In the transcription network shown in **Figure 5**, ANT and AIL6 are associated with AP3, which is a floral organ regulatory gene in Arabidopsis that is connected to the regulation of GNC-GNL. GNC and GNL were originally identified in gene expression studies with nitrate, cytokinin, and light treatments of Arabidopsis (Bi et al., 2005; Naito et al., 2007). Subsequent studies demonstrated that auxin and GA also regulate GNC and GNL expression. GNC and GNL act as negative regulators of germination, GA catabolism (GA20X2), cotyledon expansion, flowering time, senescence, and floral organ abscission (Richter et al., 2013; Behringer and Schwechheimer, 2015). Of particular interest is that over-expression of GNC and GNL share many phenotypes with *arf2* mutants (Behringer and Schwechheimer, 2015), which as mentioned above display a delay in floral organ abscission (Ellis et al., 2005; Okushima et al., 2005; Schruff et al., 2006). Moreover, ARF2 and ARF7 were demonstrated to directly bind to the promoters of GNC and GNL (Richter et al., 2013). These observations further support the putative role of auxin- and GA-mediation of GNC and GNL expression in the control of abscission.

In our soybean RNA-seq data, the expression of GNC and GNL homologs are more strongly expressed in the petiole than the LAZ (**Figure 5** and **Table 1**) and their lower expression or lack of expression in the LAZ may be important to soybean leaf abscission. Moreover, considering that ANT and AIL6 proteins, like GNC and GNL, are downstream components of the ARF2/ARF7-mediated signaling module, it is possible that the GNC-GNL and ANT-AIL6 pair of co-transcriptional regulators may play an important role in the transcriptional regulation of organ abscission in plants. This is consistent with their role described above to control the balance between plant hormone signaling pathways in plant growth and development.

TFs Associated with Organ Polarity and Growth

The transcriptional network analysis indicates that ANT-AIL6 and GNC-GNL are associated with meristem TFs affecting organ polarity and organ boundary determinants, which includes YAB-ATS/KAN4, AS1, and KNAT6 (**Figure 5**). The YABBY gene family was briefly discussed above because members in this family were prominent in TF Cluster 1. Three members of the YABBY gene family (AFO, INO, and YAB5) were also prominent in the transcriptional regulatory network generated with the ATRM data (Jin et al., 2015; **Figure 5**). In Arabidopsis, YABs regulate growth of the integuments and lateral organs, which includes leaves, sepals, petals, and carpels (Eshed et al., 1999, 2001, 2004; Kerstetter et al., 2001). In particular, the INO gene is expressed in the outer integument of the ovule (Villanueva et al., 1999) and the AFO/FIL, YAB3, YAB2, and YAB5 genes

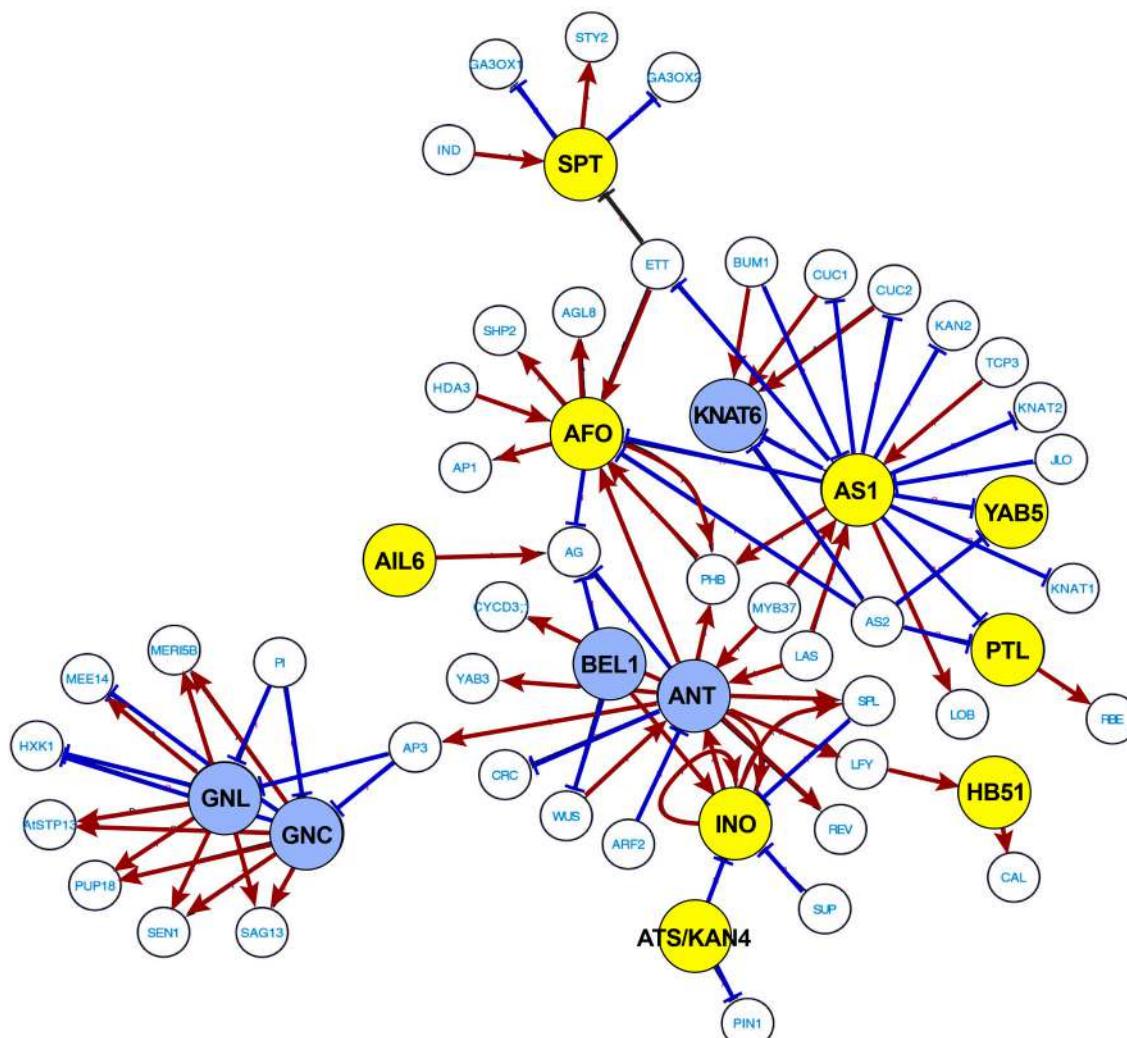


FIGURE 5 | Most extensive transcriptional network underlying soybean leaf abscission. The 188 abscission-specific soybean TFs corresponding to 133 Arabidopsis TFs (Table S2) were used to construct high-confidence transcriptional networks using the Arabidopsis Transcriptional Regulatory Map (ATRM). Of 133 Arabidopsis TF homologs, only 40 of Arabidopsis TFs were found in the ATRM data set (Table 1, Table S5). Arabidopsis TF orthologs identified in expression data of soybean leaf abscission were color-coded. Soybean TFs eight-fold higher in the LAZ/NAZ are highlighted in yellow and TFs eight-fold lower in the LAZ/NAZ are highlighted in blue. Visualization of the network was generated by Cytoscape v. 3.2.1. Red arrows indicated a positive regulation and blue bars a negative regulation of the target TFs or co-regulators generated from ATRM data set.

are expressed in the abaxial domain of developing leaf and floral organ primordia (Siegfried et al., 1999; Sarojam et al., 2010). It was concluded that YAB gene expression is critical to organ polarity and subsequent lamina growth and cell identity in developing organs (Eshed et al., 1999, 2004; Sawa et al., 1999; Siegfried et al., 1999; Stahle et al., 2009; Sarojam et al., 2010). YABs (i.e., AFO/FIL, YAB3, YAB2, YAB5) repress expression of shoot apical meristem (SAM) regulatory genes in the developing leaf primordia (Bonaccorso et al., 2012). Without YAB activity, expression of *KNOX* and *WUS* genes were up-regulated in leaves, which caused the formation of meristem-like structures on the leaves (Kumaran et al., 2002; Sarojam et al., 2010). The soybean *INO*, *AFO/FIL*, *YAB2*, and *YAB5* genes are highly expressed in the LAZ at 0 and 12 h (Figure 5, Table 1; Tables S2, S3).

In contrast, expression of a soybean *KNAT6* gene is repressed prior to and at the beginning of abscission at 0, 12, and 24 h (Figure 5 and Table 1). Their expression pattern suggests that YAB-KNAT6 may contribute to defining the organ separation boundary by suppressing the AZ cell proliferation or promoting de-differentiation of cells at the onset of abscission similar to their role in the meristem.

Also in the ATRM generated TF network is a KAN TF (*ATS/KAN4*) that can interact with the YAB protein (*INO*). Arabidopsis gene expression and mutant analysis of *ATS/KAN4* indicate that it plays a role in defining the organ boundary that separates the two integuments (inner and outer integuments; McAbee et al., 2006). It was concluded that *ATS/KAN4* gene expression regulates organ polarity and

boundary formation necessary for proper integument growth as it does for abaxial identity during leaf development. Increased expression of soybean *ATS/KAN4* genes (**Figure 5** and **Table 1**) during organ separation at 24 and 48 h may indicate that the soybean *ATS/KAN4* gene may be involved in defining the separation boundary of the AZ. Studies of *ATS/KAN4* and *YABs* in Arabidopsis and their expression patterns in soybean leaf abscission collectively emphasizes the importance of a balance among diverse polarity determinants in the control of abscission.

Other boundary determinants critical to organ polarity and cell fate identified in our study include a soybean homolog of *AS1* (**Figure 5** and **Table 1**). In Arabidopsis, *AS1* is also required for organ boundary/polarity, cell fate, and proper establishment of floral organ AZ cells (Byrne et al., 2000; Hazen et al., 2005; Gubert et al., 2014). It was suggested that Arabidopsis *AS1* controls the placement of the sepal and petal abscission zones, which appear to affect either the timing of abscission zone differentiation or the activation of cell separation (Gubert et al., 2014). The soybean *AS1* gene is most highly expressed in the LAZ at 12 h before or at the beginning of actual cell separation (**Figure 5** and **Table 1**).

In Arabidopsis, *AS1*, together with *AS2*, repress expression of *KNOTTED1-LIKE HOMEODOMAIN* (*KNOX*) genes, which are meristem-promoting genes affecting leaf development (Ori et al., 2000; Guo et al., 2008; Xu et al., 2008). Moreover, *AS1* acts upstream of *BREVIPEDICELLUS* (*BP*, *KNAT1*), *KNAT2*, and *KNAT6* homeodomain TFs that have been demonstrated to affect development of the floral organ AZ in Arabidopsis. In Arabidopsis, *BP/KNAT1* represses expression of *KNAT6* (Shi et al., 2011). Although *KNAT1* expression is not restricted to the AZ in Arabidopsis, *KNAT6* expression was higher in the AZ compared to surrounding tissues (NAZ; Shi et al., 2011). In soybean, expression of *KNAT6* (*KNOTTED1-LIKE HOMEODOMAIN 6*) was lower in the AZ compared to the petiole at 0, 12, and 24 h (**Table 1**). It appears that abscission-specificity of *KNAT6* in soybean is different than in Arabidopsis; nonetheless, the interactions of soybean *AS1*, *KNAT1*, and *KNAT6* in the development of the AZ might still be of interest because *AS1* and *KNAT6* gene expression was significantly different between the LAZ and petiole and this may influence development of the AZ or formation of the separation layer in soybean.

Also of potential interest in regard to defining the separation layer within the soybean LAZ are TFs for soybean BELL-type homeodomain and PETAL LOSS (PTL). In Arabidopsis, the BELL-type homeodomain TF was shown to repress growth in the boundary region between the floral organ and flower receptacle, and also be required for establishment of stamen AZ (Gómez-Mena and Sablowski, 2008). In addition, PTL was suggested to act as a negative regulator of cell proliferation in the floral organs of Arabidopsis. Expression of the soybean *BEL1* gene is lower in the LAZ than the petiole and in contrast the *PTL* genes were more highly expressed in the LAZ at 0 and 12 h and declined over time (**Figure 5** and **Table 1**; **Table S2**). Gene expression profiles for these TFs support their potential antagonistic role in restricting cell proliferation and organ boundary determination in the soybean AZ.

CONCLUDING REMARKS

In the current study, we identified 188 soybean TFs that were differentially regulated more than eight-fold in an abscission-specific manner. Cluster analysis of these abscission-specific TFs requiring an up or down-regulation in two consecutive time points enabled us to group soybean TFs that may share similar functions in the progression of organ abscission and development of the AZ cells. Of particular interest was TFs that were more highly expressed in the AZ relative to the petiole at 0 and 12 h (TF Cluster 1). Many of the TFs identified within this cluster are known as key determinants in maintenance of organ polarity, lateral organ growth and cell fate. The association of these TFs with organ polarity and boundary definition was further substantiated in the transcriptional networks generated using the ATRM data set (Jin et al., 2015), which suggests that these TFs may play a role in defining the separation layer within the multilayer LAZ. The most prominent TFs associated with each of the phases of abscission are listed in the abscission model depicted in **Figure 6**. We propose that the TFs listed in the model function as transcriptional regulators to balance plant hormone signaling, organ polarity, and meristem-like responses in the AZ cells prior to the onset of organ separation.

Identification of meristem-associated genes in the AZ has been noted by others (Nakano et al., 2013; Wang et al., 2013). In addition, a recent publication further substantiates the concept that meristem-associated genes that control formation of specialized domains of restricted growth known as boundaries between stem cell activity of shoot apical meristem and lateral organ growth may also underlie boundaries formed during organ abscission (Hepworth and Pautot, 2015). It seems logical that organ (leaf, flower, fruit, etc.) abscission would be an adaptation of a primal process in the meristem. Processes and signals that regulate organ polarity and boundary formation in the meristem

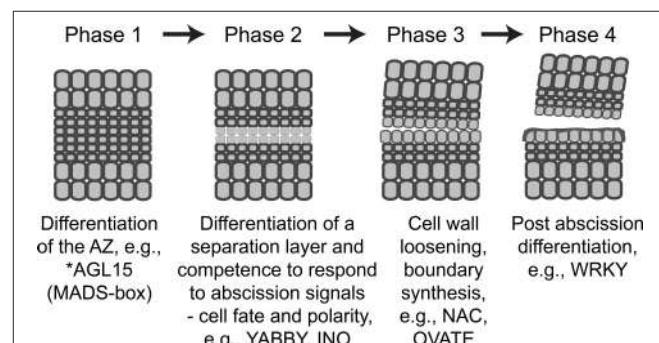


FIGURE 6 | Revised abscission model depicting each of the four phases of abscission and the most prominent TFs associated with each phase. The descriptions for phases 2 and 3 have been revised to reflect the findings in our study. * No soybean *AGL15*-like gene was expressed in our RNA-seq results; however, others have demonstrated its importance to formation of the Arabidopsis floral AZ and we have placed it into phase 1 (Fernandez et al., 2000) similar to the placement of the tomato *JOINTLESS*, *MADS-box* gene (Mao et al., 2000; Nakano et al., 2012). The up and down-regulated expression patterns for *YABBY*, *INO*, *NAC*, *OVATE*, and *WRKY* genes can be found in **Table S2**.

might therefore be similarly found in the AZ. Nonetheless, the functional relevance of these TFs in abscission awaits further experimentation. Identification of the transcriptional targets for these TFs will provide a better understanding of how these conserved gene networks control plant development. In regard to abscission, most plant AZs are comprised of several layers of small, less-vacuolated cells (Roberts et al., 2002). The signals and factors that determine where within this AZ the separation layer will form are not known (Tucker and Kim, 2015). It will be interesting to determine in the future if some of the TFs identified in this study are involved in establishing the position of the separation layer within the AZ.

AUTHOR CONTRIBUTIONS

JK and MT, plant and RNA sample preparation, data analysis and manuscript preparation; RY, plant and RNA sample preparation; JY, RS, and CC, data analysis and manuscript preparation.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2016.00125>

Figure S1 | GO term analysis of abscission-specific TF Clusters 3, 4, and 5 (Figures 3A,B).

Figure S2 | GO term analysis of abscission-specific DEG Clusters 1 and 5 for the entire transcriptome (Figures 4A,B).

Figure S3 | GO term analysis of abscission-specific DEG Clusters 2, 3 and 8 for the entire transcriptome (Figures 4A,B).

Figure S4 | Less extensive transcriptional networks underlying soybean leaf abscission.

Table S1 | Excel file listing expression and annotation of 14,654 differentially expressed genes (DEGs) that were > eight-fold abscission-specific or changed > eight-fold with time and $p < 0.015$.

Table S2 | Excel file listing expression and annotation of 188 abscission-specific soybean TFs (Figures 1D, 2).

Table S3 | Excel file listing expression and annotation of 48 soybean TFs depicted in Figures 3A,B.

Table S4 | Excel file listing expression and annotation of 520 DEGs depicted in Figures 4A,B.

Table S5 | Excel file giving additional information on ATRM genes used for transcriptional networks.

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Flower abscission in *Vitis vinifera* L. triggered by gibberellic acid and shade discloses differences in the underlying metabolic pathways

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Understanding abscission is both a biological and an agronomic challenge. Flower abscission induced independently by shade and gibberellic acid (GAc) sprays was monitored in grapevine (*Vitis vinifera* L.) growing under a soilless greenhouse system during two seasonal growing conditions, in an early and late production cycle. Physiological and metabolic changes triggered by each of the two distinct stimuli were determined. Environmental conditions exerted a significant effect on fruit set as showed by the higher natural drop rate recorded in the late production cycle with respect to the early cycle. Shade and GAc treatments increased the percentage of flower drop compared to the control, and at a similar degree, during the late production cycle. The reduction of leaf gas exchanges under shade conditions was not observed in GAc treated vines. The metabolic profile assessed in samples collected during the late cycle differently affected primary and secondary metabolisms and showed that most of the treatment-resulting variations occurred in opposite trends in inflorescences unbalanced in either hormonal or energy deficit abscission-inducing signals. Particularly concerning carbohydrates metabolism, sucrose, glucose, tricarboxylic acid metabolites and intermediates of the raffinose family oligosaccharides pathway were lower in shaded and higher in GAc samples. Altered oxidative stress remediation mechanisms and indolacetic acid (IAA) concentration were identified as abscission signatures common to both stimuli. According to the global analysis performed, we report that grape flower abscission mechanisms triggered by GAc application and C-starvation are not based on the same metabolic pathways.

Keywords: abscission, gibberellic acid, grapevine, inflorescence, metabolomics, shade, thinning

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CW, cell wall; DAB, days after bloom; E, transpiration rate; GAc, gibberellic acid; gs, stomatal conductance; IAA, indolacetic acid; PAs, polyamines; PAL, phenylalanine; Pn, net photosynthetic rate; ROS, reactive oxygen species; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; TCA, tricarboxylic acid.

Introduction

Abscission is the process by which vegetative and reproductive organs can be detached from plants as an effect of developmental, hormonal, and environmental cues. Abscission control is regarded as an important agricultural concern because it affects production yield and quality. In fruit species, fruit set is often excessive and the natural drop, which enables the plant to self-regulate its load, is not sufficient to satisfy fresh market quality standards (Bonghi et al., 2000). Fruit thinning is a common practice, particularly in table grape production, in which the reduction of berry number per bunch is mandatory to guarantee improved bunch appearance and quality and decreased diseases incidence (Dokoozlian and Peacock, 2001). Excluding labor-demanding manual thinning, the most common thinning method for table grapes uses chemical treatments with GAc sprays at bloom to induce flower abscission. The effectiveness of this practice is known to vary due to both internal (such as cultivar, phenologic stage, physiological condition, and age) and external (such as nutrient availability, irrigation, temperature, irradiation, and humidity) conditions (Weaver et al., 1962; Hopping, 1976; Looney and Wood, 1977; Dokoozlian, 1998; Dokoozlian and Peacock, 2001; Reynolds and Savigny, 2004; Reynolds et al., 2006; Hed et al., 2011). Gibberellins participate in biological processes such as cell elongation, dormancy breaking, parthenocarpy induction, and seed germination (Yamaguchi, 2008; Davière and Achard, 2013). However, despite the widespread use of GAc spraying, the mechanisms by which GAc works as thinning agent are not fully understood. According to the photosynthates competition hypothesis (Dokoozlian, 1998), GAc stimulates general organ growing, inducing competition for nutrients between flowers and shoots and/or among flowers within the inflorescence, which leads to reductions in the amount of nutrients available for berry set and growth. Alternatively, GAc can be responsible for unbalancing hormone relative concentrations, in agreement with the hormone balance hypothesis (Dokoozlian, 1998). Auxins are known to regulate gibberellin endogenous levels (Yamaguchi, 2008). A flow of inhibitory auxin in an organ destined to abscise prevents cell separation until its endogenous levels drop, de-repressing ethylene, which then activates the transcription of CW disassembly related genes (Else et al., 2004; Dal Cin et al., 2005). The effect of shade imposition to promote berry set reduction has been first investigated by Roubelakis and Kliewer (1976) and Ferree et al. (2001). The use of this practice as an alternative thinning method was successful also in other species, such as apple (Schneider, 1975; Byers et al., 1985, 1990, 1991; Corelli Grappadelli et al., 1990; Widmer et al., 2008; Zibordi et al., 2009; Basak, 2011) and involves intercepted light reduction during a short period of time after bloom. The pronounced reduction of net photosynthetic rate under shading promotes the competition for photoassimilates between vegetative and reproductive organs, leading to shedding of the later, which have less sink strength at this early stage of development (Vasconcelos et al., 2009). Hence, abscission stands as challenging biological question since it can be induced by agents that apparently act by promoting opposite changes to

the plant physiology. However, although the hormone and the assimilate theories may look contrasting, changes in assimilate availability may be the trigger required for changing hormone balances, leading to abscission. Moreover, sugars are more than an energy source as may also act as messengers operating in gene expression regulation or as signaling molecules (Lebon et al., 2008).

The mechanisms underlying organ abscission, were recently reviewed by Estornell et al. (2013), and involve signal peptides and specific receptors, mostly regulated by hormones, in which ethylene, ABA, and jasmonic acid act as accelerating signals. Conversely, auxin, gibberellins, PAs, and brassinosteroids act in abscission inhibiting signaling. The developmental phenomenon of physiological drop represents the natural reduction of fruit set and enables the plant to shed the weaker sinks, regulating the fruit load according to its capacity to produce the metabolic energy required to complete the development of reproductive and vegetative structures (Bonghi et al., 2000). Natural drop occurs after an increased ABA and ethylene production that induces a negative feedback in fruit development, as demonstrated in apple (Botton et al., 2011). In wine grapes, natural drop was showed to be related to lower sugar and PAs availability for developing flowers (Aziz, 2003; Lebon et al., 2004). Declines in the sugar supply at meiosis results in excessive flower abortion in this species (Lebon et al., 2008) which together with the expression of sucrose- or hexose-transporter genes (Davies et al., 1999), suggests a role for sugars in flower stress avoidance. Free-PA synthesis is also closely related to the onset of ovarian development and retards abscission (Aziz, 2003). Since PAs and ethylene share SAM as a common intermediate, SAM may be alternatively channeled toward the PA pathway, functioning as an alternative control. Free PAs fluctuate in parallel with sugars in the grape inflorescence, suggesting also a contribution in the modulation of their concentrations (Aziz, 2003).

Changes on the biochemical and transcriptome profiles during flower and fruit abscission triggered by growth regulators (Whitelaw et al., 2002; Dal Cin et al., 2005, 2009; Li and Yuan, 2008; Meir et al., 2010; Botton et al., 2011; Giulia et al., 2013; Peng et al., 2013; Wang et al., 2013) or by low light conditions (Aziz, 2003; Zhou et al., 2008; Li et al., 2013) have been studied in several species such as tomato (*Solanum lycopersicum*), apple (*Malus domestica*), lychee (*Litchi chinensis*), or wine grapes aiming at understanding the effect caused by chemical/environmental perturbations. The above-cited studies revealed a coordinated response of hormones, ROS, sugar metabolism, and signaling pathways to determine the downstream activation of abscission which includes increased activity of CW-modifying enzymes. Nonetheless, to our knowledge, only one publication to date (Zhu et al., 2011) reports a direct comparison of the mechanisms underlying abscission triggered by two distinct cues. The authors compared naphthaleneacetic acid (NAA) and shading treatments in inducing abscission in apple, through transcriptome analysis, and observed shared pathways involving reduction of photosynthesis, carbon transport and signaling, and hormone crosstalk. The aim of the present study was to provide a first global approach for understanding the

changes occurring in vine inflorescences and canopy that explain flower abscission in *Vitis vinifera* L. (Black Magic table grape cultivar), triggered by two contrasting abscission-inducing treatments (shade and GAc spraying) under conditions that leading to similar berry shed rates. The goal was to search for specificities and common links in metabolic pathways that control abscission.

Materials and Methods

Experimental Conditions and Design

The trials were carried out in a greenhouse in the south of Sicily, in a soilless table grape commercial production system (Di Lorenzo et al., 2014) of 'Black Magic' vines (*V. vinifera* L.) own-rooted in 2010 (Figure 1A). Black Magic is a very early seeded table grape cultivar, with high fertility and yield, released by the National Research Institute of Grape and Wine in Chisinau, Moldova. The assays were performed during the late production cycle of 2011 and the early production cycle of 2012. Plants were spaced 1.60 m between lines \times 0.40 m between plants, trained as unilateral cordon pruned with six buds and managed following integrated fertilization, irrigation, and pest-management practices (Di Lorenzo et al., 2009). The number of fertirrigations ranged between 5 and 20, judged by monitoring microclimate conditions. Nutritive solutions had the composition of 3, 1.25, 0.5, 0.65, 0.75, 0.5, 1.25, 7, 0.75, 2, and 0.5 mM of Ca, Mg, Na, K, NH₄, Si, P, NO₃, HCO₃/CO₃, SO₄, and Cl, respectively, the pH was 5.0 and the electrical conductivity was maintained between 1.5 and 2 ms cm⁻¹. Treatments were: (i) reduction of intercepted light and (ii) chemical thinning with GAc, both established at bloom (50% cap fall, stage 65 of the BBCH scale Lorenz et al., 1994). Shade treatments entailed covering the vines with green polypropylene 90% nets (Seroplast, Italy) for a period of 12 days. Chemical treatment consisted in spraying GAc (Gibberellin 1.8% GA₃, Gobbi, Italy) at 15 ppm concentration. A group that remained untreated was included as control. Experiments were designed in three randomized blocks by treatment with 14 vines each, using single vines as replicates. Climate conditions were monitored above the canopy of shaded and control vines (WatchDog MicroStation, Spectrum Tech., USA; Supplementary Figure S1).

Late Production Cycle

Plants were kept stored cold until June 2011 and the experiments started at 3rd July. The 50% cap fall stage (bloom) occurred after 34 days and harvest was carried out 67 DAB. This production cycle lasted a total of 101 days. The day (7 a.m. to 7 p.m.)/night (7 p.m. to 7 a.m.) mean temperatures registered were 32/23°C, relative humidity was 41/64% and PAR was 5/504 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Early Production Cycle

The experiments were conducted in 2012 using the same plants as in the previous year. The early production cycle started at 9 February, 50% cap fall stage occurred after 53 days and grapes were harvested 77 DAB. This cycle lasted a total of 130 days. The recorded day/night mean temperatures were 26/14°C, relative humidity was 45/79% and PAR was 17/566 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Vine Physiology Monitoring

Flower and berry drop were monitored by positioning non-woven cloth bags around bunches at 50% cap fall after the imposition of each treatment, to collect the shed flowers (Figures 1B,C). Flowers were collected and counted, 2, 4, and 12 DAB in 10 bunches per treatment. Bunches were selected taking uniformity of bloom in account. At harvest, the same bunches were collected and the final number of berries was recorded to calculate the cumulative and daily rate berry drop percentages. Net Pn, E and gs were measured in the morning period (9:00–11:00 a.m.) using a portable infrared gas analyzer (CIRAS, PPsystems, UK) on 12 mature leaves from the central part of the shoot, twice during the shade period (at 3 and 10 DAB). Shoot length, estimated leaf chlorophyll content (SPAD-502 m, Minolta, Japan) and total (sum of primary and secondary) leaf area (WinDIAS leaf area measurement system, Delta-T Devices, UK) were determined 12 DAB, before removal of the shade nets, in nine shoots per treatment.

Metabolic Analysis

Quantification of Target Metabolites

Sugar (glucose, sucrose, fructose, and stachyose), free PA (putrescine, spermine, spermidine, and cadaverine) and hormone (IAA and ABA) contents extracted from inflorescence samples collected in the late cycle, 1, 3, and 4 DAB were quantified



FIGURE 1 | Aspect of the experimental table grape vines (Black Magic cv) growing in greenhouse conditions (A), monitoring of flowers drop (B) and flowers detached from the base of flower pedicel (C) after abscission-inducing treatments application.

by high performance liquid chromatography (HPLC), aiming at determining the metabolic changes explaining flower abscission. The biochemical analyses were conducted using liquid nitrogen frozen powdered samples of whole inflorescences deprived from the rachis. Samples for soluble sugars quantification (100 mg) were extracted according to Damesin and Lelarge (2003), and samples were injected into a HPLC (Beckman Coulter, USA) and separated on a Sugar-Pak I column I (300×6.5 mm, Waters) at 90°C under a $122 \mu\text{M}$ EDTA-Ca solution and a flow rate of 0.5 ml min^{-1} . Peaks were detected by RI (Refractive Index Detector 2414, Waters). Free PAs were quantified according to Smith and Davies (1987) with modifications. Samples (100 mg) were mixed with $300 \mu\text{L}$ of a 5% perchloric acid solution, kept for 50 min in ice and centrifuged for 20 min at 20000 g at 4°C . Saturated Na_2CO_3 ($200 \mu\text{l}$) and dansyl chloride ($400 \mu\text{L}$, 5 mg ml^{-1} in acetone) were added to $100 \mu\text{l}$ of the supernatant, and mixtures were incubated in the dark at 60°C for 1 h. Proline (10 mg) was then added and further incubated for 30 min. PAs were extracted with $500 \mu\text{l}$ of toluene, the organic phase was dried under nitrogen and the residue was dissolved in $300 \mu\text{l}$ acetonitrile. The resulting samples were injected into the HPLC (Ultimate 3000, Dionex, Sunnyville, CA, USA), eluted through a C18 column (particle size $5 \mu\text{m}$, 4.6×150 mm, Thermo Scientific) at a flow rate of 1 ml min^{-1} with a mobile phase consisting of 10% acetonitrile solution, pH 3.5 (solvent A) and acetonitrile (solvent B) using a 60 to 90% of solvent A gradient, during 23 min. Peaks were detected with a diode array detector (DAD) at 346 nm . IAA and ABA were extracted according to Kelen et al. (2004) with modifications. Samples (200 mg) were extracted with $600 \mu\text{L}$ of 70% methanol and incubated at 4°C overnight. The extraction was repeated twice and the methanol evaporated under vacuum. 0.1 M phosphate buffer ($800 \mu\text{l}$) was added to the aqueous phase and partitioned with $300 \mu\text{l}$ of ethyl acetate three times. After ethyl acetate removal, the pH was adjusted to 2.5 with 1 N HCl. The solution was further partitioned three times with $450 \mu\text{l}$ of diethyl ether, passed through anhydrous sodium sulfate, evaporated at 50°C under vacuum and the residue was dissolved in $100 \mu\text{l}$ of methanol. Aliquots were injected into the HPLC (Ultimate 3000, Dionex, Sunnyville, CA, USA), eluted through a C18 column (particle size $5 \mu\text{m}$, 4.6×150 mm, Thermo Scientific) under a 30 mM phosphoric acid solution with 26% acetonitrile at 4 pH during 30 min at 0.8 ml min^{-1} and the peaks were detected with a DAD at 208 and 265 nm . In all cases, extractions were done in duplicate readings, each from three biological replicates per treatment. Standards for peak identification were purchased from Sigma-Aldrich®.

Global Metabolomic Profile

Sample points for metabolomic analysis were chosen based on the significant changes observed after target chromatography quantifications. Therefore, samples from three biological replicates (200 mg) of GAc-, shaded-treated and control inflorescences collected at 4 DAB in the late production cycle, were lyophilized, methanol extracted, and analyzed using the integrated platform developed by Metabolon® (Durham, USA)

consisting of a combination of three independent approaches: (1) ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS2) optimized for basic species, (2) UHLC/MS/MS2 optimized for acidic species, and (3) gas chromatography/mass spectrometry (GC/MS). Methods were followed as previously described (Evans et al., 2009; Ohta et al., 2009).

Evaluation of Productivity and Berry Quality Attributes

The final number of shot berries (parthenocarpic small berries that remain green at harvest) and regular-sized berries, bunch weight, rachis length and weight, bunch compactness (ratio between total number of berries and length of the rachis) and yield per plant (product of the bunch weight by number of bunches per plant) were recorded and calculated at harvest in the same 10 bunches per treatment used for flower drop monitoring. Ten berries per bunch were randomly selected to measure berries weight and diameter. The remaining berries were distributed in three samples per treatment to measure total soluble solids (TSS; in °Brix using a PR-32 refractometer, Atago, Japan) and titratable acidity (TA; by potentiometric titration with 0.1 N NaOH up to pH 8.1).

Data Imputation and Statistical Analysis

To access the significance of the differences observed between treatments and production cycles, variance analysis (one- and two-way ANOVA) and *post hoc* (Tukey's HSD with $\alpha = 0.05$) tests were conducted using Statistix 9 (Analytical Software, Tallahassee, FL, USA). To improve adjustment to the normal distribution, percentage values were arcsin sqrt(x) transformed and values concerning number of berries were square-root transformed. For global metabolomic analyses, raw area counts for each biochemical were rescaled by dividing each sample's value by the median value for the specific metabolite. Following \log_2 transformations, statistical analysis of the data was performed using Array Studio (Omicsoft). In order to visualize the results, a heat map was generated to show fold change (FC) defined as the \log_2 of the means ratio of each treatment and control for each compound (Supplementary Figure S2). Welch's two-sample *t*-tests were used to determine whether each metabolite had significantly increased or decreased in abundance. False discovery rates (FDRs) were calculated as *q*-values according to Storey and Tibshirani (2003) to account for the large number of tests. Metabolites that significantly changed in response to at least one of the imposed treatments were used to conduct correlation matrix-based principal component analysis (PCA) and hierarchical clustering. Dendograms associated with the heatmap and approximately unbiased and bootstrap probability *P*-values were computed using pvclust version 1.3.2 (Suzuki and Shimodaira, 2006) with the UPGMA method and 1000 bootstrap replications. Box plots were generated for those compounds that showed a significant increase or decrease using both the Welch two-sample *t*-test, FDR (i.e., $P < 0.05$ and $q < 0.10$) significance values and $|FC| \geq 1$. Mapping of named metabolites was performed onto general biochemical pathways, provided in the Kyoto Encyclopedia of

Genes and Genomes (KEGG¹) and Plant Metabolic Network (PMN²).

Results

Effect of GAc and Shade on Flower Abscission

The purpose of the treatments was to induce flower abscission, triggered by two distinct stimuli, with distinct physiological basis. In the late production cycle, both shade and GAc treatments resulted in higher cumulative percentages of berry drop (95.9% in the shade and 94.3% in the GAc treatment) comparing to the natural drop values observed in control bunches (81.0%; Table 1). Similarly, the average daily number of berries drop was highest in the shade treatment (115 ± 20 berries dropped per bunch per day), followed by GAc (62 ± 14 berries dropped per bunch per day) and lower in the control (28 ± 4 berries dropped per bunch per day) between 2 and 4 DAB (Figure 2A). In the early production cycle, shade imposition was the treatment that promoted the highest percentage of berries drop (49.4%; Table 1). This effect was reflected by an average higher daily number of dropped berries during 2–4 and 4–12 DAB intervals (13 ± 5 and 104 ± 26 berries dropped per bunch per day, respectively),

when compared to control (1 ± 0.5 and 29 ± 10 berries dropped per bunch per day, respectively) and GAc treatments (0.3 ± 0.2 and 10 ± 3 berries dropped per bunch per day, respectively; Figure 2B). Based on these results, the metabolic composition of samples collected in the late cycle, treated with hormonal and light stress abscission-inducing signals, was analyzed.

Impacts on Vine Physiology

Natural flower drop was significantly affected by environmental factors, exerting a significant effect on fruit set (Table 1). A higher drop rate occurred in the late production cycle (81%) when compared to the early cycle (16.9%). Comparing shaded with unshaded conditions, a 90% PAR reduction was observed, while no significant differences in temperature and relative humidity were perceived (Supplementary Figure S1). On clear sunny day conditions, the 90%-interception shade cloth provided approximately a maximum PAR of 157 and $170 \mu\text{mol m}^{-2} \text{s}^{-1}$ in late and early cycles, respectively, which demonstrates the strong net Pn reduction achieved under shaded conditions, in the magnitudes of 90 and 99%, in the late and early cycle, respectively. Transpiration rate (E; not shown) and gs decreased under shade, only during the early production cycle, by 23 and 54%, respectively, when compared to controls (Table 1). No differences in shoot length and total leaf area were observed between treatments. Nevertheless, in

¹www.genome.jp/kegg/

²www.plantcyc.org/

TABLE 1 | Effect of shade and GAc treatments on the average percentage of flower drop, total leaf area, and estimated leaf chlorophyll content at 12 DAB, on net photosynthetic rate (Pn) and gs during the shade period in 'Black Magic' vines in late and early cycles.

Production cycle	Treatment	Cumulative flower drop (%)	Leaf area ($\text{m}^2 \text{ vine}^{-1}$)	Shoot length (cm)	Leaf chlorophyll content (spad units)	Pn ($\mu\text{mol m}^{-2} \text{s}^{-1}$)	gs ($\text{mol m}^{-2} \text{s}^{-1}$)
Late	Control	81.0 b	0.52	79.4	30.5	2.72 a	227.57
	GAc	94.3 a	0.62	96.3	30.3	2.12 a	251.81
	Shade	95.9 a	0.58	86.8	31.7	0.26 b	153.4
Early	Control	16.9 b	1.86	174.6	28.7 ab	3.23 a	576.96 a
	GAc	5.4 b	1.83	183.4	28.1 b	3.18 a	613.69 a
	Shade	49.4 a	1.92	158.7	31.2 a	0.04 b	268.89 b

Within each column, different letters indicate significant differences ($P < 0.05$) among treatments, independently in each production cycle, according to Tukey's HSD test.

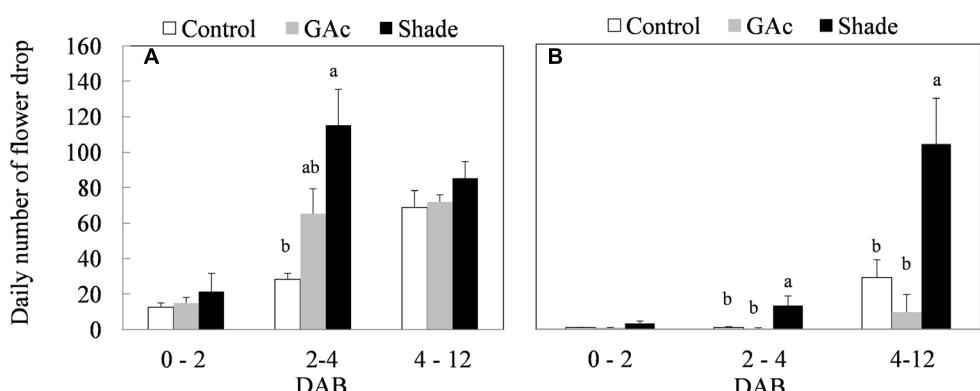


FIGURE 2 | Average daily number of flower drop in summer (A) and spring (B) production cycles as effect of GAc and shade treatments on 'Black Magic' vines (mean \pm SE). Within each sampling date, different letters indicate statistically significant differences ($P < 0.05$).

TABLE 2 | Effect of shade and GAc treatments on bunch and berries characteristics at harvest in Black Magic table grape cultivar in the late and early cycles.

Production cycle	Treatment	Yield (kg plant ⁻¹)	Bunch weight (g)	No berries	No shot berries	Rachis length (cm)	Rachis weight (g)
Late	Control	1.9 a	315.9 a	96.8 a	22.3	15.0 ab	7.7 b
	GAc	1.1 b	193.2 b	62.1 b	29.1	17.4 a	10.4 a
	Shade	0.9 b	148.3 b	46.2 b	14.3	12.0 b	4.2 c
Early	Control	8.9 a	879.8 a	173.0 a	188.3 b	24.1 a	12.9 a
	GAc	5.6 b	555.0 b	105.5 b	407.1 a	23.2 a	10.5 ab
	Shade	5.7 b	562.3 b	93.4 b	117.6 b	20.2 b	7.8 b
		Bunch compactness	Berry diameter (cm)	Berry weight (g)	TSS (°Brix)	TA (g L ⁻¹)	
Late	Control	8.0 a	14.1 a	3.83 a	12.5 b	5.7	
	GAc	6.1 ab	13.2 c	3.47 b	14.1 ab	5.1	
	Shade	5.1 b	13.7 b	3.36 b	15.5 a	5.4	
Early	Control	15.5 b	17.2 ab	5.15 c	13.9	3.8	
	GAc	22.1 a	16.6 b	5.18 b	14.3	4.7	
	Shade	10.5 c	17.8 a	5.78 a	15.8	3.8	

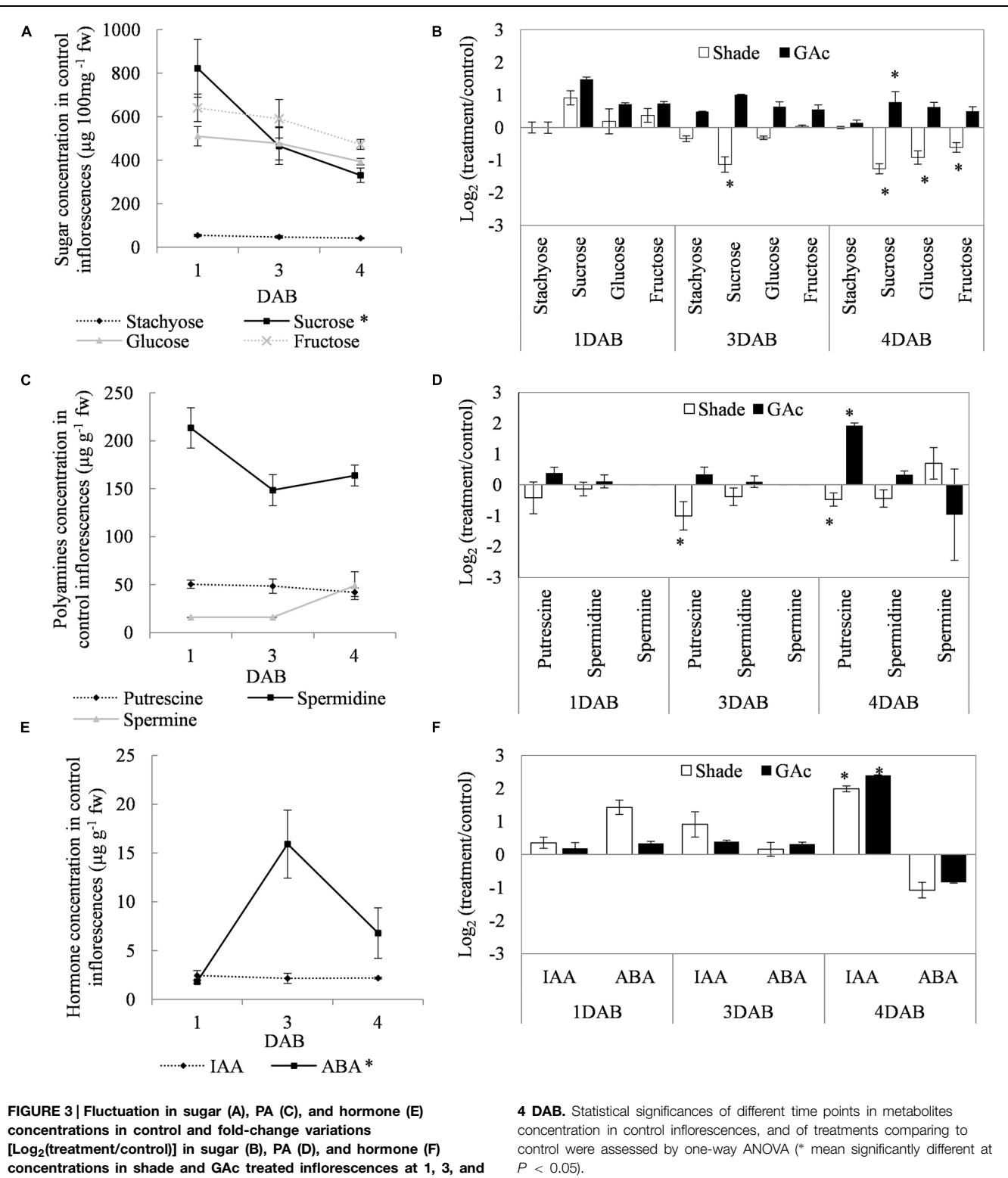
Values represent the average of the appropriate number of replicates. Within each column, different letters indicate significant differences ($P < 0.05$) among treatments individually in each production cycle according to Tukey's HSD test.

the early cycle, a higher estimated leaf chlorophyll content was perceived in shaded plants (31.2 spad units) when compared with plants treated with GAc (28.1 spad units; **Table 1**). Production cycles and the interaction production cycles \times treatment were statistically significantly different regarding cumulative flower drop and gs ($P < 0.01$). Production cycle also affected leaf area, shoot length and leaf chlorophyll content ($P < 0.01$; **Table 1**), impacting final bunch morphology and berry quality (**Table 2**).

Impacts on Metabolite Content

Regarding the metabolites analyzed in inflorescences sampled from untreated vines 1, 3, and 4 DAB during the late cycle, the results showed reduced sucrose levels between 1 and 4 DAB (**Figure 3A**) and increased ABA concentrations, peaking at 3 DAB (**Figure 3E**). Conversely, compared to the control, in shade-treated inflorescences, sucrose concentration decreased at 3 and 4 DAB and fructose and glucose at 4 DAB. In GAc-treated inflorescences, sucrose concentration was highest at 4 DAB (**Figure 3B**). A significant increase of putrescine content was also observed in the same samples, 4 DAB. In samples submitted to the shade treatment, this PA decreased 3 and 4 DAB (**Figure 3D**). Cadaverine was not detected. Concerning hormones, IAA concentration was significantly increased in result of both treatments 4 DAB and no differences in ABA levels were observed between treated inflorescences and controls (**Figure 3F**). From the 215 metabolites investigated by the global metabolic analyses conducted in samples collected 4 DAB, a total of 211 were detected (Supplementary Figure S2) and 48 showed to be differentially changed in abundance ($P < 0.05$) in inflorescences induced for abscission. A total of 34 and 23 metabolites showed differential abundance in shade and GAc treatments, respectively, of which 9 metabolites were

common in the different treatments (**Table 3**). Hierarchical clustering (**Figure 4A**) showed the association between samples according to the metabolite profile. Samples resulting from each treatment were significantly clustered together. Oleonate, the only metabolite that highly decreased with GAc treatments (FC = -2) was separated from the other metabolites. Raffinose, sucrose and benzoyl-O-glucose, showed a distinct pattern according to the imposed treatment, and were grouped in a different cluster. PCA (**Figure 4B**) showed that all samples could be separated according to the treatment to each they were submitted to. The first component allows distinguishing inflorescences developing under shade from all the other samples. GAc samples were separated from controls by the second component. Differentially quantified metabolites were mapped onto general biochemical pathways, and categorized into functional classes as showed in **Figure 5**. Among the 34 metabolites significantly altered in abundance in shaded inflorescences, those assigned to carbohydrates composed the most prevalent class (38%), followed by products of secondary metabolism (26%), amino acid (15%), nucleotide (9%), peptide (7%), cofactors (3%), and lipids (3%). Among the 23 metabolites that significantly changed in response to GAc, products from carbohydrate metabolism was also the most prevalent class (52%), followed by amino acid (18%), secondary metabolism (13%), nucleotide (9%), cofactor (4%), and hormone (4%). A list of all metabolites significantly affected by GAc and shade treatments ($P < 0.05$), assigned functional categories, KEGG compound number and respective fold-change is provided in **Table 3**. Shade and GAc treatments were responsible for a decreased concentration of 24 and four metabolites, respectively, sharing two metabolites derived from the carbohydrate pathway, namely myo-inositol tetrakisphosphate and erythrulose. On the opposite trend, the imposed treatments induced increased concentration of 10 and 19 metabolites, in shade and GAc,



4 DAB. Statistical significances of different time points in metabolites concentration in control inflorescences, and of treatments comparing to control were assessed by one-way ANOVA (* mean significantly different at $P < 0.05$).

respectively. N6-carbamoylthreonyladenosine, a metabolite from the nucleotide class, was common to both sample sets. Six metabolites concurrently increased in response to GAc and decreased under shade. Four were derived from

carbohydrates metabolism, namely sucrose, glucose, raffinose, and malate and the other two were derived from secondary metabolism, and included benzyl alcohol and benzyl-O-glucose. Regarding amino acid pathway, decreased quinate,

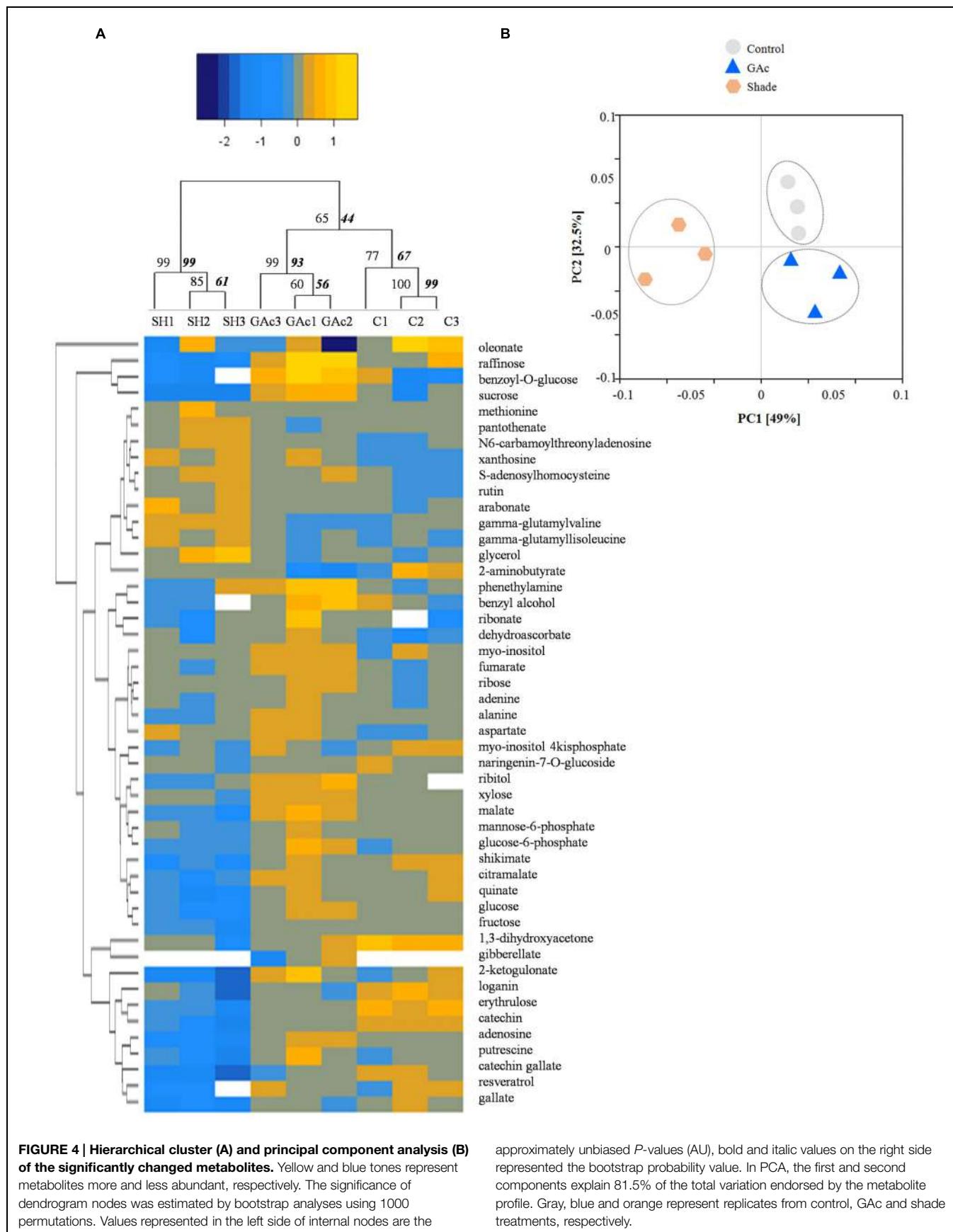
TABLE 3 | List of metabolites significantly affected by GAc and shade treatments ($P < 0.05$), functional categories, KEGG compound number and respective fold-change.

Super pathway	Compound	KEGG	\log_2 (GA _c /control)	\log_2 (shade/control)
Amino acid	2-Aminobutyrate	C02261	-1.0	
	Phenethylamine	C02455	1.6	
	Quinate	C00296		-1.1
	Shikimate	C00493		-1.2
	Putrescine	C00134		-1.3
	Alanine	C00041	0.5	
	Aspartate	C00049	0.6	
	Methionine	C00073		0.7
	S-adenosylhomocysteine (SAH)	C00021		0.6
	2-Ketogulonate	C02261		-1.8
Carbohydrate	Ribonate		1.3	
	Raffinose	C00492	1.4	-1.6
	Glucose	C00031	0.3	-0.9
	Glucose-6-phosphate (G6P)	C00668		-0.5
	Fumarate	C00122	0.8	
	Malate	C00149	0.7	-0.8
	Arabonate	C00878		0.8
	Ribitol	C00474	0.8	
	Xylose	C00181	0.5	
	Ribose	C00121	0.5	
	Sucrose	C00089	1.3	-1.7
	Erythrulose	C02045	-0.8	-1.6
	Fructose	C00095		-0.8
	Mannose-6-phosphate	C00275		-0.5
	Citramalate	C00815		-0.8
	1,3-Dihydroxyacetone	C00184		-1.5
	Myo-inositol	C00137	0.5	
	Myo-inositol 4-kisphosphate (1,3,4,6/3,4,5,6/ 1,3,4,5)	C01272	-0.7	-1.1
Lipids	Glycerol	C00116		1.3
Coenzyme	Dehydroascorbate	C05422	1.0	
Nucleotide	Pantothenate	C00864		0.7
	Adenosine	C00212		-1.3
	Adenine	C00147	0.5	
Hormone	N6-carbamoylthreonyladenosine		0.4	0.9
	Xanthosine			0.8
	Gibberellate	C01699	3.3	
Peptide	Gamma-glutamylisoleucine			0.7
	Gamma-glutamylvaline			0.7
Secondary metabolism	Oleanolate		-2.0	
	Benzyl alcohol	C00556	1.1	-0.8
	Benzoyl-O-glucose		2.0	-1.4
	Catechin	C06562		-1.6
	Naringenin-7-O-glucoside			-0.5
	Rutin	C05625		0.4
	Catechin gallate			-2.3
	Gallate	C01424		-1.7
	Resveratrol	C01424		-1.9
	Loganin	C01433		-1.5

Bold letters correspond to the highly significant different metabolites $FC(|\log_2(\text{treatment/control})|) \geq 1$.

shikimate, and putrescine concentrations and increased metabolites derived from aspartate family (methionine and SAH) were observed in shade-derived samples. In the GAc

treated samples, an increase of phenethylamine, aspartate, and alanine and a decrease of 2-aminobutyrate occurred. All metabolites from the carbohydrate pathway were reduced



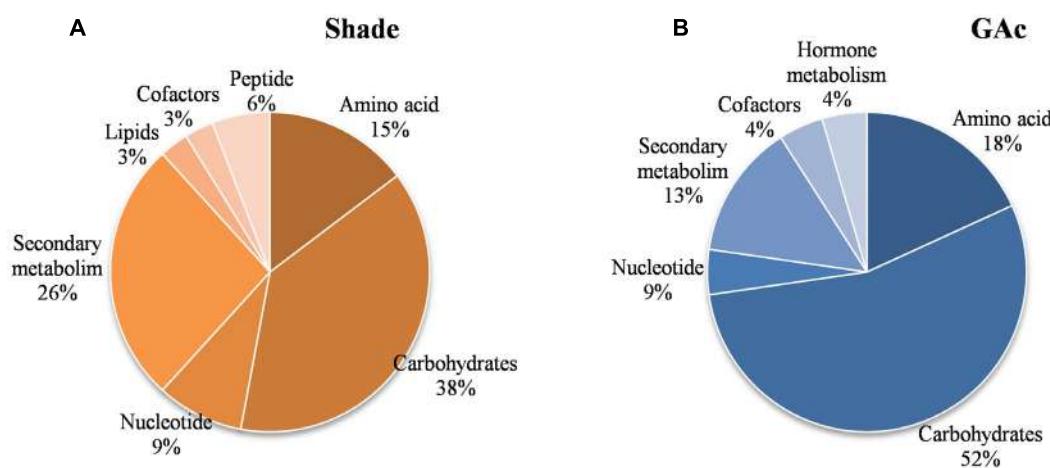


FIGURE 5 | Functional categorization of the 48 metabolites that showed significantly changes ($P < 0.05$) in abundance. In shaded inflorescences (A), 34 metabolites from carbohydrate, secondary metabolism, amino acid, nucleotide, peptide, cofactor, and lipid functional pathways were significantly

affected. In GAc-treated inflorescences (B), 19 metabolite contents from carbohydrate, amino acid, secondary metabolism, hormone, cofactor, and nucleotide pathways changed. Nine metabolites were changed in both the treatments.

in shaded inflorescences except arabinose. Conversely, in GAc-treated samples, all metabolites increased except myoinositol tetrakisphosphate and erythrulose. Glycerol, a product from the lipids metabolism, highly increased in result of shade conditions. Gibberelate was detected only in the GAc-derived samples, probably as the result of the exogenous application. Several metabolites from coenzyme and nucleotide metabolisms increased in both treatments except adenosine that was reduced in shade. Likewise, gamma-glutamylisoleucine (gamma-glu-lleu) and gamma-glutamylvaline (gamma-glu-val), from peptides metabolism, increased in the shade treatment. The concentration of metabolites derived from secondary metabolism was reduced in both treatments except two aromatic benzenoids (benzyl alcohol and benzyl-O-glucose) that increased in the GAc treatment and rutin that was increased in the shaded samples. Focusing on the metabolites with more pronounced changes [$FC (|\log_2 (\text{treatment/control})|) \geq 1$], it was observed that raffinose, inositol, glycolysis, TCA cycle, shikimate, PAL and PA pathways were involved in the changes that occurred in inflorescences treated to enhance abscission rates (Figure 6). Sucrose and raffinose amounts changed in opposite directions in shade and GAc treated inflorescences, and a down- and up-regulation of the raffinose family oligosaccharides (RFOs) pathway was found in shade and GAc, respectively. Inositol and metabolites from the shikimate pathway (quinate and shikimate) were reduced in shade. Erythrulose and 1,3-dihydroxyacetone derived from glucose and glyceraldehyde-3-P in the glycolysis pathway were also reduced in this treatment. Concerning compounds associated with the TCA cycle, 2-ketogulonate, derived from oxaloacetate, was reduced in shaded samples and 2-aminobutyrate, derived from α -ketoglutarate (via glutamate), was reduced in response to GAc spraying. PA metabolism, likewise derived from glutamate, was reduced in result of the shade treatment. Compounds from benzenoids family increased in GAc treated inflorescences

whereas oleanolate was decreased. Flavonoids (catechin and catechin gallate), phenylpropanoids (resveratrol and gallate), benzyl-O-glucose and loganin were reduced in response to shade.

Impacts on Bunch and Berry Quality

Shade and GAc treatments reduced yield per plant, bunch weight and number of regular sized berries, in both cycles, when compared to untreated vines (Table 2). In the late production cycle, no differences in shot berries number were observed while in the early cycle, GAc promoted a higher number of these berries (407.1 shot berries), which was reflected in the increased number of total berries (512.6 berries per bunch) measured. Rachis length was shorter in bunches from vines submitted to shade and GAc, in the early production cycle. Nevertheless, bunch compactness was lower in plants that were shaded during flowering in both production cycles (5.1 and 10.5 berries cm^{-1}) and higher in GAc treated plants in the early production cycle (22.1 berries cm^{-1}) when compared with control. Rachis weight was lower in both production cycles in bunches from shaded vines, and was higher in GAc treated bunches in late cycle. Regarding berry quality parameters, the weight and transversal diameter of the berries were reduced in grapes from GAc treated and shaded vines in late cycle, when compared with controls. In the early cycle, no significant differences were observed in berry diameter but shade lead to increased berry weight. Berry TSS content was higher in shaded vines comparing to the control in the late cycle, while differences in titratable acidity were not observed (Table 2). Both production cycle and the interaction production cycles \times treatment significantly affected yield per plant, number of berries, number of shot berries, bunch compactness and berry diameter and berry weight ($P < 0.05$). Production cycle also affected average bunch weight and titratable acidity ($P < 0.001$).

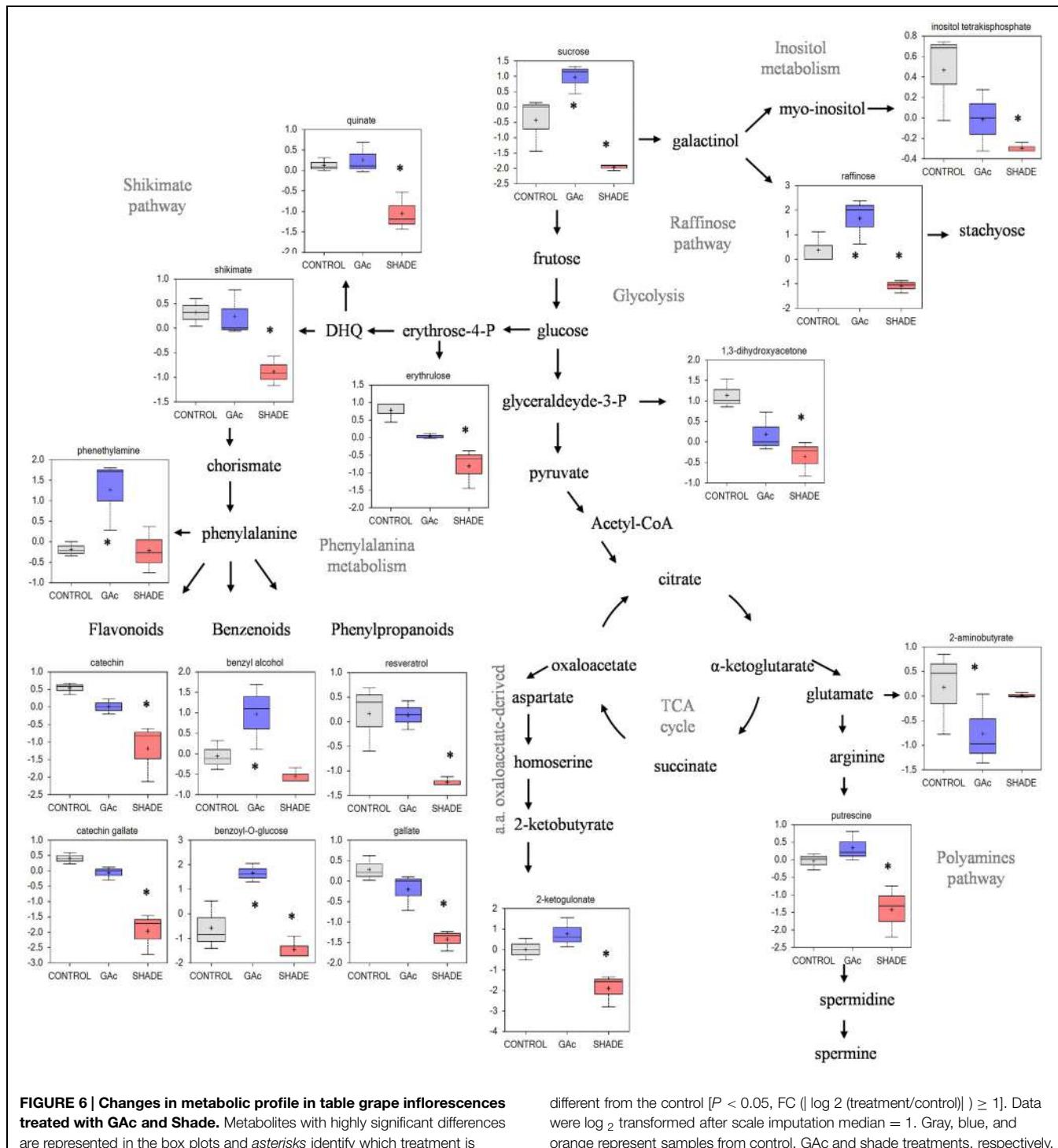


FIGURE 6 | Changes in metabolic profile in table grape inflorescences treated with GAc and Shade. Metabolites with highly significant differences are represented in the box plots and asterisks identify which treatment is

different from the control [$P < 0.05$, $\log_2 (\text{FC}) \geq 1$]. Data were \log_2 transformed after scale imputation median = 1. Gray, blue, and orange represent samples from control, GAc and shade treatments, respectively.

Discussion

Flower Abscission Induced by Hormonal and C-Starvation Stimuli

The direct comparison of the changes in *V. vinifera* L. inflorescences metabolite abundance that resulted from the imposition of two different abscission-triggering treatments

was possible due to controlled conditions allowed from the experimental model used. Using potted plants growing under soilless greenhouse conditions, it was possible to apply both treatments to homogenous biological material. Moreover, this system allowed achieving improved plant growth and grape productivity, extending the harvest schedule and, relevant to the objectives of this work, obtaining more than one

production cycle in the same agronomic year (Di Lorenzo et al., 2009).

The significant effect of climatic conditions on fruit set, revealed by the differences observed in natural flower drop rates between the two production cycles, can be explained by the influence exerted by the maximum temperatures registered during bloom in the late cycle that exceeded 35°C in the majority of the days during the bloom period (Supplementary Figure S1). Under these range of temperatures, fruit set is known to decrease due to reduction of ovule fertility (Kliener, 1977) and pollen germination rates (Vasconcelos et al., 2009).

The Black Magic table grape cultivar showed to be sensitive to shade imposed during bloom, resulting in increased flower drop percentages in both production cycles while the response to GAc application showed to be dependent of microclimate conditions. Under this treatment, fruit set was impaired in the late production cycle while an increase was observed in the early cycle, which agrees with previous results (Reynolds and Savigny, 2004; Reynolds et al., 2006) in 3-years trials. The significant reduction of fruit set induced by the 12-days period shading during bloom (**Table 1**) suggests that this approach can be exploited as an effective method for thinning in table grape production, relying on the pronounced decline of net photosynthetic rate, which promotes a decrease on carbon resources available to both vegetative and reproductive sinks and increases the competition between them (Corelli Grappadelli et al., 1990; Byers et al., 1991; Zibordi et al., 2009). The moment of shade imposition matched a stage during which the vine carbon reserves reached a minimum, which coincides with the onset of bloom in grapevines (Zapata et al., 2004). During this sensible period, interruptions or partial sugar supply declines are known to promote flower abortion (Lebon et al., 2008). In the present study, monitoring daily rate of berry drop during the shade imposition period enabled us to verify that the maximum rate of berries drop depends on the global environmental conditions, occurring 2–4 DAB in late cycle and between 4 and 12 DAB in early cycle, indicating precocity in C-shortage in the former cycle. Shading did not affect leaf area nor shoot growth, confirming previous observations that indicate that reproductive growth is more sensitive to environmental stress or limitation of resources than vegetative growth (Chiarello and Gulmon, 1991). The increased estimated leaf chlorophyll content in result from intercepted light reduction when compared with GAc treated vines, agrees with Ferree et al. (2001), and suggests an adaptability of the grapevine to low light intensity by increasing the PAR trapping efficiency (Cartechini and Palliotti, 1995).

The evidence that disturbances in growth regulators internal concentrations have an important influence on fruit set has been exploited in table grape production. In fact, GAc exogenous bloom application is commonly used as a mean to achieve cluster loosening (Dokoozlian and Peacock, 2001). Nonetheless, environment was demonstrated to play a major role in modulating the responses to growth regulator treatments, in particular the temperature. Low temperatures lead to sub-optimal response while, under high temperature conditions, the response may be excessive (Wertheim and Webster, 2005). Thus, we suggest that the observed differences on GAc effectiveness

to induce flower abscission and increase shot berries number was related to the environmental conditions and physiological stage of the vines. During the late production cycle, vines are developing under more intense stress conditions, and had a smaller leaf area and shoot length than in the early cycle. Plants are expected to have lower carbohydrates and endogenous GA levels, resulting in a higher sensitivity to exogenously applied GAc and a reduction of fruit set comparing to control. Sensitivity to exogenously applied GAc was reported to be inversely related with endogenous gibberellins levels (Boll et al., 2009).

Sugar Metabolism and Other Energy Sources

Sucrose, glucose, and fructose are the major phloem sap sugars which feed the developing vine inflorescences (Lebon et al., 2008). The reduction on the sucrose content in inflorescences developing under control conditions observed 4 DAB, at the onset of natural drop, agrees with previous observations (Glad et al., 1992) reporting that this sugar, predominant in this stage, represents 85% in sap flow at full bloom and declines thereafter to 60% at the end of fertilization, explaining natural drop. Our results were expected in confirming that decreased light intensity inhibits photosynthesis and sugar accumulation in inflorescences but showed that, in contrast, GAc treatment did not affect photosynthesis and even increased the inflorescence sugar content. Noticeably, both treatments resulted in similar rates of flower abscission (**Table 1** and **Figure 3**). Shade induced more pronounced effects than GAc spraying concerning the number of changed metabolites (**Table 3**). Essentially, all carbon metabolites identified showed to be present in lower amounts in shaded and in higher levels in GAc samples, including sucrose and glucose, as well as TCA intermediates (malate, citramalate, and fumarate), and intermediates of the RFO pathway, such as raffinose (**Table 3** and **Figure 6**). The decline of carbohydrate transport metabolism that occurred in shade agrees with abscission modulation induced by NAA and by shade in apple (Zhu et al., 2011). It was also verified that under shade, as in other stress conditions, the synthesis of glycerol may be favored via starch degradation, as an energy resource, and decrease of the carbon flow into TCA cycle (Xia et al., 2014). Regarding amino acid pathways, in shaded samples, the concentration of quinate, shikimate, and putrescine decreased while methionine and SAH increased (**Table 3** and **Figure 6**). In addition, adenosine, which plays an important role in biochemical processes as energy transfer [adenosine triphosphate and diphosphate (ATP and ADP) and in signal transduction cyclic adenosine monophosphate (cAMP)], also decreased. Shade conditions led to a signature of carbon/nitrogen (C/N) imbalance with lower energy and carbon metabolites, biosynthetic precursors such as shikimate and nitrogen-rich compounds associated with anabolic activity such as putrescine, and higher proteinogenic amino acid such as methionine that may result from protein turnover to free up amino acid carbon backbones for energy utilization. Likewise, the increased amount of pantheonate (vitamin B₅) whose biosynthetic pathway involves valine and alanine amino acids (Raman and Rathinasabapathi, 2004) observed in shade-derived inflorescences support the hypothesis of proteinogenic amino acids abundance from protein turnover. On the other hand, since all Calvin cycle metabolites

were present in lower amounts in shaded samples, the pathway of pantheonate functioning as CoA biosynthesis precursor needs a more detailed evaluation. Our results are in accordance with Baena-González and Sheen (2008) which review physiological and molecular responses associated with plant energy deficit, including activation of catabolic pathways to provide alternative nutrient, metabolite and energy sources, and a decline in the activity of biosynthetic enzymes to preserve energy, and with Aziz (2003) showing that shading the vines at full bloom causes a decrease in both sugars and free PAs and leads to a substantial increase of abscission.

Gibberellins are involved in pathways of regulation of flowering and fruit-set in grapes, as active GAs, mainly GA₁, peaks at anthesis and decrease thereafter (Perez et al., 2000; Giacomelli et al., 2013). GAc is commonly applied during bloom to reduce fruit set but the molecular mechanisms underlying this process are largely unknown. In *Arabidopsis thaliana*, GAc induces increased 3-P-glycerate and promotes plant growth rate (Meyer et al., 2007; Ribeiro et al., 2012). In this study, GAc application led to generalized up-regulation of both primary (carbohydrates, amino acid, coenzyme, and nucleotide pathways) and secondary metabolisms (**Table 3** and **Figure 6**). Since no changes in photosynthetic rate (source) were detectable in samples submitted to this treatment, we hypothesize that an increase on inflorescences sink strength occurs after GAc treatments, resulting in the formation of king berries, with higher potential to compete for carbohydrates and other metabolites and higher growth rate, inhibiting the development and inducing abscission of later flowers. Regarding TCA cycle-derived metabolites, only 2-aminobutyrate from glutamate family decreased as a result of GAc while, on the other hand, metabolites derived from aromatic amino acid phenylalanine and from aspartate family (alanine and aspartate) showed the opposite trend (**Table 3** and **Figure 6**). Glutamate derives from α -ketoglutarate and can be involved in the biosynthesis of 2-aminobutyrate or, alternatively, in the biosynthesis of arginine and PAs biosynthesis. According to our results, it can be hypothesized that the pathway from glutamate to PAs is favored when vines are treated with GAc, in contrast with biosynthesis of 2-aminobutyrate.

Cell Wall Modifications

The recorded increase of CW monosaccharides in samples from both abscission-triggering treatments (**Table 3**) sounds with the known CW disassembly and remodeling processes that occur during pedicel AZ formation as part of the coordinated series of modifications that ultimately lead to CW loosening, cell separation and differentiation of a protective layer on the proximal side after organ detachment (Lee et al., 2008). The increased arabinose concentration, which is a metabolite derived from arabinose, as consequence of the shade treatment contrasts with the observations in GAc treated inflorescences where xylose was the increased monosaccharide (**Table 3**). These differences are likely to reflect differences on target CW polymers, with pectins and xyloglucans more affected by shade or GAc, respectively. Pectin changes depends on the type of substitutions and branches in their backbone and

are considered a central event (Fukuda et al., 2013) since the continuity between AZ cells is preserved by the middle lamellae, which is rich in this class of polymers, responsible for cell-cell adhesion. Pectins are additionally responsible for modulating the CW porosity and, in so, controlling the enzymes access to their substrates (Baron-Epel et al., 1988). Augmented arabinose levels may also indicate a higher substitution of pectic polysaccharides with arabinosyl residues which can work as plasticizers (Harholt et al., 2010) and be involved in the formation of the protective layer in the proximal area. In fact, during abscission, CWs of the proximal area are relatively richer in cellulose, arabinose-rich polymers and pectin, and poorer in xylan-rich polysaccharides and lignin when compared with AZ CWs (Lee et al., 2008). Regarding the detection of increased concentrations of xylose in samples from GAc-treated inflorescences, it may similarly reflect CW loosening processes needed for organ shed or CW strengthening requirements, but through action on cellulose-xyloglucan contact points. Xyloglucans are closely intertwined with cellulose at limited sites designed as “biomechanical hotspots,” promoting selective targets majorly modulating CW loosening (Park and Cosgrove, 2012). Our results confirm the putative role of xyloglucans in providing CW strength for attachment of organs and its dynamic metabolism in mediating abscission, in response to some triggering signals. These assumptions are further supported by gene expression assays since it has been demonstrated that the activation of the abscission molecular machinery involves alterations of genes encoding CW remodeling enzymes acting on structural polysaccharides leading to the middle lamellae breakdown, accompanied by distortion and dissolution of primary CWs along the abscission plane (Lashbrook and Cai, 2008; Lee et al., 2008; Agusti et al., 2009; Meir et al., 2010; Zhu et al., 2011; Peng et al., 2013; Wang et al., 2013) and glycosyl hydrolysis (Lashbrook and Cai, 2008; Singh et al., 2011, 2013). The pattern of differential temporal regulation of distinct classes of CW-related genes (Lashbrook and Cai, 2008) additionally suggests that the differences observed between treatments may be the result of triggering their action at different stages of the process. It should be noted that the samples here investigated include cells other than AZ. Hence, as CWs represent primarily communication between the plant and the environment, a role in adaptation to the imposed abiotic stress can be discussed. The observed difference in CW composition are known to be related to events such as localized cell division, arrestment of elongation and modifications in the differentiation status, to impact anatomy and development (Braidwood et al., 2013).

Markers of Oxidative Stress

Likewise, both abscission-triggering stimuli lead to oxidative stress related metabolism, but the results suggest that different pathways are tracked. Some of the significant increases observed are related to metabolites associated with oxygen stress remediation. Gamma-glutamyl amino acids, observed in shaded samples, are intermediates in the glutathione synthesis cycle (**Table 3**) and dehydroascorbate, observed in GAc treated samples, indicates responses to elevated oxidative

stress conditions related to the ROS scavenging coupled ascorbate/dehydroascorbate cycle (**Table 3**). During abscission a continuous increase of ROS production is known to occur. ROS role in abscission encompasses multiple steps of signaling (Sakamoto et al., 2008) associated with ROS-sugar-hormone cross talk (Botton et al., 2011) and ROS-mediated oxidative damage/cleavage on CW components leading to cell separation (Cohen et al., 2014). Regulation of excessive ROS by the free radical scavenging systems comprises essential enzymatic components and non-enzymatic molecules such as ascorbate and glutathione. Glutathione and ascorbate play important roles individually or through the ascorbate glutathione cycle, having specific functions besides interchangeable antioxidants (Bohnert and Sheveleva, 1998). Our results suggest that distinct metabolite-dependent responses are triggered by each treatment agreeing with the independence and interdependence of glutathione and ascorbate in peroxide metabolism model proposed by Foyer and Noctor (2011).

Hormone Regulation

The occurrence of an ABA peak 3 DAB in control inflorescences (**Figure 3E**) preceding the rise of natural flower drop (4–12 DAB; **Figure 2A**) is in accordance with previous works describing hormones as mediators of the AZ cell responses to abscission signals (Estornell et al., 2013). Interplay between a decrease of sugar, increases levels of ABA, ethylene, and ROS in organ predicted to abscise were verified, taking place before the onset of abscission (Botton et al., 2011). Our results confirm ABA as a component of the self-regulatory mechanism that adjusts fruit load to carbon supply occurring under natural conditions or following treatments (Gomez-Cadenas et al., 2000).

The increase of inflorescence IAA (auxin) concentration registered in both treatments (**Figure 3F**) may suggest that IAA was accumulated on the proximal side of abscission and the auxin flux to the distal organ predict to abscise was interrupted. It has been showed that a constant auxin transport through the AZ is needed to prevent abscission (Taylor and Whitelaw, 2001) and a auxin depletion linked with acquisition of ethylene sensitivity within AZ cells is needed to its induction (Meir et al., 2010; Basu et al., 2013). Our results are also consistent with the auxin gradient theory (Addicott et al., 1955) based on the evidence that auxin application in the proximal end of AZ explants accelerates abscission whereas when applied at the distal end delays it, and suggesting that changes in auxin gradients may act in signaling the onset of senescence and abscission. Ethylene and auxins are critical factors that regulate the onset of abscission (Basu et al., 2013) in a mechanism where the auxin depletion inside AZs and an altered expression of auxin-regulated genes induce the acquisition of sensitivity to ethylene and AZ activation. The increase of methionine and SAH, which are intermediates in the ethylene biosyntheses, observed in shaded-treated samples (**Table 3**) can be associated with the increase of ethylene, acting as a trigger in the abscission process (Meir et al., 2010). SAM, derived from methionine, is also the precursor of the spermidine and spermine biosynthesis pathway or alternatively can be used on the synthesis of ACC which is the immediate precursor of ethylene (Wang et al., 2002).

Secondary Metabolism

In shade, decreased loganin content, which is a monoterpenoid intermediate in the production of indole alkaloids, and several phenylpropanoids, benzenoids, and flavonoids was observed (**Table 3** and **Figure 6**), indicating suppression of biosynthesis of secondary metabolites and a slowdown of biochemical reactions in the AZ and neighboring tissues (Wang et al., 2013). This significant reduction can also mean an initial delay in fruit set and development under these conditions due to drastic reductions in carbon supply during this period, when compared to control samples. In this later situation, the accumulation of compounds characteristics of berry development, mainly in red and black varieties as ‘Black Magic,’ is known to be already started (Braidot et al., 2008). The decreased catechin can be also the result of the condensation of such flavanols, as observed after ethylene exogenous application (Rizzuti et al., 2015). Among the metabolites analyzed, flavonoid rutin was the exception in the general trade, showing a slightly increase in shade, probably due to its potential as strong radical scavenger and inhibitor of lipid peroxidation (Kumar and Pandey, 2013). On the other hand, GAc application led to a general advance in berry development in this stage and can have the opposite effect in ripening, depending on the cultivar (Teszlák et al., 2005). Comparing to the control, the aromatic compounds (benzenoids) showed increased accumulation in GAc treated samples (**Table 3**). Also in GAc, the decreased terpenoid oleonate levels measured suggests a reduction of steroids synthesis, which are membrane components that appears to control membrane fluidity and permeability and, in some plants, have a specific function in signal transduction (Piironen et al., 2000).

Final Development of Reproductive Structures

The treatments imposed to produce biological samples enriched in abscission signals affected final yield and quality in both production cycles and some implications can be ascertain with relevance for table grape production (**Table 2**). The reduction of the number of berries in the late production cycle in shade and GAc treatments lead to reductions of bunch weight and yield per plant, indicating that the two approaches were efficient in inducing abscission. However, in the late cycle, both treatments affected berry weigh and diameter in a detrimental way, which can be the result of a decreased seed number and weight (Reynolds et al., 2006). In early cycle, shade resulted in a successful thinning method reducing total berries number and improving berry weight and diameter. The shade treatment affected bunch characteristics, reducing rachis length and weight and still reducing the number of berries per centimeter of rachis, in both cycles. The observed effect on the rachis can result from competition for photoassimilates favoring vegetative growth in detriment of the development of reproductive organs (Chiarello and Gulmon, 1991). In the early cycle, GAc treatment showed to be ineffective as thinning method due to the increased number of shot berries, total berries number and bunch compactness. The high number of shot berries observed as a negative effect of GAc application was also described by Dokoozlian and Peacock (2001). Recently, in a study performed by Abu-Zahra and Salameh (2012) aimed at evaluating the impact of GAc spray

(50 ppm at the end of bloom, 18 and 40 days after end of bloom) on 'Black Magic' grape quality, an increase of berries number, berry size, TSS, titratable acidity, and decreased color intensity was observed. Although, according to Cartechini and Palliotti (1995), shade during flowering has no effect on final berries sugar content, under our conditions, the shade treatments increased TSS in the late production cycle, which can be a direct result of the reduction of berries weight and diameter.

A Mechanistic View of Flower Abscission

Control in *Vitis vinifera* L.

The analysis performed in the late cycle, when both treatments were efficient in inducing abscission, showed that GAc responses comprised a relatively low numbers of significant changes, while the shade treatment conducted to more dramatic physiological and metabolomic alterations (Table 3). These results allowed us to propose a mechanistic model to explain differences and common links for flower abscission determination in response to two stimuli (Figure 7). Comparing the composition in metabolites of grapevine inflorescences treated with the different abscission inducers (shade and GAc) and the control, we can conclude that abscission mechanisms triggered by hormonal application and via C-starvation are not based in the same pathways. A new insight on the mode of action of GAc during bloom is here provided, showing that it is based on a generally stimulation of cell metabolism and gene expression

revealed by ribose and its derived metabolites, sugar, amino acid and PA metabolisms in the whole inflorescence and a highly significant inhibition of a glutamate sub-pathway related with 2-aminobutyrate (Table 3), which can be a key step in the GAc metabolism in inflorescences at bloom stage and may participate in a cross talk between IAA and gibberellate. In other biological processes (Yamaguchi, 2008), it has been described that bioactive gibberellins and auxins can positively regulate flower abscission triggered by GAc spraying. On other hand, shade induced abscission through energy deprivation mechanisms showed by the decline of photosynthesis, carbon metabolism and biosynthetic activity. The increased accumulation of ethylene precursors suggests that these events may participate together with ethylene production (Table 3). The common markers of abscission were increased IAA concentration in inflorescences, which can be a result of an auxin gradient change through the AZ, and increases in oxidative stress marker metabolites agreeing with previous studies in other species (Estornell et al., 2013). Despite the grapevine economic value and scientific relevance as a model species, this study provides the first mechanistic view of the metabolomic changes responsible for the flower abscission regulation in this species (Figure 7), triggered by exogenous GAc application and reduction of the intercepted light, unraveling the complexity of its opposite effects and contributing to the advance in knowledge that will ultimately may lead to improved control of grapevine fruit set.

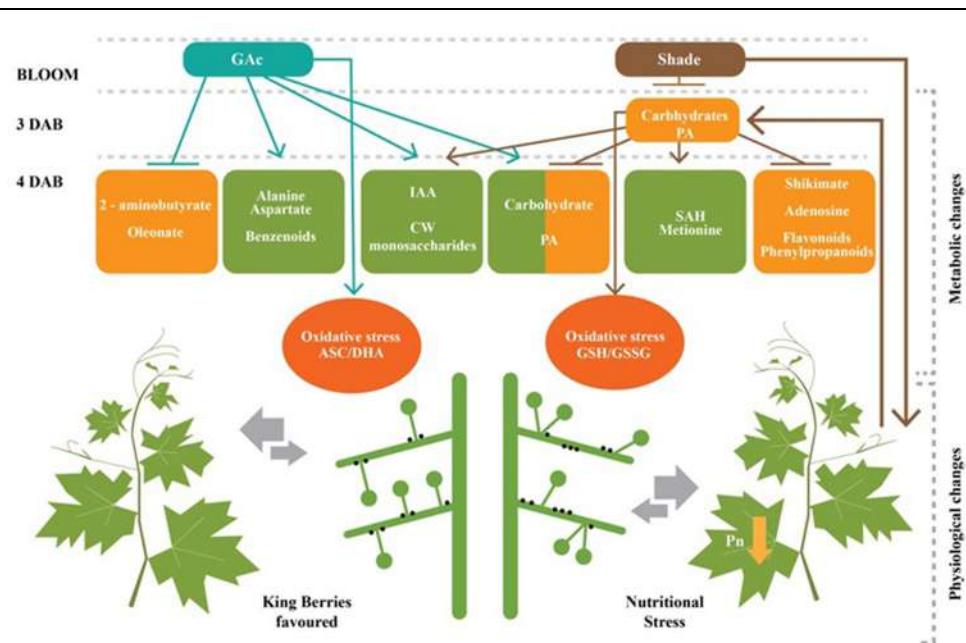


FIGURE 7 | A proposed mechanistic model for flower abscission in *Vitis Vinifera* L. inflorescences triggered by GAc and shade. Shade treatment reduced net photosynthetic rate which lead to significant alterations 3 and 4 DAB, including global carbohydrates starvation, reduction on shikimate, PA (putrescine) and secondary metabolisms and increase of oxidative stress, revealed by glutathione remediation cycle. GAc induced an increase on carbohydrates, PA (putrescine), amino acids and secondary metabolisms and oxidative stress, revealed by ascorbate/dehydroascorbate remediation couple at

4 DAB. Both treatments induced IAA and CW monosaccharide accumulation. The thickness of the arrows related to inter-organ competition is proportional to the sink strength at bloom stage. According to this model, flower abscission in shade is due to a general nutritional stress and, in GAc treatment to the growth of king berries which inhibits the development of lateral flowers. Abscission layer in plant side is represented by black dots. Green and orange boxes indicate the increase and decrease on metabolite concentrations, respectively, as response of imposed treatments.

Author Contributions

SD, PS, CO, and LG were responsible for the conception and design of the experiments, SD and PS were responsible for acquisition of data, SD, VC, and AL performed the laboratory analyses, SD, PS, RD, CO, and LG interpreted the data. All authors drafted and approved the manuscript. RD, CO, and LG steered the whole project.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00457>

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Bimodal effect of hydrogen peroxide and oxidative events in nitrite-induced rapid root abscission by the water fern *Azolla pinnata*

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In the genus *Azolla* rapid abscission of roots from floating fronds occurs within minutes in response to a variety of stresses, including exposure to nitrite. We found that hydrogen peroxide, though itself not an inducer of root abscission, modulates nitrite-induced root abscission by *Azolla pinnata* in a dose-dependent manner, with 2 mM H₂O₂ significantly diminishing the responsiveness to 2 mM NaNO₂, and 10 mM H₂O₂ slightly enhancing it. Hypoxia, which has been found in other plants to result in autogenic production of H₂O₂, dramatically stimulated root abscission of *A. pinnata* in response to nitrite, especially for plants previously cultivated in medium containing 5 mM KNO₃ compared to plants cultivated under N₂-fixing conditions without combined nitrogen. Plants, including *Azolla*, produce the small signaling molecule nitric oxide (NO) from nitrite using nitrate reductase. We found *Azolla* plants to display dose-dependent root abscission in response to the NO donor spermine NONOate. Treatment of plants with the thiol-modifying agents S-methyl methanethiosulfonate or glutathione inhibited the nitrite-induced root abscission response. Synchrotron radiation-based Fourier transform infrared spectromicroscopy revealed higher levels of carbonylation in the abscission zone of dropped roots, indicative of reaction products of polysaccharides with potent free radical oxidants. We hypothesize that metabolic products of nitrite and NO react with H₂O₂ in the apoplast leading to free-radical-mediated cleavage of structural polysaccharides and consequent rapid root abscission.

Keywords: root abscission, apoplast, free radical cleavage, FTIR spectromicroscopy, hydrogen peroxide, nitric oxide, nitrite, plant cell wall loosening

Introduction

Small floating water ferns of the genus *Azolla* are model systems for studies of abscission (Cohen et al., 2014), dropping roots within minutes in response to heat (Ueda et al., 1999) and to various chemical exposures, including nitrite (Ueda and Kitoh, 1994; Gurung et al., 2012).

To assess the abscission response intact plants can be placed onto a test solution and observed for root dropping. Alternatively, roots may be pulled from the frond, immersed into test solution

and then observed for expansion and separation of abscission zone cells. Studies on pulled roots of *Azolla filiculoides* found that exposure to the protease papain nearly abolished the abscission response, demonstrating an essential function for extracellular proteins (Uheda et al., 1994), and that neither actinomycin D nor cyclohexamide inhibit the abscission response, indicating that such proteins are preformed (Uheda and Kitoh, 1994). An increase in apoplastic pH, which is typically in the range of pH 5–6 in plants (Palmgren, 2001), is likely to be a proximate stimulus for abscission since immersion of pulled roots in buffered solutions having pH ≥ 6.7 results in near immediate cell separation at the abscission zone (Uheda et al., 1994). Consistent with this, immersion in acidic buffers abolishes responsiveness to abscission-inducing compounds (Uheda and Kitoh, 1994) while treatment with various protonophores rapidly induces cell separation (Uheda and Kitoh, 1994).

The finding that the dissolution of middle lamella of abscission zone cells cannot be reproduced by exogenously provided cell wall-degrading enzymes (Uheda et al., 1994; Fukuda et al., 2013), as well as the rapid speed of the process, led to the hypothesis that free radical-mediated breakage of cell wall polysaccharides occurs during rapid abscission (Fukuda et al., 2013). Such oxidative non-hydrolytic cleavage of cell wall polysaccharides has been observed in other processes that involve cell wall loosening, including seed germination, cell elongation (Schopfer, 2001; Müller et al., 2009), and fruit ripening (Fry et al., 2001). Supporting this hypothesis, histochemical evidence of oxidative reactions was found in the abscission zone of abscised roots of *A. filiculoides* and, furthermore, abscission could be induced by treatment of plants with agents that generate hydroxyl radicals (·OH) via a Fenton-type reaction (Yamada et al., 2015).

Plant cell wall associated peroxidases are capable of generating ·OH that can cleave (Liszakay et al., 2003) and carbonylate cell wall polysaccharides (Fry, 1998; Fry et al., 2001; Marnett et al., 2003). Recent advancements in synchrotron radiation-based Fourier transform infrared (SR-FTIR) spectromicroscopy have allowed for detection of functional groups including carbonyls *in situ* rather than by the traditional analysis of extracts (Holman et al., 2010; Lacayo et al., 2010). One limitation in applying this technique is that samples must be thin enough to allow for transmission of the light beam. In this regard the thinness of *Azolla* roots presents an advantage since there is no need to carry out thin-sectioning of samples prior to spectroscopic analysis.

Here we report pharmacological studies that indicate a role for thiol-targeted oxidative events in regulating the abscission process as well as SR-FTIR spectromicroscopic evidence of free radical attack in the abscission zone of dropped roots.

Materials and Methods

Plant Material and Surface Sterilization

Laboratory cultures of *Azolla pinnata* were established from plants collected in December 2012 and May 2014 from a taro field in Ginowan, Okinawa, Japan. The plants were thoroughly washed to remove attached mud and debris. The plants were then treated

with a solution of 0.12% sodium hypochlorite and 0.01% Triton X-100 for 30 min followed by repeated washings in a large volume of distilled water and finally transferred into nutrient medium (Peters and Mayne, 1974).

Nutrient medium and culture conditions

A. pinnata was cultured in a two-fifth strength cobalt-supplemented nitrogen-source-free Hoagland's E-medium (Gurung et al., 2012). Medium pH was adjusted to 5.8 with potassium hydroxide. Plants were grown in a plant growth chamber (Type FLI-2000H, Eyla, Japan) maintained at 27 ± 1°C, 80% humidity, 16:8 h light:dark photoperiod and 50 μmol m⁻² s⁻¹ (at plant level) provided by fluorescent lamps (Type FL 40 SBR-A, NEC, Japan). For experiments 15 to 20 fronds were randomly selected from the culture stock and de-rooted manually using forceps. Rootless fronds were cultured in nutrient medium after rinsing in distilled water. The plants were transferred to a fresh medium every 4 days.

Synchrotron Radiation-Based Fourier Transform Infrared Spectromicroscopy

Plant samples were prepared for SR-FTIR spectromicroscopy by freeze drying. Roots from *A. pinnata* were selected under a visible microscope and enclosed between two CaF₂ infrared crystals in order to assure the planarity of the sample. For each treatment at least two regions were selected for measurement, one close to the abscission zone and one few millimeters apart, as an internal control. IR maps were collected at infrared beamlines of the Advanced Light Source (<http://infrared.als.lbl.gov/>), mid-infrared photons (~2.5–15.5 μm wavelength λ, or ~4000–650 cm⁻¹ wavenumber) emitted from the synchrotron bending magnet are focused down to a 0.76λ diffraction-limit spot size with a Nicolet Continuum infrared microscope, coupled to a Nicolet 6700 FTIR Spectrometer (Thermo Fisher Scientific Inc.), using a 32X objective with 0.65 numerical aperture. Each map was acquired with a spatial resolution of 5 μm. Spectra were corrected for water vapor and CO₂ and converted in ENVI format. An average spectrum was calculated for abscission and control zones from all maps and treatments, and then a cluster analysis was performed calculating the Euclidian distances of the second derivative of vector normalized spectra in the range from 1800 to 1200 cm⁻¹ using the Ward's method for the agglomerative hierarchical clustering procedure. Wavenumber FTIR peak identities were assigned based on Socrates (2004). Moreover seasonal differences were identified by analyzing the spectral difference of the controls. The same approach was used to highlight chemical differences in the samples due to the specific treatment, considering the difference between treated and control from the same batch, and chemical differences of the abscission zone from the control zone in the same root. The difference spectra were calculated after applying vector normalization on the whole 1800–1200 cm⁻¹ interval.

Chemical Treatment of *A. pinnata*

Abscission assays were carried out using roots of equal age (i.e., from fronds that had been de-rooted at the same time, 9–12 days prior to performing the assay). 2 to 3 fronds (20–30

roots) were suspended in a beaker containing 20 ml 10 mM potassium phosphate buffer at pH 7. The chemicals to be tested, sodium nitrite (hereafter referred to as nitrite), H₂O₂, 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazole-1-oxyl-3-oxide (carboxy-PTIO), and spermine NONOate (SNN; Cayman Chemicals Company, Ann Arbor, MI, USA) were subsequently supplied as concentrated stock solutions. The total number of dropped roots following addition of the chemicals was recorded every 10 min for up to 2.5 h. The abscission response was quantified as the ratio of the detached to the initial number of roots.

For some trials the plants were co-incubated or pre-incubated with other chemicals in 10 mM potassium phosphate buffer. 20 mM glutathione (GSH) was added to 2 mM nitrite-treated plants at three time intervals: at the same time ($t = 0$) and 30 and 60 min after initiation of nitrite treatment. Nitrite-treated plants without any GSH addition served as controls. For trials testing the effect of S-methyl methanethiosulfonate (MMTS), which covalently sulfenylates the sulfur of cysteine residues, plants were pre-incubated with 1 mM MMTS for 60 min followed by addition of 4 or 6 mM nitrite. We used higher levels of nitrite for this set of experiments because this batch plants showed less than typical responsiveness to 2 mM nitrite.

Statistics

The means of abscission responses to treatments, quantified as percent of roots dropped, were compared by ANOVA and subjected to a Tukey-Kramer test of significance using SAS/STAT(R) 9.22 (SAS Institute Inc., Cary, NC, USA).

Results

Combinatorial Effect of Nitrite and Hydrogen Peroxide on *A. pinnata* Root Abscission

In other plant systems Sakamoto et al. (2008) have described a H₂O₂ burst coinciding with leaf abscission that would presumably provide substrate for ·OH generation (Liszka et al., 2003). This H₂O₂ burst is apparently distinct from the lower level of H₂O₂ production that functions in the abscission signaling process. We have previously reported that treatment of *A. pinnata* plants with up to 10 mM H₂O₂ alone does not induce root abscission (Gurung et al., 2012). However, in combination with the known abscission-inducing agent nitrite, treatment of plants with H₂O₂ exhibited a pronounced effect that depended on the concentration and timing of the H₂O₂ treatment relative to exposure to nitrite (Figures 1A,B). The inhibitory effect of 2 mM H₂O₂ on root abscission induced by 2 mM nitrite could be overridden by the further addition of nitrite (>4 mM nitrite; Figure 1C).

Fluorescence, indicative of phenolics accumulation (Yamasaki et al., 1995), in the root tip region formerly inserted in the frond was not discernibly different in roots dropped from plants treated with 2 mM nitrite and 10 mM H₂O₂ compared to the same region of untreated pulled roots (Figure 2). Release of bubbles following addition of H₂O₂ (presumably due to catalase activity) was qualitatively stronger at the root tip than other portions of

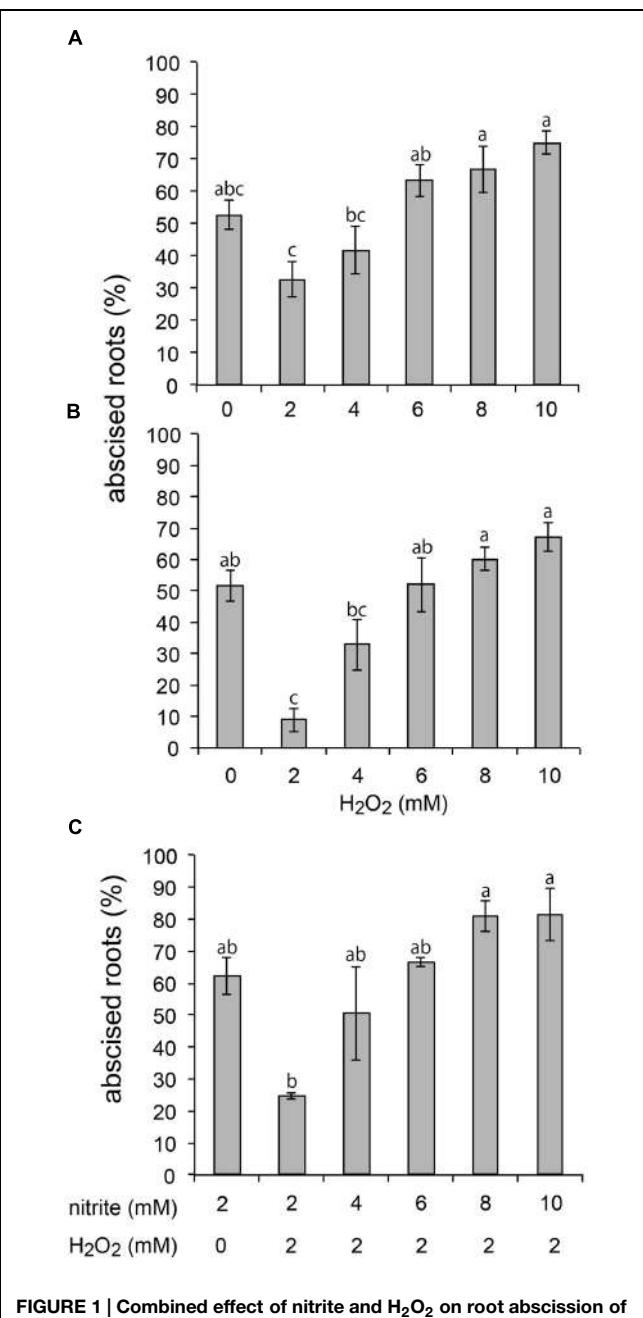


FIGURE 1 | Combined effect of nitrite and H₂O₂ on root abscission of *Azolla pinnata* plants. Abscission was measured 90 min after 2 mM nitrite was added. 2, 4, 6, 8, or 10 mM H₂O₂ were added (A) simultaneously with or (B) 60 min before addition of nitrite. (C) 2 mM H₂O₂ was added simultaneously with 2, 4, 6, 8, or 10 mM nitrite (A, n = 7; B, n = 8, C, n = 2, mean ± SE; means with the same letter are not significantly different).

the root and was noticeably diminished in roots that had been abscised by following exposure to 5 mM nitrite (Supplementary Figure S1).

Exposure of plants to hypoxia induces generation of H₂O₂ (Blokhina et al., 2001). We observed that hypoxia in combination with 2 mM nitrite induced abscission to a greater extent than did treatment with 2 mM nitrite alone (Figure 3); hypoxia without

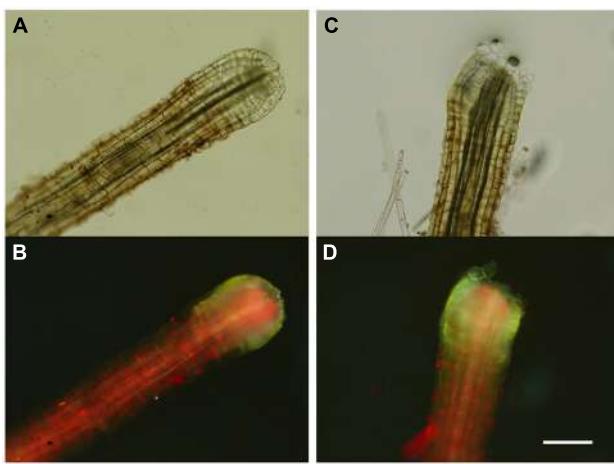


FIGURE 2 | Effect of combined treatment with nitrite and hydrogen peroxide on proximal root ends. Representative proximal ends of *A. pinnata* roots from plants were **(A,B)** untreated and removed from the frond by pulling or **(C,D)** dropped following simultaneous treatment with 2 mM nitrite and 10 mM H₂O₂ for 120 min. **(A,C)** Light, **(B,D)** fluorescence (excitation 380 nm) microscopic images; scale bar, 200 μ m.

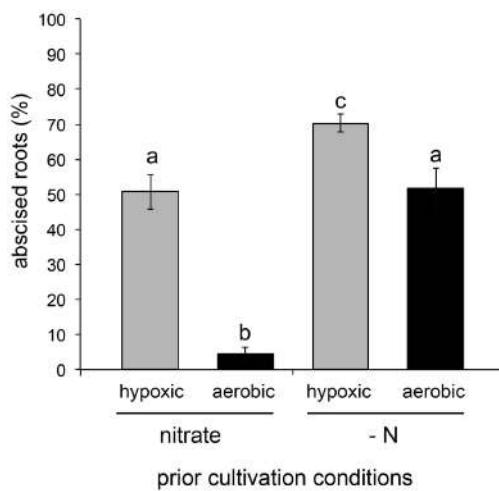


FIGURE 3 | Effect of hypoxia on abscission induced by 2 mM nitrite treatment of plants with or without prior cultivation in medium containing 5 mM nitrate. Abscission under hypoxic or aerobic conditions was measured 150 min after addition of nitrite (means \pm SE; hypoxia, $n = 4$; aerobic, $n = 6$).

nitrite supplementation did not induce abscission (results not shown). Plants that had been cultivated in medium containing 5 mM potassium nitrate prior to the assay showed significantly less responsiveness to hypoxia (**Figure 3**).

Plants, including *A. pinnata*, can convert nitrite to nitric oxide (NO; Yamasaki, 2005; Gurung et al., 2012), from which a variety of reactive nitrogen species (RNS) can be generated (Fukuto et al., 2012). A role for NO in nitrite-induced root abscission was implied by the reduction in abscission observed of nitrite-treated plants in the presence carboxy-PTIO, which oxidizes NO; the

inhibitory effect of carboxy-PTIO was substantially diminished by increasing the concentration of nitrite (Supplementary Figure S2). Consistent with this finding, treatment of plants with the NO donor SNN elicited a strong dose-dependent abscission induction response (**Figure 4**).

Thiol-Active Treatments Suppressing Induction of Abscission

Nitrite-induced abscission was suppressed by treatment of plants with MMTS (**Figure 5**) or GSH (**Figure 6**). 20 mM GSH nearly prevented the abscission response to 2 mM nitrite and when added at 30 or 60 min following nitrite prevented further abscission (**Figure 6**).

SR-FTIR Demonstration of Abscission Zone Carbonyl Formation

The chemical maps presented in **Figure 7A** show the SR-FTIR intensity of the ratio of the C=O (non-peptide) signal (1780–1705 cm⁻¹) to the C–O–R signal (1705–1550 cm⁻¹); the ratio was chosen in order to not be affected by difference of thickness between the measured areas. The tips encompassing the abscission zone of dropped roots are richer in C=O than other root parts. By using agglomerative cluster analysis on the average spectra of *A. pinnata* exposed to different conditions, we can observe that there always is a segregation of tips (red in **Figure 7B**) and internal parts (blue in **Figure 7B**) of the roots. This phenomenon means that the spectral features, hence chemical composition, of the tips are more similar between themselves than the other internal parts of the same roots and the tips of the untreated control roots (green in **Figure 7B**). Looking at the difference spectra presented in **Figures 7C,D** it can be seen that the abscised tips show increases in signal at \sim 1740 cm⁻¹ in the SR-FTIR spectrum, indicative of carbonyl groups that are formed upon ·OH attack of cell wall polysaccharides (Fry, 1998; Fry et al., 2001; Marnett et al., 2003). This increase occurs on top

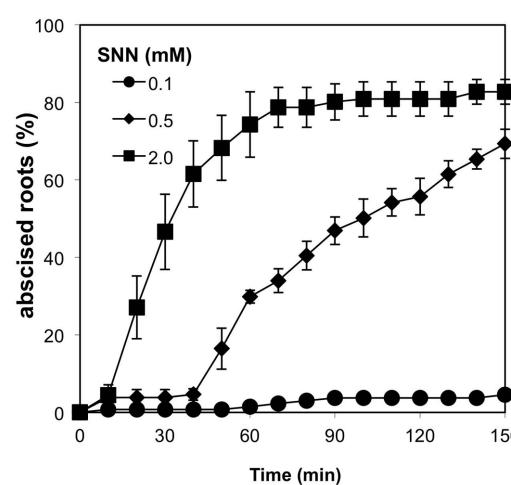
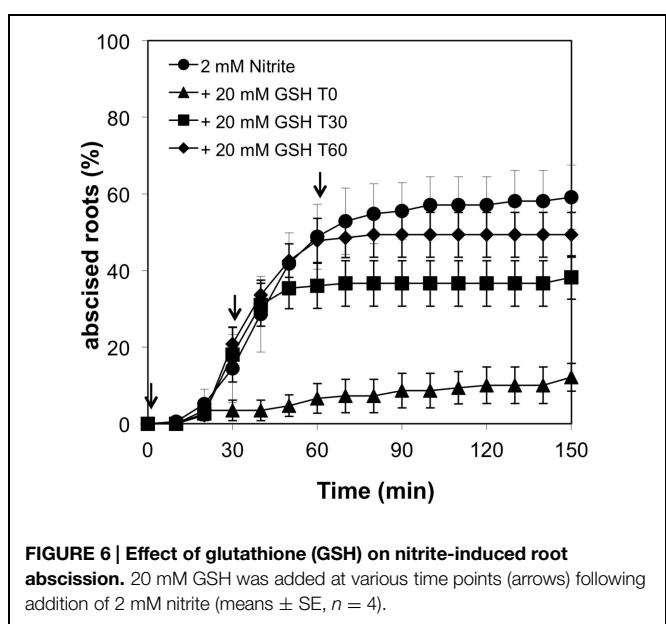
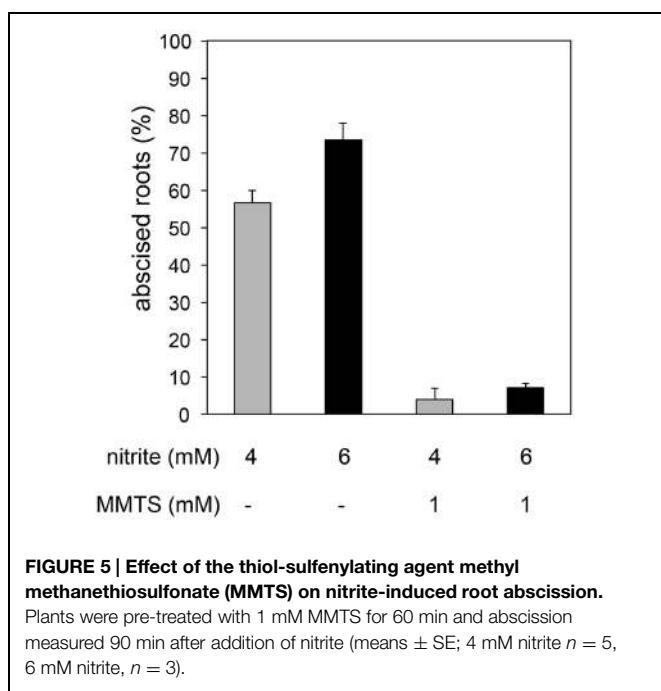


FIGURE 4 | Dose-dependent induction of *A. pinnata* root abscission by spermine NONOate (SNN). 0.1 mM ($n = 3$), 0.5 mM ($n = 4$), 2.0 mM ($n = 4$; means \pm SE).



of a pre-existing peak in this range arising from C=O containing molecules in the plant tissue, including carboxylic acid groups from methylated pectin (Szymanska-Chargot and Zdunek, 2013). Importantly, in the frond attachment point of untreated roots this characteristic peak is from 4 to 6 times less intense. Appearance of carbonyl groups would not be expected if only enzymatic hydrolysis of polymers were occurring in the cell wall. Thus, these results support the model for ·OH-mediated cell wall dissolution. Furthermore, evidence of increased oxidation in the treatment can be seen in peaks within the range 1430–1335 cm⁻¹ due to

the symmetric stretching of CO₂⁻ in carboxylates and 1320–1210 cm⁻¹ from the stretching of C-O groups, which can result from ·OH attack of biological molecules (Nappi and Vass, 1998). Lastly, we can assign the signal at 1460–1430 cm⁻¹ to peroxy acids, whose O-H bending shifts toward 1430 cm⁻¹ for long-chain linear acids that may originate from the oxidation of cell wall carbohydrates. Other important signals that are clearly detectable in the difference spectra of the nitrite treatments in Figures 7C,D are those arising from NO₃⁻ and NO₂⁻, respectively, at 1380 and 1254 cm⁻¹ (Mallard, 2015).

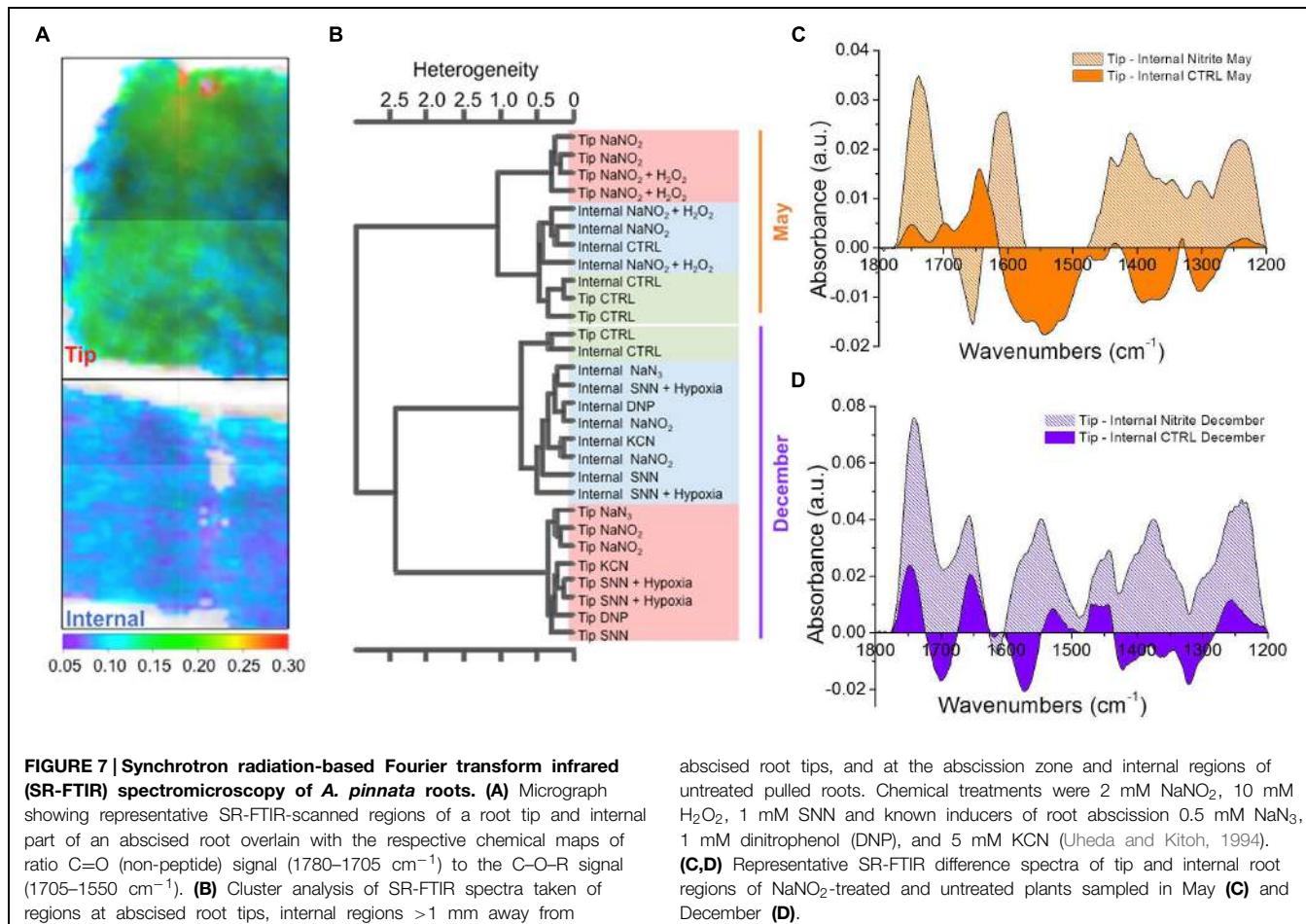
Seasonal differences are apparent in Figure 7B, which shows primary clustering by sampling date (December and May), and in comparing the distinct difference spectra of tips of untreated roots in Figures 7C,D. Large-scale seasonal changes in tissue concentrations deoxyanthocyanins, carotenoids, and chlorophyll known to occur in *Azolla* species (Cohen et al., 2002; Kösékal, 2014) may account for these seasonal spectral differences.

Discussion

Previous reports have pointed to the functioning of preformed extracellular proteins in the rapid root abscission process of *Azolla* (Ueda and Kitoh, 1994) with evidence of free radical, rather than hydrolytic enzyme, mediated cleavage of apoplastic polysaccharides (Fukuda et al., 2013; Yamada et al., 2015). Our results show the abscission process to be influenced by agents that react with or generate compounds that react with thiols, including GSH, MMTS, and H₂O₂ in combination with nitrite (Heinecke and Ford, 2010). The effect of H₂O₂ on nitrite-induced abscission, from inhibitory at <4 mM to stimulatory at >8 mM, is consistent with the bimodal action of H₂O₂ on leaf abscission in other systems, behaving as a regulator at low concentrations and as an agent of cell wall dissolution at higher concentrations (Nappi and Vass, 1998). The greater inhibition of nitrite-induced abscission following 60 min pretreatment with H₂O₂ (Figure 1B) compared to concomitant addition (Figure 1A) could imply H₂O₂ acting as a signal to activate the antioxidant capacity, including upregulating biosynthesis of thiol compounds like GSH, as has been shown in other plant systems (Foyer and Noctor, 2011). Higher concentrations of H₂O₂ may favor conditions that generate ·OH-like potent oxidants with consequent cell wall loosening events (Supplementary Figure S3; Cohen et al., 2014 and references therein).

The positive effect of hypoxia on nitrite-induced abscission may be due to hypoxia-stimulated production of H₂O₂; transition into hypoxia is known to result in H₂O₂ production in plants (Blokhina et al., 2001) but was not measured in our experiments. The lower responsiveness of nitrate-grown plants to nitrite may be due to competition of nitrate with nitrite for the active site of nitrate reductase, thereby lowering the production of NO by this enzyme (Rockel et al., 2002). Consistent with this observation, tungsten, a non-specific inhibitor of nitrate reductase, also significantly lowered abscission responsiveness to nitrite (Gurung, 2014).

The putative thiol in the target protein may be positioned such that it can only be modified via reactions that occur at the nearby



iron rather than through external nitrating agents like NO_2 gas. The finding that, of the many RNS molecules and donors tested, only the polyamine NO donor SNN induced abscission may indicate a necessity for generating near the site of action; the affinity of polyamines for plant cell walls is well documented (Zepeda-Jazo et al., 2011). Spermine can itself serve as an inducer of root abscission but the response does not initiate until after 50 min of exposure and does not require the activity of the H_2O_2 -producing polyamine oxidase (Gurung et al., 2012). NO may also be involved in the abscission-inducing mechanism of the most potent inducer of *Azolla* root abscission, sodium azide (Uheda and Kitoh, 1994), which is converted to NO (presumably via HNO) by catalase (Gurung et al., 2014).

The presumptive apoplastic protein target of chemical abscission inducers is most likely already positioned in the apoplast before the abscission-inducing event, based on the rapidity of the response and its insensitivity to cycloheximide (Uheda and Kitoh, 1994). In citrus, Gaspar et al. (1978) demonstrated that activity of preformed peroxidases are upregulated in abscission zones in response to ethylene treatment. Cell wall peroxidases are capable of catalyzing the production of ·OH from H_2O_2 (Liszakay et al., 2003) and could conceivably be a regulatory target in *Azolla* abscission zones (Cohen et al., 2014). Several lines of evidence implicate free

abscised root tips, and at the abscission zone and internal regions of untreated pulled roots. Chemical treatments were 2 mM NaNO_2 , 10 mM H_2O_2 , 1 mM SNN and known inducers of root abscission 0.5 mM NaN_3 , 1 mM dinitrophenol (DNP), and 5 mM KCN (Uheda and Kitoh, 1994). **(C,D)** Representative SR-FTIR difference spectra of tip and internal root regions of NaNO_2 -treated and untreated plants sampled in May **(C)** and December **(D)**.

radical attack in the dissolution of pectin seen during abscission in *Azolla* and other plants (Fry et al., 2002; Fukuda et al., 2013; Yamada et al., 2015) and are consistent with the SR-FTIR spectromicroscopic results reported here.

An increase in the FTIR signal at $\sim 1740\text{ cm}^{-1}$ as we have observed in the *Azolla* root abscission zone has been observed of other oxidative plant cell wall degradation processes that are known to result in formation of C=O groups, including UV-irradiation (Müller et al., 2003) and microbial degradation of wood (Gilardi et al., 1995) and periodate oxidation of cellulose (Spedding, 1960).

Our findings provide more evidence for ·OH formation in cell wall cleavage events during the abscission process of *A. pinnata* and lay a foundation for use of SR-FTIR spectromicroscopy for abscission studies. The demonstration of this application for SR-FTIR microscopy is important because tools for investigating the role of hydroxyl radicals in the abscission process are currently limited to ^3H fingerprinting (Müller et al., 2009). In spite of the rapidity of their abscission response and ease of experimental manipulation *Azolla* species have been considered to be “not suitable for biochemical studies on abscission because the abscission zone is very small” (Uheda and Nakamura, 2000). With the advent of SR-FTIR microscopy the small size of *Azolla* roots can now be seen as an advantage for study since their

thinness allows for the penetration of the light beam, allowing us to examine biochemical changes more readily in *Azolla* than in the thicker abscission zones of other species.

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Supplementary Material

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Primary and Secondary Ablission in *Pisum sativum* and *Euphorbia pulcherrima*—How Do They Compare and How Do They Differ?

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Ablission is a highly regulated and coordinated developmental process in plants. It is important to understand the processes leading up to the event, in order to better control abission in crop plants. This has the potential to reduce yield losses in the field and increase the ornamental value of flowers and potted plants. A reliable method of abission induction in poinsettia (*Euphorbia pulcherrima*) flowers has been established to study the process in a comprehensive manner. By correctly decapitating buds of the third order, abission can be induced in 1 week. AFLP differential display (DD) was used to search for genes regulating abission. Through validation using qRT-PCR, more information of the genes involved during induced secondary abission have been obtained. A study using two pea (*Pisum sativum*) mutants in the *def* (*Developmental funiculus*) gene, which was compared with wild type peas (tall and dwarf in both cases) was performed. The *def* mutant results in a deformed, abission-less zone instead of normal primary abission at the funiculus. RNA *in situ* hybridization studies using gene sequences from the poinsettia differential display, resulted in six genes differentially expressed for abission specific genes in both poinsettia and pea. Two of these genes are associated with gene up- or down-regulation during the first 2 days after decapitation in poinsettia. Present and previous results in poinsettia (biochemically and gene expressions), enables a more detailed division of the secondary abission phases in poinsettia than what has previously been described from primary abission in *Arabidopsis*. This study compares the inducible secondary abission in poinsettia and the non-abscising mutants/wild types in pea demonstrating primary abission zones. The results may have wide implications on the understanding of abission, since pea and poinsettia have been separated for 94–98 million years in evolution, hence any genes or processes in common are bound to be widespread in the plant kingdom.

Keywords: induced abission, primary abission, secondary abission, pea, poinsettia, *def* mutants, Differentially expressed genes, RNA *in situ* hybridization

Abbreviations: AZ, abission zone; DD, differentially expressed genes.

INTRODUCTION

Abscission is a beneficial process for plants themselves, since this is the mechanism for plants to discard unwanted or superfluous organs in a highly orchestrated manner. However, this developmental process cause seed shattering, fruit drop, flower abscission, and other loss of value for crops valuable to man. It is not surprising that prevention of seed shattering probably was one of the first characters selected for when man started to cultivate plants and selected for cereal plants where he could harvest more seeds (Harlan et al., 1973). Abscission is a complicated process, it is not clear the orchestrated manner by which abscission is controlled in plants. The process is important to understand, since agricultural and horticultural production is increasingly more sophisticated and facilitates precise control of the growth conditions, in greenhouses and increasingly also in the field.

Cells in an abscission zone (AZ) are typically small, square-shaped with dense cytoplasm (Sexton and Roberts, 1982) and clearly distinguishable from surrounding cells. The number of cell layers in an AZ is fixed for a species, but is highly variable between species, with tomato as an example of two discrete cell layers, which split between them (Valdovinos and Jensen, 1968; Tabuchi et al., 2001). The AZ of *Sambucus nigra* on the other hand, is composed of up to 50 cell layers (Taylor and Whitelaw, 2001). The term secondary abscission zones was first introduced by Lloyd (1913–14). He reported on injury-induced abscission in *Impatiens sultani*. Secondary abscission has also later been described as a zone which occurs in a position where a zone would not normally form in an intact plant (Webster, 1970; Pierik, 1973). Having termed these adventitious AZ as secondary, the predestined AZ occurring at particular sites of positional differentiated cells have since been given the term primary (Huang and Lloyd, 1999) to distinguish between the two.

Abscission can be affected by environmental factors and is a highly coordinated biological mechanism (Brown and Addicott, 1950; Osborne, 1955; Addicott, 1982; Patterson, 2001; Roberts et al., 2002). It has been reported that low light conditions might trigger cyathia abscission in poinsettia (*Euphorbia pulcherrima*) (Bailey and Miller, 1991; Moe et al., 1992) but environmental regulations, as well as the biological background of abscission has not been fully investigated. Although the abscission process is a natural biological process to dispose of redundant organs, premature abscission results in the loss of yield and value in agriculture and horticulture.

Valdovinos and Jensen demonstrated the cell wall disintegration in the AZ allowing separation in tomato and tobacco (Valdovinos and Jensen, 1968). Reviews have followed with more insight into the process (Sexton and Roberts, 1982; Osborne and Morgan, 1989; Taylor and Whitelaw, 2001; Bosca et al., 2006). Our own results have clearly demonstrated and confirmed that abscission is controlled by inter-organ signaling events, yet it is still not clear how these signals co-ordinate the events. Cell wall modifications in the AZ of poinsettia, visualized using antibodies during the course of an induced abscission process, is one way we have chosen to elucidate upon

the abscission process (Lee et al., 2008). Some of the other approached will become clear in the present article.

Poinsettia is not the obvious choice for fundamental studies since the molecular tools available for other model plants are not available. Secondly, the life span is much longer than for *Arabidopsis*. Thirdly, it is vegetatively propagated, does not readily set seed and segregation studies would be difficult to perform. Lastly, it has no available non-abscising mutants. However, poinsettia is an important ornamental plant worldwide during Christmas time. It is by far the most important potted plant crop in Norway with more than five million plants produced each year, for a population of about the same number. In addition, Norwegian poinsettia growers have pointed out that poinsettia suffers from premature flower abscission, which can result in severe losses in value. Therefore, there are economic reasons for being able to control this. A method for induction of abscission to investigate the abscission process has been developed using this plant species (Munster, 2006). This makes the study of abscission in poinsettia very precise and predictable. Poinsettia flower pedicels have no pre-destined AZ, and hence they are defined as having secondary abscission. This inducible abscission system in the poinsettia flower resembles systems in other plant species (Webster, 1970; Hashim et al., 1980; Oberholster et al., 1991; Kuang et al., 1992), especially the model plant tomato (pedicel abscission) and thus provides a reliable, synchronized system for studying the abscission process in general. Poinsettia (*E. pulcherrima*) belongs to the large family of *Euphorbiaceae*, with about 300 genera and 7500 species. A number of plants of this family are of considerable economic importance. Prominent plants include cassava (*Manihot esculenta*), physic nut or Barbados nut (*Jatropha curcas*), castor oil plant (*Ricinus communis*), and the Para rubber tree (*Hevea brasiliensis*). Amongst several of these, genomic and molecular tools are becoming available, because of their economic importance for producing biofuels.

Previously, this inducible system has been used to study the turnover of carbohydrates in the abscission zone (Lee et al., 2008), and the effect of the cut position on hormones in the bud (Munster, 2006). This article reports on the genes differentially displayed during the 7 day period from induction to abscission from the thesis of Munster (2006). We have since further verified the gene expressions in poinsettia through quantitative RT-PCR and RNA *in situ* hybridizations, all of which is included in this article.

Poinsettia has no mutants for abscission, in order to study gene expression. However, there are other model systems with numerous mutants. There are two *def* (*Developmental funiculus*) mutant peas in the John Innes Pea Collection, one dwarf and one tall type (JI184 and JI3020). These mutants were the tools to elucidate upon the process of abscission in peas (Ayeh, 2008). That study concluded that the *def* gene is a single locus gene (Ayeh et al., 2011) and the abscission zone between the funiculus and the pea was characterized (Ayeh et al., 2009) in both mutants (with abscission-less zones) and tall and dwarf wild types. Pea has primary abscission, with the AZ clearly defined from the onset in the wild types and only the distorted abscission-less zone in the mutants.

Pea and poinsettia are separated by 94–98 million years (Bennett et al., 2000). Hence, any genes they share during the abscission process will most likely be universal throughout the plant kingdom. This paper summarizes the interesting comparable results in pea and poinsettia with respect to cell wall alterations (Ayeh, 2008; Lee et al., 2008) as well as gene expression during the abscission process from induction to abscission. Our hypothesis is that the primary abscission in pea (*Pisum sativum*) and secondary abscission in poinsettia (*E. pulcherrima*) are more similar than different. Pea represents primary abscission where the abscission zones are clearly defined from the development of the organs. Poinsettia represents secondary abscission, where the abscission zones can develop upon induction. This paper presents results, which tests the hypothesis comparing the developmental stages in poinsettia abscission with the *def* mutants and wild types in pea, discussing the similarities and differences between these two systems as models for abscission.

MATERIALS AND METHODS

Plant Material—Poinsettia

Poinsettia (*Euphorbia pulcherrima*) ‘Lilo’ were grown as previously described in Lee et al. (2008). Plants were grown under long day condition (20 h photoperiod at $150 \mu\text{mol m}^{-2}\text{sec}^{-1}$) and the plants were kept under short day conditions (10/14 h photoperiod) at 20°C to induce flowering.

Induction of Abscission in the Flower Pedicel (Secondary Abscission)

Cyathia of the third order (all male flowers) were used for analyses to standardize abscission zone development, since this gives six flowers in the same inflorescence of the same developmental stage (3rd order; Figure 1). When third order flowers began to open, they were decapitated with a razor blade just below the floral organs, with the floral bottom still intact, cut position 2 (cp2) in Figure 2A (Munster, 2006). The flowers developed abscission zones (AZs) under short day conditions, with 7 ± 1 days to complete abscission. AZs were dissected from the decapitated pedicels every 24 h, and harvested on the same time to create the complete series from Day 0 (control) to Day 7 and obtain comparable gene expressions. Figures 2B–E shows the development of the AZ from Day 0 to Day 7 (day of abscission). Figure 3 shows micrographs of poinsettia comparing a pedicel with no AZ (A) with induced (B), and natural (C) abscission.

Plant Material—Pea

The four lines of pea (*P. sativum* L.) seeds (JI 116, JI 2822, JI 1184, and JI 3020) in this study were selected based on the presence of specific alleles at the *Def* locus, which control the detachment of the seed from the funiculus (Ayeh et al., 2009, 2011). Two wild types (WT) with the *Def* locus and two *def* mutant pea seeds were kindly supplied from the John Innes Pisum Collection Ayeh et al. (2009). Tall wild type (JI 116) and dwarf wild type (JI 2822) develop normal abscission events and therefore abscise the seed from the funiculus through the intervening hilum region. The tall *def* mutant (JI 1184) and the dwarf *def* mutant (JI 3020)

both lack the abscission event and therefore fail to abscise the seed from the funiculus. These lines have a deficient abscission zone, which we have given the name abscission-zone-less (AZL; Ayeh, 2008). Seeds of each line were sown in pots with fertilized peat and grown under greenhouse conditions at 22°C and 16/8 h photoperiod with a photon flux of $110 \mu\text{mol m}^{-2}\text{ s}^{-1}$ [400–700 nm Photosynthetic Active Radiation (PAR)] and a daylength extending light provided from incandescent lamps (OSRAM, Germany).

Definition of Growth Stages in Pea

We used young and mature developmental stages in both the wild and the *def* mutant pea plants. For the tall wild type JI 116, developmental stage 10.1 indicates young seed. For the tall *def* mutant type JI 1184, developmental stage 8.1 indicates young seed for a comparable developmental stage. The developmental stage 2.1 indicates mature seed for both JI 116 and JI 1184. For the dwarf wild type JI 2822 and the dwarf *def* mutant JI 3020, developmental stages 4.1 and 3.1 indicate young seeds, respectively. Developmental stage 1.1 indicates mature seed for both dwarf wild type JI 2822 and dwarf *def* mutant JI 3020.

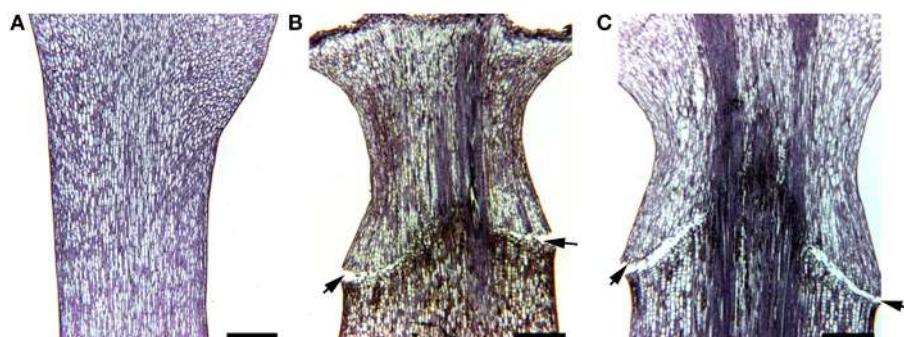
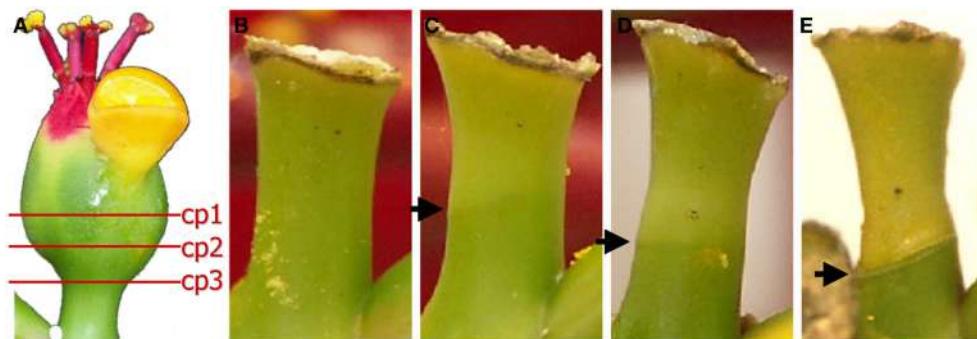
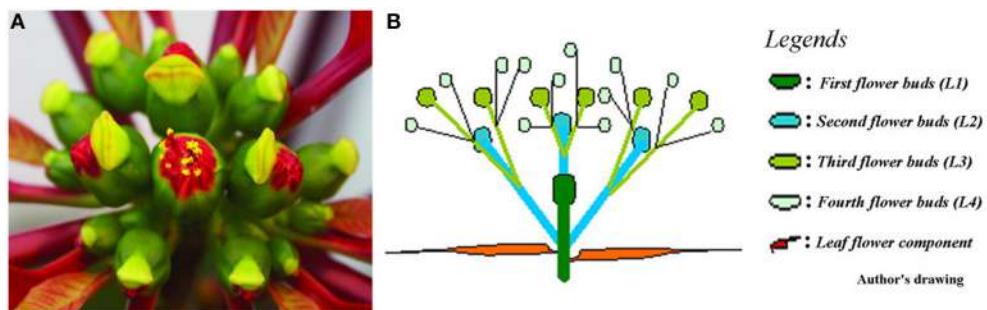
Differential Display (DD) in Poinsettia

AZs were dissected from the area of the pedicel from Day 0 until Day 7 as described in Munster (2006). As soon as the AZ could be defined visually, samples of the distal part were harvested as an internal control to eliminate senescence related genes from the bands picked from DD. The pedicel slices from AZ tissue were stored immediately in RNAlater (0.1 g/ml) at -20°C until use.

RNA Extraction, Differential Display, and Sequencing

RNA was extracted from the AZ tissues by a time course according to instruction of the Qiagen RNeasy Plant Kit. RNA was treated using RNase-Free DNase I Set (Qiagen). The quantity and quality of the RNA was measured by Nano Drop ND-1000 spectrophotometer (Nano Drop Technologies, USA).

Fluorescent DD was performed with the RNAspectra kit (GenHunter, Nashville, TN, USA) in duplicates as all reactions were performed with both red (rhodamin) and green (fluorocin) fluorescence in parallel. The mRNA in the total RNA samples was converted into DNA by reverse transcriptase with anchor primers (H-T11A, H-T11G, or H-T11C). The resulting cDNA was template and PCR products were amplified by DNA Polymerase DyNAzymeII (Finnzyme, Espoo, Finland) using the three different anchor primers, respectively, for each arbitrary primer in separate reactions (for details see Supplementary Table 1). The RNA spectral kits used were red and green no. 1, 4, 5, and 8. Four primers were in addition designed on conserved areas of polygalacturonase and β -1,3-glucanase and used in the AFLP DD analyses. These primers were added since these two enzymes are associated with cell wall breakdowns during abscission. These primers were PG-A 5'-AAGCTTATTATGGAGC-3', PG-T 5'-AAGCTTATTGGAGC-3', GluC 5'-AAGCTTAT GGAATG-3', and GluC 5'- AAGCTTATGGCATG -3'. The amplification products were separated on 6% denaturing polyacrylamide gels casted between low fluorescence glass plates (Amersham Bioscience). Parallel amplification products



(fluorocin or rhodamin) were separated on different gels and scanned on Typhoon 8600 (Amersham Bioscience, UK) using the following laser settings: fluorocin; excitation 495 nm, emission 520 nm, green laser (532 nm), emission filter 526 SP and rhodamin; excitation 570 nm, emission 590 nm, green laser (532 nm), emission filter 580 BP 30. Kapton tape (Amersham Bioscience, UK) was used for gel orientation. The digital gel image was printed on paper size 1:1 and used for gel orientation and band identification. The AFLP gels were scored visually

in duplicates, and the differentially expressed bands excised from the gel. DNA from the fragments was eluted in distilled water, precipitated and reamplified by PCR as described by the fluorescent DD kit manufacturer (GenHunter, USA). The PCR product was purified on an agarose gel. Single bands larger than 200 bp were excised and subcloned into plasmid pCR 2.1-TOPO (Thermo Fisher Scientific, USA) and chemically transferred into Top10 *Escherichia coli* cells as described by the manufacturer. Twelve positive *E. coli* colonies were selected,

restreaked, and analyzed by colony PCR. The inserts were confirmed by separating the PCR products on an agarose gel. Plasmids from eight *E. coli* clones were prepared using Montage plasmid miniprep 96 (MERK Millipore, Germany) and Jetquick Plasmid Purification Spin Kit (Genomed, Germany). The insert was sequenced with BigDye Terminator Cycle Sequencing Kit v3.1 and ABI prism 3100 (MERK Millipore, USA). Sequences were visualized and processed in BioEdit sequence alignment editor (Hall, 1999).

Bioinformatics and Putative Homology Identification of Sequences

To identify homologous sequences of those differentially expressed during induced secondary pedicel abscission in poinsettia, different Blast [BlastN 2.2.30+, Database GenBank no (All GenBank + EMBL + DDBJ + PDB sequences) and standard settings] methods were used (Altschul et al., 1990). We also used Blast2Go to examine the ontology. All the Blast searches were repeated on August 30 2015. Most commonly used was Discontinuous MegaBlast (Morgulis et al., 2008), BlastN, and BlastX 2.2.32+.

Real-Time qRT-PCR for Verification and Quantification

Real-Time qRT-PCR primers were constructed using Primer Express (Thermo Fisher Scientific, USA; Supplementary Table 1). The primers were tested on both cDNA and genomic DNA. Real time qRT-PCT analyses for the short sequences were performed with a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific,) using SuperScript III Platinum Two-Step qRT-PCR Kit (Thermo Fisher Scientific). Transcript levels were normalized using poinsettia 18S primer pair to make correlative gene expression measurements (Table 1). All reactions were done in triplicate using two different biological preparations.

qRT-PCR reactions for the whole gene sequences were performed with a 7700 Real time PCR system (MERK Millipore) used Platinum® SYBR® Green qPCR SuperMIX-UDG with ROX according to the manual (Thermo Fisher Scientific). The qRT-PCR was carried out in 25 μ l reactions using 2.5 μ l of diluted template, 0.5 μ l of each primer (stock 10 μ M- final 0.2 μ M) and 1x SYBR Green reaction mix. Template, cDNA, were diluted 10^{-1} and 10^{-4} for the reactions included RACE-primers and 18s primers, respectively. Triplicate repeats of each reaction and a template control of nuclease free water was carried out. Amplifications were performed with the following program: 95°C for 2 min followed by cycles of 95°C for 15 s and 60°C, 30 s. After amplification a melting curve analysis was performed. An internal reference dye, ROX, was included in the Platinum SYBR Green buffer to normalize the fluorescent reporter signal in real-time quantitative RT-PCR.

Whole Gene Sequencing by 5'Rapid Amplification of cDNA Ends (5'Race)

The total RNA from the AZ-tissue in poinsettia was used as template to synthesize first strand cDNA in a reverse transcription reaction using modified oligo (dT) primer. Gene-specific primers (GSP) were constructed from seven of the

DD-sequences, using Primer 3 Software (<http://frodo.wi.mit.edu>). For the GSP to find the correct cDNA-sequences the RACE reaction was optimized to isolate the complete gene sequence. The seven sequences were picked on the basis of showing interesting DD differences, but too short for qRT-PCR and Blast searches initially. The primers used are shown in Table 2.

The 5'-RACE was performed according to BD SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, USA). The RACE products were characterized by cloning and sequencing. The 5'RACE products were cloned into the pCR®4-TOPO vector and transformed into competent TOP10 *E. coli* cells (Supplementary Table 2). The inserts were sequenced to verify that the amplified product had a segment of the same sequence as in the DD product and to obtain sequence information from the RACE product and its orientation in the 4-TOPO vector (Supplementary Table 3).

RNA *in situ* Hybridization of Poinsettia and Pea

Flower buds of poinsettia ‘Lilo’ induced for abscission and control plants were cut into small pieces (2–3 mm-thick) which were immediately fixed using 4% paraformaldehyde in sodium phosphate buffer pH 7.0 and 0.1% (v/v) Tween 20, under vacuum for 1 h, and left overnight at 4°C. After fixation, samples were washed in saline, dehydrated through a graded ethanol series, and embedded in paraplast (Sakura, Japan) using Tissue-Tek VIP Jr automatic embedding machine (Sakura, Japan). The 10 μ m-thick sections were collected on poly-L-lysine coated slides.

Similarly, the primary abscission zones of the wild type peas (JI 116 and JI 2822), as well as the two *def* mutant peas (JI 1184 and JI 3020) were harvested and given the same fixation and embedding as the poinsettias above. The *def* gene is a single locus gene (Ayeh et al., 2011) and the abscission zone between the funiculus and the pea was characterized (Ayeh et al., 2009) in both mutants (with abscission-less zones) and both tall and dwarf wild types.

The 20 selected sequences for hybridization were reamplified from the pCR2.1TOPO constructs using their respective AFLP DD primers (Supplementary Table 4) and inserted into the pCR4 TOPO plasmid using the TA overhang cloning technology (Thermo Fisher Scientific, Germany). Single-stranded RNA probes were synthesized after linearization of plasmid DNA, using *NotI* and *SpeI* restriction enzymes for sense and antisense probes, respectively. Sense and antisense probes labeled with digoxigenin (DIG). dUTP were prepared using T3 and T7 RNA polymerases (Roche, Germany), respectively.

The 10 μ m-thick sections on poly-L-lysine coated slides were dewaxed using Histoclear (Cell Path, UK). The sections were treated with Proteinase K (1 μ g ml⁻¹) and acetylated using 0.5% acetic anhydride (Sigma Aldrich, Switzerland) in 0.1 M triethanolamine and followed by washing in PBS solutions and dehydrations using a graded ethanol series. Per slide, 100–200 ng labeled antisense and sense riboprobes were applied in 40 μ l hybridization solutions in a humid chamber for 16 h at 50°C. Hybridization was performed using hybridization solution

TABLE 1 | Real-time RT-PCR results and similarity searches of AFLP DD clones from poinsettia pedicel secondary abscission.

DD#	Primers used	GenBank Acc. #	Size (bp)	Real time RT-PCR results ^a							Discontinuous MegaBlast, BlastN, or BlastX						
				Days after abscission induction							Accession no.	Description	Species	Id (of 100%)	E-value		
				0	1	2	3	4	5	6							
025a	29A	EB647682	194	Yellow	Red	Yellow	Yellow	Orange	Orange	Orange	GQ856147	Unknown mito. genome region	Citrus <i>lunatus</i>	91 ^b	9e-50		
006a	33G	EB647681	269	Orange	Orange	Orange	Orange	Orange	Orange	Orange	XP_007012396	Photosystem II subunit X	Theobroma cacao	86 ^d	3e-12		
220_	29C	EB647701	515	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	AY340642	UVB-repressible protein	Trifolium pratense	79 ^b	5e-31*		
084_	40A	EB647705	456	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002528833	Transcription factor	<i>Ricinus communis</i> *	81 ^b	5e-26		
090a	26A	EB647690	255	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	AEJ07931	Opioid polypeptide	Zea <i>mays</i>	41 ^d	4e-24		
038b	62C	EB647683	233	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_011094475	Organ-specific protein	Sesamum <i>indicum</i>	71 ^c	0.020		
304b	PGTC	EB647707	217	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	AY188755	Atypical receptor-like kinase	Zea <i>mays</i>	86 ^c	8.2 ^{**}		
057b	34A	EB647687	412	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	CP011890	Unknown region of chr. 5	Ovis canadensis	86 ^c	0.005		
208_	27A	EB647706	443	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	HQ874649	Unknown mito. genome region	<i>Ricinus communis</i> *	96 ^b	1e-61		
136b	GlcG	EB647697	447	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	YP_002720125	Cytochrome f subunit (PetA)	Jatropha <i>curcas</i> *	96 ^d	1e-65		
045c	1C	EB647684	347	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	AY794600	Chloroplast tRNA-Leu	<i>E. pulcherrima</i> *	99 ^b	3e-98		
140b	FG0	EB647699	306	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002526333	fK56-binding protein	<i>Ricinus communis</i> *	81 ^b	3e-47		
103_	GlaG	EB647693	434	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002510884	Uridylate kinase	<i>Ricinus communis</i> *	83 ^b	2e-35		
003a	33A	EB647680	279	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002532624	eIF3E (translation initiation)	<i>Ricinus communis</i> *	84 ^b	2e-28		
301_	PGA	EB647702	448	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_012213718	CASP-like protein	Jatropha <i>curcas</i> *	77 ^b	8e-36		
060c	34A	EB647688	304	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	FJ228477	α -tubulin	Betula <i>pendula</i>	92 ^b	2e-21		
320b	PGTG	EB647704	218	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002512412	RNA binding protein	<i>Ricinus communis</i> *	74 ^b	1e-20		
082a	40G	EB647689	269	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_010526012	V-ATPase G subunit 1	Tarenaya <i>hassleriana</i>	83 ^b	2e-09		
101_	GlaG	EB647692	426	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	BT092277	Unknown mRNA	Glycine <i>max</i>	87 ^b	6e-07		
140a	FG0	EB647698	307	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002523025	Putative β -glucosidase	<i>Ricinus communis</i> *	72 ^c	1e-06		
091b	26G	EB647691	247	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002511291	Histone deacetylase	<i>Ricinus communis</i> *	71 ^c	6e-06		
047b	6C	EB647685	236	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	AM932356	Partial tRNA-Leu gene	Typhonium <i>giganteum</i>	73 ^c	1e-05		
130_	GlcG	EB647696	297	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	CP001685	Glucan endo-1,3- β -D-glucosidase	Leptotrichia <i>buccalis</i>	90 ^c	0.019		
304a	8C	EB647686	299	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	XM_002305663	Proteasome beta subunit B family protein	Populus <i>trichocarpa</i>	82 ^b	7e-08		
	GlaG	EB647694	343	Yellow	Yellow	Yellow	Yellow	Orange	Orange	Orange	AY792209	NADH dehydrogenase SU 4L	Ceratitis <i>neosilvatica</i>	81 ^c	3.5		
												No significant matches	—	—	—	—	
												No significant matches	—	—	—	—	

(Continued)

TABLE 1 | Continued

DD#	Primers used	GenBank Acc. #	Size (bp)	Real time RT-PCR results ^a							Discontinuous Megablast, BlastN, or BlastX				
				0	1	2	3	4	5	6	7	Dist ^e	Accession no.	Description	Species
304a	GicG	EB647695	268									-	-	No significant matches	-
304a	GicG	EB673117	249									-	-	No significant matches	-
204c	27G	EB647700	214									-	XM_012216503	Proteasome subunit alpha type-1-B-like	Jatropha curcas*
304a	PGA	EB647703	463									-	-	No significant matches	-

^aSimilarities found in DNA sequences from *chloroplast*, *mitochondria*, *chloroplasts* or *bacteria*. List of primers in Supplementary Materials.

^bVisualization of Real-time RT-PCR results relative to 18S expression of two individual experiments. The highest expression is black and the lowest expression is white, showing decreasing nuances of orange for descending steps of equal size (8 nuances/steps).

^cDiscontinuous Megablast [BlastN 2.2.32+], Database GenBank no (All GenBank + EMBL + DDBJ + PDB sequences) and standard settings 30 August 2015.

^dBlastN [BlastN 2.2.32+], Database GenBank no (All GenBank + EMBL + DDBJ + PDB sequences) and standard settings 30 August 2015.

^eInternal control: Distal part of the abscising pedicel above the abscission zone at Day 0.

*Euphorbiaceae family member.

^fFrom BlastX in Sept 2012, incl because they give an additional indication of function.

TABLE 2 | AFLP DD and RACE primers, results and similarity searches for full-length genes in poinsettia secondary abscission.

DD#	Primers used for DD	Primers used for 5' RACE	AFLP DD results ^a							BlastX ^b					
			0	1	2	3	4	5	6	7	Days after abscission induction	Accession no.	Description	Species	Id (of 100%)
135	5'gacttccgtccatccatccatc 3'	+	-	-	-	-	-	-	-	-	XP_002518733	Polyadenylylate-binding protein 2	<i>Ricinus communis</i> *	86	8e-155
133a	5'cgagtgccatgcacctgaaacct 3'	-	+	-	-	-	-	-	-	-	NP_176471	Lys-specific histone demethylase 1-1	<i>A. thaliana</i>	73	8e-138
											Predicted	Lys-specific histone demethylase 1-1	<i>Prunus nume</i>	79	0.0
108	5'ccccaggcaacaataaataaggc 3'	+	-	-	-	-	-	-	-	-	XP_002530011	V-SNARE protein	<i>Ricinus communis</i> *	91	2e-126
122	5'catcccggatggatccatatacg 3'	-	+	-	-	-	-	-	-	-	XP_012085745	Bidirectional sugar transporter N3	<i>Jatropha curcas</i> *	76	4e-136
82b	5'ggaaacaacccggagaatgtctac 3'	-	+	-	-	-	-	-	-	-	XP_002522811	Glycine-rich RNA-binding protein	<i>Ricinus communis</i> *	74	1e-44
105	5'ggccatggcaatcacaaaccatc 3'	+	-	-	-	-	-	-	-	-	NP_198917	DNA-directed RNA Pol. II su. K	<i>A. thaliana</i>	88	6e-24
32a	5'gtcttagtcatcaaccccaaag 3'	-	+	+	+	+	+	+	+	+	NP_001077782	DVL3	<i>A. thaliana</i>	58	1e-5

^aSimilarities found in DNA sequences from *chromosomes*.

^bReconstruction of visual screening of the result where + is upregulated and - is downregulated.

^cBlastX [BLASTX 2.2.32+] Database no and standard settings 30 August 2015. Reading frame (RF) +2 of the Acc. # NP_198917, NP_001077782, XP_002522811 and XP_002518733; RF +1 of the Acc. # XP_012085745 and NP_176471; RF +3 of the Acc. # XP_002530011.

*Euphorbiaceae.

containing 50% formamide, dextran sulfate, Denhardt's solution, tRNA, and 10 × hybridization buffer (3 M NaCl, 0.5 M Na₂HPO₄, 10 mM EDTA). After hybridization, sections were washed in SSPE and NTE buffer. After RNase treatment, sections were blocked and probe was detected using anti-digoxigenic-alkaline phosphate-coupled antibody. The hybridized DIG-labeled probe and target was detected by anti-DIG antibodies conjugated with alkaline phosphatase (Butler et al., 2001). Sections were visualized by applying 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and nitroblue tetrazolium salt (NTB; Promega, USA) diluted in alkaline phosphate buffer. Sections were photographed using bright field optics (Leica, Germany).

RESULTS

Genes Differentially Expressed during Secondary Abscission in Poinsettia and their Putative Identity

To elucidate on secondary abscission related genes, AFLP DD analysis provided a database of poinsettia cDNA sequences involved in AZ development and the induced secondary abscission process. The total RNA samples used in the AFLP DD screening were prepared from AZ tissue at eight different stages: every 24 h from Day 0 (control) and until abscission on Day 7. After 3–4 days, the first visible signs of the forthcoming abscission event could be seen as a lighter green band at the place of the AZ and the onset of senescence related color changes of the distal end of the flowers (Figures 2B–E). On Day 7 the distal end normally abscise after a coordinated separation of the cell layers in the AZ. Generally, the induction of flower abscission in poinsettia by decapitation (Figure 2A) has an accuracy of ±1 day (data not shown).

Our screen gave us 126 gene transcripts, either up- or downregulated, an example gel from the DD is shown in Figure 4. The sequences which were >200 bp (74 sequences) were also run in a Real-Time quantitative assay (qRT-PCR), a selection of the most interesting are presented in Figure 5. Seven others, which were of particular interest due to their expression profiles in the DD, were then put through a 5'RACE and whole gene sequencing to include them in assay; making a total of 81 sequences tested. Almost half of the first 74 (35) were confirmed to be significantly differentially expressed relative to the ribosomal subunit 18 (18S) in the qRT-PCR assay. BLAST analysis gave a decent similarity hit for 29 sequences, where 18 matched beyond an expectation value of E⁻⁴ (Table 1). Eight of the sequences had no matches in databases, some of these were strongly up- or downregulated as early abscission events. They are putatively very interesting gene sequences to study further to find their specific functions, as potentially unknown AZ-genes. From the 5'-RACE-extended sequences we obtained matches beyond E⁻⁵ for all seven, some these also highly relevant (Table 2).

Blast “Top-hits” species distribution chart obtained by performing Blasts to NCBI, using Blast2go program, showed only two hits to *Theobroma cacao* and one hit to the following species: *Aeglops tauschi*, *Nicotiana sylvestris*, *Zea mays*, *R. communis*,

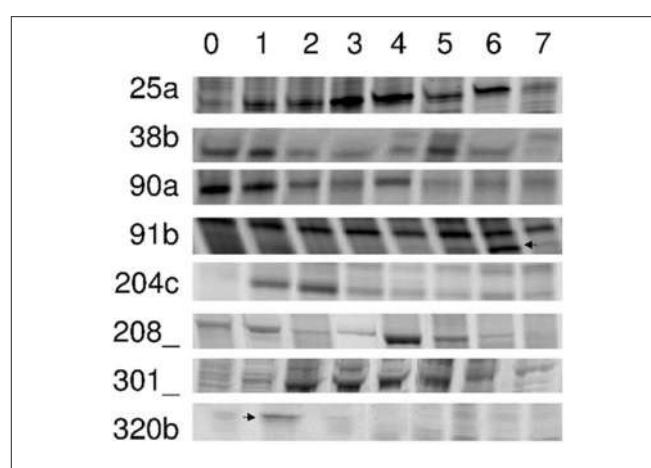


FIGURE 4 | DD-PAGE sections cDNA PCR products that were differentially expressed. Poinsettia flower abscission zone total RNA samples were prepared from Day 0 to Day 7(0–7). Left column indicate specific clone numbers. Arrows are indicating specific bands isolated.

Datura stramonium and *Medicago truncatula*. Of these species, *R. communis* belongs to the same family as poinsettia, namely Euphorbiaceae. Examining Table 1, *Jatropha curcas* also belongs to the same family. Although many exact hits in Tables 1, 3, Jatropha does not reach as high overall as the abovementioned species, and does not have more than one hit in Blastx.

Table 3 provides the percentage of up- and down-regulated genes belonging to each proposed phase in abscission of poinsettia. The majority of the significant differentially expressed genes were upregulated during the first or second day after the induction of abscission, in phase I. At this stage, it is not possible to see any changes in the anatomy of the pedicel or any color changes. Many of the genes can be functionally classified to energy metabolism, cell growth and division.

We have also identified two sequences similar to cell wall degrading enzymes (glucan endo-1,3-β-glucosidase and another putative β-glucosidase), some sequences, which can be associated with endoreduplication (fk506-binding protein and α-tubulin), as well as a putative signal transducer.

Our specific primers toward polygalacturonase and β-1,3-glucanase, showed differences in the AFLP screens, but could not be identified as such through the BLAST searches. We thus consider them as successful arbitrary primers that were all able to produce DD bands picked up for identification. Regarding the 32 arbitrary primer from GenHunter, large differences could be observed in their ability to give differentially displayed bands, only 10 out of 32 primers did. The 36 different arbitrary primers used would statistically give ~75% of all expressed cDNAs, based on calculations made by Yang and Liang (2004). In our hands, fluorescent probed primers were just as effective as radioactive labeled primers (data not shown), corresponding to that the two methods have comparable sensitivity (Ito et al., 1994). The rhodamin signal was generally slightly stronger than the fluorocin signal. Figure 4 shows eight examples of selected DD bands. From all DD bands, we obtained 126 sequences after cloning the reamplified DD bands into a pCR 2.1-TOPO vector.

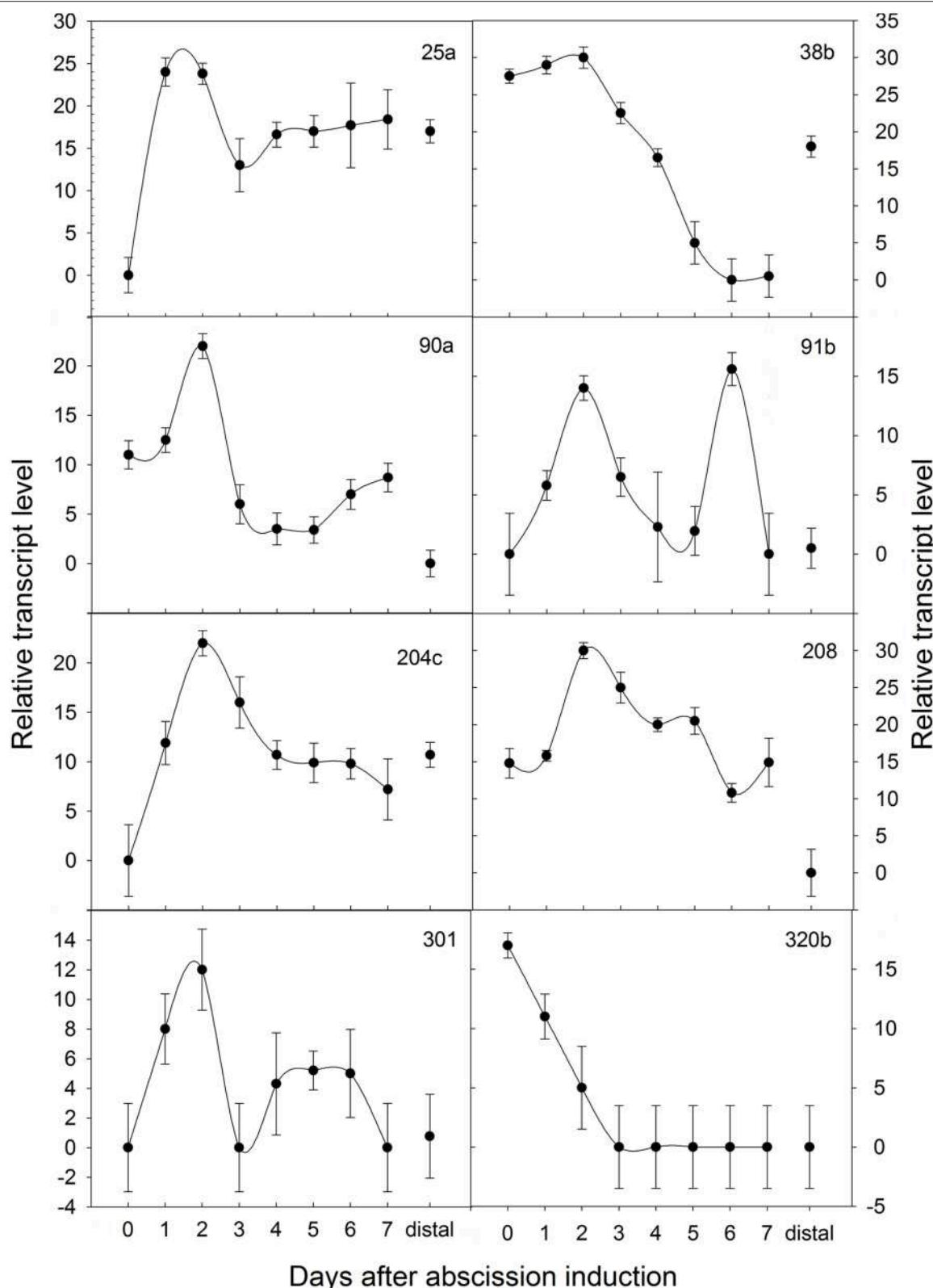


FIGURE 5 | Temporal expression patterns of poinsettia DD clones monitored by Real-Time qRT-PCR. $\Delta\Delta Ct$ on the y axis refers to the fold difference of a particular DD clone mRNA level relative to its lowest expression. Expressions were normalized to the 18S ribosomal RNA endogenous control during an induced abscission process of poinsettia pedicel, whereas distal is an internal control sample dissected from above the AZ.

TABLE 3 | Proposed division of abscission phases in poinsettia (0-IV), the corresponding # of days after decapitation and the percentage (%) of the 225 DD bands, which fall into each phase, either up- or downregulated.

Corresponding days after decapitation	0 (Control)	0-2	2-4	4/5-7	7
Phases in abscission	0	I	II	III	IV
% sequences Upregulated	6	3	8	16	2
			4		
			19		
			11		
% sequences Downregulated	29	3			

126 of these were successfully cloned and sequenced.

When sequenced, product lengths were between 180 and 520 bp (Munster, 2006). Some bands where reamplified as a shorter sequence than the one initially picked out from the AFLP DD gel, this can be explained by the fact that the primers used are arbitrary and the PCR conditions are changed from AFLP to reamplification.

An overview of all bands picked up in the AFLP DD (225 in total) shows that 29% (65/225) of the clones derived from DD bands are downregulated after Day 0, in Phase 0.3% (7/225) are down-regulated after Day 2, after Phase I. Approximately 6% (13/225, Phase 0) and 3% (7/225) of the bands were upregulated at Day 1 and 2, respectively, taken together about 9% of the DD bands were upregulated in Phase I. During Phase II (Days 2–4), 8% (18/225) of the DD bands were upregulated. On Days 3–5 (Phase II-III), 4% (10/225) of the DD bands were upregulated. During Phase III (Days 4/5–7), 16% (35/225) of the DD bands were upregulated. Two percent (5/225) of the DD bands were upregulated at Day 7, in Phase IV. In addition, 19% (43/225) and 11% (24/225) of the bands were seen upregulated at Days 1–7 (after Phase 0) and Days 2–7 (after Phase I), respectively. Of these bands, 126 were successfully cloned and sequenced.

For all sequences, similarity to known protein and DNA sequences were found using different Blast methods (Discontinuous MegaBlast, BlastN and BlastX) and standard settings (Tables 1, 2). Twenty-two of 29 sequences generated putative similarities, out of which 13 have a very high similarity (*E*-value < e⁻²¹), five have high similarity (*E*-value e⁻¹⁰–e⁻⁴), and four have a less high similarity (*E*-value e⁻³–8.2). The latter ones are also reported due to the fact that short sequences can show relevant hits with high *E*-value (Information, T.N.C.F.B., 2012). This can be illustrated by the similarity hit on Glucan endo-1,3-β-D-glucosidase in *Leptotrichia buccalis* by sequence 91b (Acc.# EB647691) with an *E*-value of 8e⁻³ (Table 1). Two of the sequences did not have a significant similarity, while two were annotated as unknown mRNA and one as unknown mitochondrial region. In addition to the possibility of the two with no match, being a new and unknown sequence, a contributing factor might be the use of poly A selective primers, amplifying the non-translated UTR-3' region.

The identified DD genes from Tables 1, 2 can be grouped according to the following biochemical functions: (1) **Transcription** [Transcription factor (XM_002528833)

and Opie3 pol protein (RTV_2, AEJ07931) and DNA-directed RNA Pol. II su. K (NP_198917)]. (2) **Signal transduction** [Organ-specific protein/Atypical receptor-like kinase (XM_011094475/ AY188755), DVL3 (NP_001077782)] and (3) **translation/protein synthesis** (eIF3E (translation initiation (XM_002532624), Chloroplast tRNA-Leu (AY794600), RNA binding proteins (XM_002512412 and XP_002522811), Lys-specific histone demethylase 1-1 (NP_176471), and Polyadenylate-binding protein 2 (XP_002518733), Proteasome subunit alpha type-1-B-like (XM_012216503)]. (4) **Energy** (V-ATPase G subunit 1 (XM_010526012) and Bidirectional sugar transporter N3 (XP_012085745), energy metabolism (photosynthesis; Cytochrome f subunit (YP_002720125), respiration; NADH dehydrogenase SU 4L (AY792209), and unknown mitochondrial DNA regions (GQ856147 and HQ874649) and proteasome metabolism (Proteasome subunit β type-7-B-like (XM_002305663)). (5) **Cell growth and division** [α -tubulin (FJ228477), V-SNARE (XP_002530011), and Histone deacetylase (XM_002511291)] and (6) **Cell structure** [cell wall degradation (Glucan endo-1,3-β-D-glucosidase (CP001685 and XM_0022523025)]. (7) **Defense/Disease** [Photosystem II subunit X/UVB-repressible protein (XP_007012396/AY340642) and an uncharacterized membrane protein (CASP-like protein, XM_012213718)]. Sequence 136b with the best similarity on the Cytochrome f subunit (1e⁻⁴⁷) also has an alternative and interesting similarity in immunophilin (NP196845 (4e⁻²⁴).

It is noteworthy that the length of the sequence did not seem to have an influence on the *E*-value (Table 1). Only a few sequences a little longer than 200 bp were not able to give any similarities to the GenBank databases using different available programs (August 2015).

RNA *in situ* Hybridization of Selected Sequences

Twenty of the DD sequences from Table 1 were also run through a RNA *in situ* hybridization to investigate further the spatial and temporal gene expression for these. The twenty were picked on the basis of which of the 29 gave good probes for RNA *in situ*. All the RNA *in situ* results verified them being expressed in the AZ and not elsewhere (data for six of them are shown in Figure 6). These six have been chosen based on the results from the other RNA *in situ* experiments, where these riboprobes were hybridized with sections of the four pea accessions from John Innes Centre, UK. These six riboprobes are on top of Table 1 and the DD # are in bold.

The six genes (6a, 25a, 38b, 84, 90a, and 220) are all differentially expressed both in poinsettia and in pea. The positive gene expression in antisense samples (Figures 6A,C,E, 7A,B,E,F) are seen as a dark blue coloring on the cells compared to no expression (light blue cells) in sense samples (Figures 6B,D,F, 7C,D,G,H). Ayeh (2008) showed that poinsettia riboprobes could be expressed in the AZ or the ALZ of the wild type and *def*-mutants (both the tall and the dwarf accessions). We found that five of these riboprobes were present only in the mutant peas (6a, 25a, 38b, 84 and 220), while only one was expressed in the wild type (90a).

Similarly, Rasolomanana (2008) was able to demonstrate through RNA *in situ* hybridization on sections of poinsettia pedicels that two of these six riboprobes (90a and 38b) are

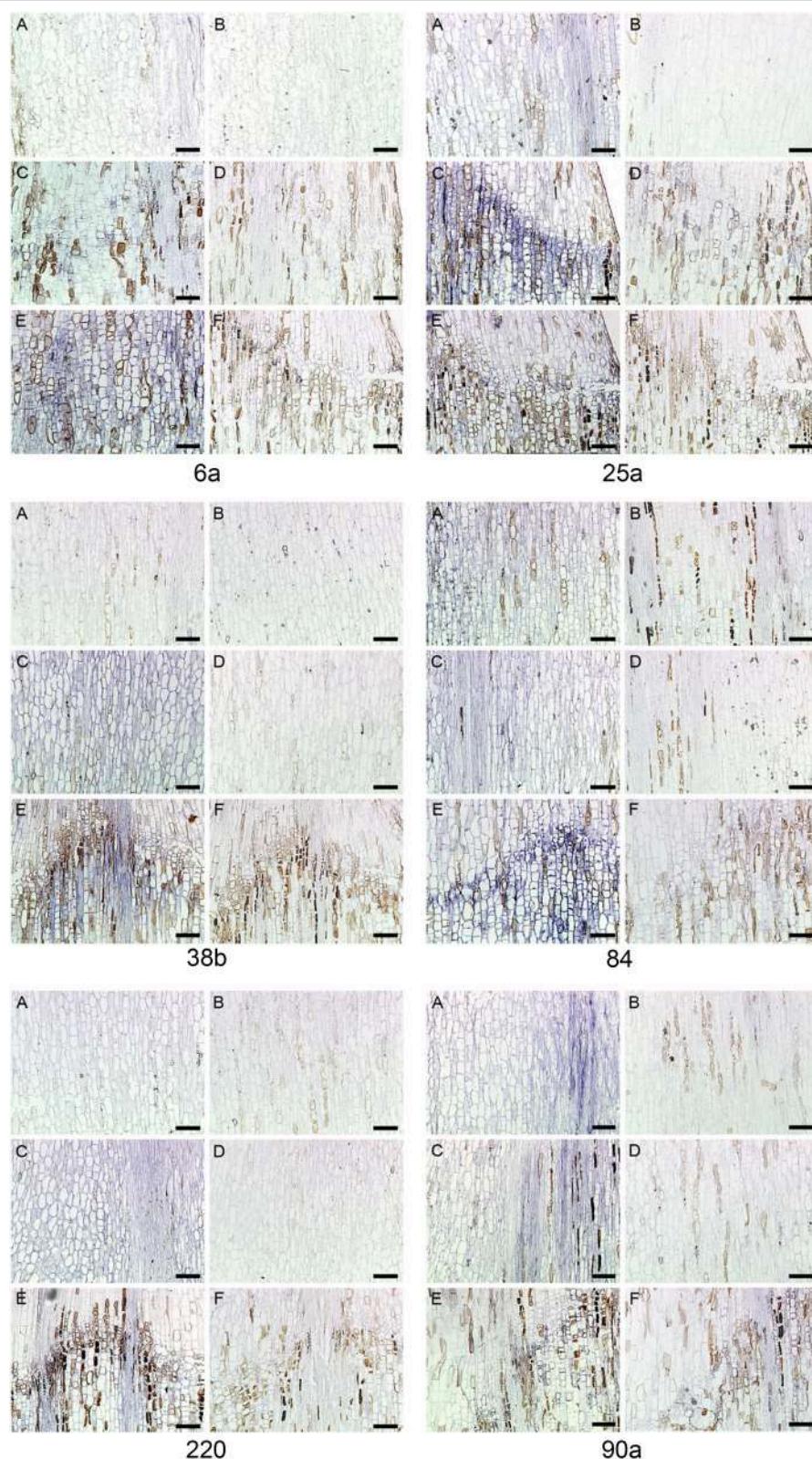
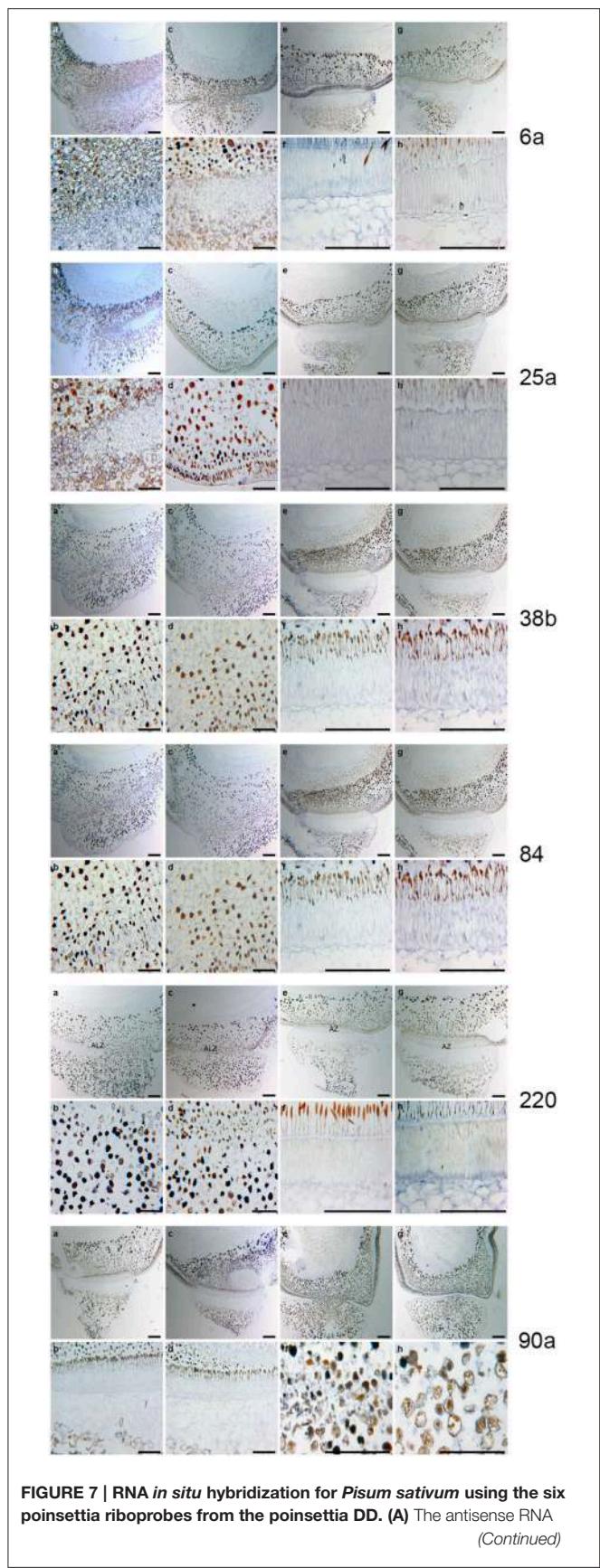


FIGURE 6 | RNA *in situ* hybridization six transcripts in *Euphorbia pulcherrima* (poinsettia) flower pedicels. Control Day 0 (**A, B**), Day 5 (**C, D**), and Day 7 (**E, F**) after abscission was induced by decapitation. Longitudinal sections were hybridized with antisense (**A, C, and E**) or sense (**B, D, and F**). The six DD riboprobes # 6a, 25a, 38b, 84, 220, and 90a. Bars: 100 μ m.

**FIGURE 7 | Continued**

localization of the *def* mutant Jl 1184 for each of the six DD riboprobes # 6a, 25a, 38b, 84, 220, and 90a. **(B)** are the higher magnification of the As. **(C)** are the sense (control probe) of *def* Jl 1184. **(D)** higher magnification of **(B)**. **(E)** antisense *Def* wild type Jl 116. **(F)** higher magnification of **(E)**. **(G)** sense *Def* wild type Jl 116. **(H)** higher magnification of **(G)**. Scale bars: **A, C, E, and G**, 12.5 μ m; **B, D, F**, and **G**, 25 μ m.

involved with the onset of abscission during the first 2 days. Another two (84 and 220) are associated with early-mid-term expression of the process. The last two (6a and 25a) were also expressed toward the end of the separation.

DISCUSSION

E. pulcherrima (poinsettia) can be used as a model system to elucidate on secondary abscission, induced by decapitation of the flower bud and studied through the 7-day period before abscission. When examining the natural abscission of a poinsettia bud, (Figure 3C) with that of a decapitated bud (Figure 3B), we can clearly see the similarities. The abscission process starts from the epidermis around the whole pedicel, and as the AZ develops toward the core of the pedicel, we see that the pedicel has continued to grow through cell division and cell elongations of the surrounding tissues, and the AZ is pushed upwards, forming a cone shaped stub as the bud falls off on Day 7. This cone shaped stub is very characteristic of poinsettia abscission. The control bud (Figure 3A) has not been decapitated and is the same age and from the same third order inflorescence as C. This bud has not reached the mature stage of the flower in the natural abscission (Figure 3B) yet, and shows no sign of developing AZ.

Although many plants have flowers and/or other plant parts that abscise, we know of few systems as accurate and foreseeable as this inducible poinsettia model system. Other model systems, as pea leaves (McManus et al., 1998), impatiens (Warren Wilson et al., 1986), and tomato (Kalaitzis et al., 1995), are all prepared explants *in vitro* and not *in planta*, as for this poinsettia model system. This study is also unique in the sense that we have never seen a comparison between so distantly related species showing consistent results with respect to elucidating upon the genes involved in abscission, and at the same time comparing a system for primary abscission using mutants with an inducible system for secondary abscission. There is a large potential for further elucidation into the significance of the genes in this study, since poinsettia and pea are so far apart evolutionarily (Bennett et al., 2000). We have revealed at least 18 putative genes involved in the poinsettia secondary abscission process, many of which are expressed differentially also for primary abscission in peas. Earlier studies have tried to unravel the primary abscission process on a broader level using *Arabidopsis* as model (Wang et al., 2006), recently also González-Carranza et al. (2012).

Abscission is a complex process to unravel and we have compared poinsettia abscission with abscission-less (AZL) mutants first in *Arabidopsis*, with the interesting gene *inflorescence deficient in abscission* (*ida*) T-DNA mutant, identified as a novel putative ligand in plants (Butenko et al.,

FIGURE 7 | RNA *in situ* hybridization for *Pisum sativum* using the six poinsettia riboprobes from the poinsettia DD. (A) The antisense RNA (Continued)

2003). The 35S:IDA line overexpressing *IDA* results in earlier abscission of floral organs and additionally abscission of organs that are not normally shed, like the whole silique. There is evidence that *IDA* encodes for arabinogalactan (Stenvik et al., 2006), a protein also found in naturally abscission surfaces. In our hands, poinsettia does not have any *ida* or *ida*-like genes. Although similar abscission processes take place in both species, the organs that abscise are not the same, since *Arabidopsis* sheds its petals, while poinsettia abscises the whole floral organ by developing an AZ on the pedicel. In *Arabidopsis* the pedicels do not abscise and there is only a rudiment of an AZ left at the base of the pedicels in this species, showing that AZ in *Arabidopsis* petals are primary (pre-defined). When we failed to find *ida* in poinsettia, we turned to the AZL *def* mutants in pea (Ayeh et al., 2011). The pea system demonstrated expression of highly conserved poinsettia sequences involved in pea abscission. Although poinsettia and *pea* are distantly related and the sequence homology is not expected to be high, we used our poinsettia *in situ* probes on pea *def* mutant tissue and obtained positive hybridization results (Ayeh, 2008).

Gene Sequences in Common for Pea and Poinsettia during Abscission

We will briefly discuss the genes with sequences common to poinsettia and pea abscission, i.e., common for primary and secondary abscission in our models. The potentially most important gene sequence could be 90a (XM_011094475/AY188755). This riboprobe is only expressed in the wild type pea, and is upregulated during the first 2 days after poinsettia decapitation. This is a strong indicating that this gene is necessary for abscission to takes place, both in pea and poinsettia. Blast search revealed that 90a is close to an accession encoding an organ-specific protein (XM_011094475). Additional information, from the Blastx we did in 2012, gave a high similarity also to an atypical receptor-like kinase (AY188755). Since this is such a prominent gene sequence in our results, with a possibility that this may be one of the genes controlling abscission in plants, it would be very interesting to follow up on this.

DD 38b is a riboprobe expressed from the start of the abscission process and then downregulated from Day 3 in poinsettia. It is also expressed in the non-abscising mutants of pea. This suggests that this gene could be prohibiting abscission from taking place and the early expression pattern suggests a putative equally important, but opposite role as 90a in starting the cascade of events leading to abscission.

DD 6a (description Photosystem II subunit X) is found in both poinsettia and pea. Liao and Burns (2012) have found that Photosystem II subunit Q, X, P, and K had a two-fold increase in huanglongbing (HLB)-infected *Citrus sinensis* trees, where this infection was highly associated with both leaf and fruit abscission. They speculate whether this could be due to a breakdown of the photosynthetic apparatus since phloem plugging leads to starch accumulation in the leaves. In poinsettia, this gene is upregulated in Day 1 (after decapitation), before any visible signs of the AZ. In an AZ there is certainly a stop in the transport of photosynthetic products as the detachment is starting from the outside of the pedicel and moves inwards as the abscission process progresses

during the week from decapitation to detachment, as can be seen in **Figure 2**, however, this is not happening until Days 4–6. Our results suggest that it is the gene for Photosystem II subunit X which is shutting down photosynthesis as an early event, and then there is a breakdown of the transport of sugars, causing the pedicel to start to yellow and senesce after another 2–4 days.

DD # 084 is annotated to Opie3 pol protein found in *Zea mays*. It is a receptor kinase, belonging to a large gene family in plants with more than 600 members in *Arabidopsis*. This gene family is said to be involved in a broad range of developmental processes, including abscission (Yu et al., 2003).

DD # 220 has a high similarity (81% and 2e-26) to a putative transcription factor from *R. communis*, also belonging to the *Euphorbiaceae* family, making this similarity even more likely. It has a strong upregulation in Day 2 and may be another putative key regulator. Transcription factors are often the key to the whole cascade of events for such complicated processes as abscission. As yet, we do not know which transcription factor it could be.

DD # 6a is expressed all through the abscission event, but is at its strongest upregulated on Day 1 of all the riboprobes, except # 82a, which also has its peak on Day 1. This is very early on, only 24 h after decapitation. It resembles a UVB-repressible protein from *Trifolium pretense*. This may have implications for the handling of stress in plants, as it is well-known that UVB induces stress and DNA damage in organisms.

DD # 25a is barely expressed on the day of decapitation, but expression increases strongly the day after. It resembles a gene from an unknown mitochondrial region in *Citrullus lanatus*. Very hard to say anything more on what this may imply, but the sequence being expressed in both poinsettia and wild type pea, suggests a closer look at the function.

A Proposed New Phase Division for Secondary Abscission

The pioneers of using the model system in *Arabidopsis* for studying abscission (Bleecker and Patterson, 1997), proposed to divide the abscission process into phases. They described this very well for primary abscission in *Arabidopsis*. However, we find that the development and morphology during induced secondary abscission in poinsettia needs to be divided with a higher resolution. We describe in a previous study the sequential transformations in the detection of cell wall polysaccharides during this induced abscission event (Lee et al., 2008) in the poinsettia model system. Based upon these results, as well as the present results on gene expression and the sequence of events here, we propose six phases: from Phase 0 to Phase V (**Table 3**). The induction phase (phase 0) is the induction and localization of the secondary abscission zone position, the following phase I is the where the cells become receptive to the signaling of the onset of the abscission process and where we observe the first biochemical changes (Lee et al., 2008). Phase II is characterized by cell divisions and the onset of rounded cells and continued biochemical changes, as can be seen by color changes in the distal part of the pedicel. Major biochemical restructuring characterize Phase III, where the ring marking the AZ is clearly visible and the analysis show de-esterification of homogalacturonan and the breakdown of cellulose, arabinose and other pectins preparing

for the separation of the organ (Lee et al., 2008). Following the previously described breakdowns, the cell walls are enriched with xylan and lignin, probably to seal off the AZ once the distal part has been removed. We propose to call this Phase IV. Finally, Phase V represents the detachment of the organ. We have related the annotated gene sequences below to these proposed, more detailed phases of abscission.

Annotated Gene Sequences Upregulated at the Onset (Phase I)

The genes differentially regulated just after the time of decapitation are putatively the key regulators of abscission. Naturally, these are of utmost interest. Main indications from the gene expression analysis (DD + qRT-PCR + RNA *in situ*) gives the following zone specific regulations: The energy state is especially upregulated on Day 1 and 2, indicated by several hits on photosystem II [XP_007012396 and XM_002512633 (fk506-binding protein)] and mitochondrial genome (GQ856147 and HQ874649) related genes/proteins. Protein metabolism is specifically upregulated during Day 2 and 3, indicated by hits on a transcription factor (XM_002528833), transportRNA (AY794600), translation initiation (XM_002532624), and RNA binding protein (XM_002512412) related genes/proteins. There is initially (Days 0–2) an increase in cell divisions going on, but this is followed by downregulation as indicated by Histone deacetylase (XM_002511291) and α -tubulin (FJ228477). All these up-and downregulations are as expected from previous observations in the microscope on cells changing shape as the ordinary pedicel cells are initiated to form AZ cells, which are smaller, more dense and less vacuolated (**Figure 3**).

From **Tables 1, 2**, Day 2 is regulatory very important, where overall central proteins (strongly upregulated) can be the proteasome-like genes upregulated Day 2 (XM_012216503 and XM_002305663). We also find the V-ATPase G subunit 1 (XM_010526012) really strongly upregulated on Day 2. Burr et al. (2011) support that V-ATPase is associated with abscission in *Arabidopsis*.

Another strong upregulation is the Atypical receptor-like kinase from *Z. mays* (AY188755). Receptor-like kinases regulate a range of signaling pathways, many of which have been shown to be involved in abscission, as reviewed by Taylor and Whitelaw (2001). Since then, numerous groups have shown the importance of receptor-like kinases in abscission, examples can be: in *Arabidopsis* (Wagner and Kohorn, 2001; Butenko et al., 2003; Diévert and Clark, 2003; Cai and Lashbrook, 2008; Cho et al., 2008; Liljegren et al., 2009; González-Carranza et al., 2012) and in tomato (van der Hoorn et al., 2005).

We had another hit for an Organ-specific protein from *Sesamum indicum*. This up-regulation is extreme and it is bound to be of importance. This is one of the common genes for poinsettia and peas described earlier (90a) and hence extremely interesting.

A much better described hit is the Lysine-specific histone demethylase 1-1 (**Table 2**) from *Arabidopsis* (DD # 133a). This enzyme is closely associated with the gene Flowering Time Locus (FLC), which is in the core of events for *Arabidopsis* flowering in the complex pathway involving particularly temperature and day

length. The enzyme is involved in H3K4 methylation of target genes, including FLC and FWA. We also had a predicted hit ($e = 0.0$) with *Prunus mume* with the same enzyme, confirming it.

Annotated Gene Sequences Downregulated at the Onset (Phase I)

The downregulated proteins can be equally important, such as Cytochrome f subunit (PetA; YP_002720125). Cytochrome F is a crucial component of the photosynthetic electron transport chain of higher plants. The subunit PetA is one of four major subunits. It seems like photosynthesis is restricted already after Day 0, the day of decapitation. This makes sense as the bud has started on its road to doom, and no longer needs to spend energy on photosynthesis. The yellowing of the pedicels become visible around Days 3–4 and could be due to the downregulation of this gene.

Another downregulated gene (DD #103) codes for CASP-like protein (XM_012213718). Roppolo et al. (2014) recently reported on *AtCASPL1D2*, a gene encoding a CASP-like protein expressed in the AZ of *Arabidopsis*. Another gene, *AtCASPL2A2* is reported to be expressed in the floral organ abscission zone (González-Carranza et al., 2012). Roppolo et al. (2014) states that: “*AtCASPL5C3* is expressed in the floral organ abscission zone as well, but its early expression in floral buds precedes the activation of the abscission zone and the expression of most of the genes known to be involved in floral organ shedding.” This supports our hit on XM_012213718 from *Jatropha curcas* (in the same family as poinsettia, *Euphorbiaceae*) as important for organ abscission and the sequence of events precedes the activation of the AZ. We deduce from this that CASP-like proteins may be important for the AZ to be able to be activated, a prerequisite for abscission, perhaps.

The combination of protein biosynthesis and degradation suggests a protein change context during the early steps of poinsettia pedicel leaf abscission. (Agustí et al., 2012) found expression in the lateral abscission zone for three putative E2 ligase proteins. Uridylate kinase (XM_002510884) is an enzyme in pyrimidin metabolism. The Unknown protein (CP011890) DD # 45c) has a very strong downregulation.

The last sequence downregulated is a riboprobe with resemblance to the V-SNARE proteins (DD # 108, from **Table 2**). These are proteins involved in membrane assembly and hence important in growth and development. **Table 2** shows that this gene is downregulated just after Day 0, which is a remarkable quick response to decapitation.

Other Annotated Gene Sequences Involved during Later Phases II–V

There is much evidence that glucanases are involved in the cell wall degradation necessary to break down the middle lamella and enable cell separation (Roberts et al., 2002). It is, therefore, hardly surprising to find Glucan endo-1,3- β -D-glucosidase and a Putative β -glucosidase (DD # 82a) popping up in the DD analysis. The putative Putative β -glucosidase (DD # 82a) is upregulated on Days 1–2, very early on, possibly to start the dehiscence of the cells. Then, there is a downregulation for the phases II–IV, before the

final expression level increases. The final is possibly to degrade the walls in phase V prior to abscission. The other DD # 91b seems to have two peaks, one on Day 2 and another on Day 6, in a similar fashion.

The hits for genes associated with endoreduplication (DD # 136b—fk506-protein and DD #3a— α -tubulin) are of particular interest. Endoreduplication is the process of preparation for cell division with the division of nuclei, but without the follow-up of a cell division. The results are cells with multiple nuclei, which are larger than cells with only a single nucleus (Sugimoto-Shirasu and Roberts, 2003). We have seen the enlarged cells toward the end of the abscission process, very much in line with what Wong and Osborne reported for *Echallium elaterium* (Wong and Osborne, 1978). These enlarged cells probably aid the plant in pushing the unwanted organ off. These genes are higher in Days 0–2, then downregulated and end up being upregulated again in Day 7 and in the distal parts. We have run a cell sorting of poinsettia cells from the various days after decapitation in a flow cytometer and find that indeed, the cells have increasing number of nuclei as the days pass (data not shown).

DD # 208 with our only hit for a poinsettia sequence (*E. pulcherrima*, **Table 1**) is close to a gene sequence coding for chloroplast tRNA-Leu. This sequence codes for tRNA involved in Leucine assembly in the chloroplasts, thus another gene important for photosynthesis. # 208 has its peak expression on Days 2–3 and then downregulated toward the end of events on Day 6.

Gene Sequences Strongly Differentiated, with Unknown Function

The riboprobes # 304a, although with a length of 463 bp, has no significant matches in the databases as of end of August 2015. It is strongly upregulated on Day 2 and then downregulated again, indicating a putative gene in a signaling pathway. The riboprobes # 113, #125b, and # 125c are all upregulated on Day 2, albeit not as strongly as # 304. However, they are all very interesting as putative regulating genes as well. Riboprobe # 50a behaves differently from the other unknown sequences; it is the only one with no significant matches which is downregulated on Day 1, culminating at the lowest level on Day 2, only to be strongly upregulated again from Day 3 and onwards. This is probably a gene coding for an inhibitory gene product in abscission. Our group has also compared the results from immunolabeling poinsettia pedicels using antibodies (JIM5, JIM7, LM5, and LM6) to describe changes in arabinogalactan, galacturonan, and esterification of homogalacturonan (HG) in poinsettia. The earliest detected temporal change (Day 2) in poinsettia was a loss of LM5 [(1→4)- β -D-galactan] epitope in the distal region. On Day 5, the AZ lost the JIM5 (partially methyl-esterified/unesterified HG) in the distal part of the pedicel. The FT-IR analysis (Fourier-Transform Infra-Red microscopy) indicated that lignin and xylan were abundant in the AZ and that lower levels of cellulose, arabinose and pectins were present at Day 7 compared to the initiation phase I. The observations in poinsettia indicate that the induction of a secondary abscission event results in a temporal sequence of cell wall modifications involving the spatial regulatory loss,

appearance and/or remodeling of distinct sets of cell wall epitopes. LM6 [(1→5)- α -L-arabinan] epitopes in the AZ cells disappeared at Day 7 (Lee et al., 2008). If we compare these findings with cell wall changes in *Def* wild type AZ using monoclonal antibodies LM5 and LM6; we observed changes in cell wall epitopes of the AZ from young to mature seeds. The apparent absence of (1→4)- β -D-galactan and (1→5)- α -L-arabinan epitopes in the AZ of mature *Def* wild type seeds may reveal the involvement of the action of hydroxyl ions (OH^-) produced by peroxidases (Cosgrove, 2005) which is known to cleave wall polysaccharides. In *def* mutant pea seeds, the absence of an abscission event at the seed/funiculus junction may be due to a structural defect in forming the AZ rather than changes in the cell wall epitopes (Ayeh, 2008).

CONCLUSIONS

The majority of the significant differentially expressed genes were upregulated during the first or second day after the induction of abscission. At this stage, it is not possible to see any changes in the anatomy of the pedicel or any color changes in poinsettia. Many of the genes found can be functionally classified to energy metabolism, cell growth and division. We have also identified two sequences similar to cell wall degrading enzymes (glucan endo-1,3- β -glucosidase and another putative β -glucosidase), as well as some sequences, which can be associated with endoreduplication (fk506-binding protein and α -tubulin), and a putative signal transducer. Our results are very much in alignment of the proposed model for abscission in Citrus (Agustí et al., 2012). The comparability with Citrus strongly supports the poinsettia model system to be suitable for further insight into gene regulations of the secondary abscission process. Based on our molecular results, it seems appropriate to modify the previous model of phases in abscission, by introducing a higher resolution for secondary abscission.

We have demonstrated that poinsettia and peas share at least six genes involved in abscission, two at the onset, two in phase II-III and the last two genes allocated to phase VI-V. It would be of great interest to use our two systems to find key regulatory gene(s) for abscission conserved throughout the plant kingdom. A universal abscission induction signal for AZ now seems more likely to find, since the six genes involved in abscission of both poinsettia and pea must be highly conserved during evolution. Further studies on our sequence data might reveal key regulatory genes, as well as more genes involved in the complicated abscission process for the plant kingdom.

AUTHOR CONTRIBUTIONS

AKHE devised and participated in all aspects of the studies. AKHE and CAM coordinated the logistics of the DD study, while AKHE and YKL did the same for the RNA *in situ* hybridization experiments. CMM and CAM contributed to designing poinsettia experiments, growing of the plants, harvested materials and carried out the DD experiments. CMM did the validations through Real-Time qRT-PCR. KOA executed the pea experiments through growing, harvesting and preparing

the pea material, as well as the RNA *in situ* in pea. PR executed the same in the poinsettia RNA *in situ* experiments. YKL performed the in structural analysis in the microscope together with KOA and PR and improved some of the *in situ* experiments through new hybridizations. TM performed the RACE experiments and the subsequent Real-Time qRT-PCR of the full-length sequences. AKHE, YKL, CAM, CMM, TM, KOA, and PR participated at various times in the data analysis. CAM, CMM, YKL, and AKHE participated in writing the article. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.01204>

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Auxin is a long-range signal that acts independently of ethylene signaling on leaf abscission in *Populus*

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Timing of leaf abscission is an important trait for biomass production and seasonal acclimation in deciduous trees. The signaling leading to organ separation, from the external cue (decreasing photoperiod) to ethylene-regulated hydrolysis of the middle lamellae in the abscission zone, is only poorly understood. Data from annual species indicate that the formation of an auxin gradient spanning the abscission zone regulates the timing of abscission. We established an experimental system in *Populus* to induce leaf shedding synchronously under controlled greenhouse conditions in order to test the function of auxin in leaf abscission. Here, we show that exogenous auxin delayed abscission of dark-induced leaves over short and long distances and that a new auxin response maximum preceded the formation of an abscission zone. Several auxin transporters were down-regulated during abscission and inhibition of polar auxin transport delayed leaf shedding. Ethylene signaling was not involved in the regulation of these auxin transporters and in the formation of an abscission zone, but was required for the expression of hydrolytic enzymes associated with cell separation. Since exogenous auxin delayed abscission in absence of ethylene signaling auxin likely acts independently of ethylene signaling on cell separation.

Keywords: *Populus*, auxin, ethylene, abscission, cell separation, PIN proteins

Introduction

In contrast to animal cells, most plant cells are tightly glued together by the middle lamella, such that even subtle positional changes to neighboring cells are impeded. During the life cycle of plants, however, many developmental processes require cell separation, e.g. anther dehiscence, floral organ abscission, pod shatter, radicle emergence and leaf abscission (Roberts et al., 2002; Lewis et al., 2006). Although the enzymatic activities required for the hydrolysis of middle lamellae and cell walls in various separation phenomena are thought to be similar, the triggers and therefore the upstream signaling are obviously diverse (Taylor and Whitelaw, 2001; Roberts et al., 2002; Fischer and Polle, 2010). While stress-induced and developmentally regulated organ abscission is relatively well studied little attention has been paid to the understanding of how seasonal cues are integrated to trigger the separation of organs from the plant body. In temperate climates the most remarkable cell separation process is the autumnal shedding of leaves from trees in fall. Despite of the paramount importance of leaf shedding as an adaptation to freezing and for nutrient cycling in forest ecosystems (Aponte et al., 2013; Zanne et al., 2014) our understanding of autumnal leaf abscission is limited.

Important roles in timing of abscission have been assigned to the plant hormones ethylene and auxin. La Rue (1936) showed that removal of the leaf blade induces abscission; but when auxin is applied to the site of removal, abscission is inhibited. Addicott and Lynch (1955), Addicott et al. (1955) and Louie and Addicott (1970) applied auxin to decapitated stems of various annual species, which resulted in premature abscission of cotyledons or leaves. They found that not the absolute concentration of auxin but the ratio between distal and proximal auxin was relevant for the timing of abscission (Addicott et al., 1955; Louie and Addicott, 1970). Lower auxin concentrations on the distal than on the proximal side of the abscission zone favored abscission, whereas relatively higher auxin concentrations on the distal side delayed abscission (Louie and Addicott, 1970). From these experiments, it was concluded that an auxin gradient spans the abscission zone and regulates the induction of abscission (Addicott et al., 1955). Although testing of the auxin gradient model has been proven difficult in absence of highly resolved auxin concentration measurements or appropriate auxin response reporters the importance of auxin has further been strengthened by genetic evidence from the model plant *Arabidopsis*. Since petioles and pedicels *Arabidopsis* do not develop functional abscission zones (Patterson, 2001), research has focused on abscission of floral organs and dehiscence. Mutations in the auxin response factors *ARF1* and *ARF2* cause delayed petal abscission (Ellis et al., 2005). Similarly, expression of *iaaL*, which inactivates auxin, or *AXR3-1*, which is a stabilized Aux/IAA variant causing auxin resistance, under the control of an abscission zone specific promoter delays abscission (Basu et al., 2013).

Ethylene has been shown to play an antagonistic role to auxin in abscission of various organs. In the ethylene-insensitive *Arabidopsis* mutants *ein2* and *etr1-1* abscission is delayed (Bleecker and Patterson, 1997; Patterson and Bleecker, 2004), while application of ethylene hastens abscission in various organs and species. In line with a promotive role of ethylene in cell separation, ethylene levels often increase shortly before organ separation and ethylene is sufficient to induce the expression of a polygalacturonase required for cell separation in tomato petioles (Hong et al., 2000; Jiang et al., 2008). Interestingly, the same polygalacturonase is inhibited by the exogenous application of auxin, underlying the suggested antagonistic effects of auxin and ethylene in abscission (Hong et al., 2000). A broadly accepted model of hormonal interaction during organ separation suggests that a depletion of auxin levels in the abscission zone renders cells more sensitive to ethylene, which in turn induces secretion of middle lamellae hydrolyzing enzymes (Estornell et al., 2013).

Although the physiology and transcriptional changes related to autumnal leaf abscission in trees have been subject to intense studies (Chen et al., 1997; Keskitalo et al., 2005; Street et al., 2006; Giovannelli et al., 2007), mechanistic understanding of autumnal abscission signaling, especially the role of auxin, is limited. Here, we describe an experimental system to induce leaf abscission in *Populus* synchronously and report that auxin is a plausible long-range signal regulating abscission that acts independently of ethylene signaling.

Materials and Methods

Plant Material and Growth Conditions

PttPIN1b::GUS, *PttPIN5b::GUS*, *PttWAT1::GUS* constructs were transformed into hybrid aspen (*Populus tremula* L. X *P. tremuloides* Michx.; clone T89). The *in vitro* growth conditions were according to Love et al. (2009). Briefly, trees were grown in clear polypropylene containers (height, 14 cm; diameter, 10 cm) with OS140ODS140 gas-exchange spore filters (Combiness) and cultured on Murashige and Skoog medium (2.2 g·l⁻¹, pH 5.6, Duchefa) with Phytagel P8169 solidifying agent (2.7 g·l⁻¹; Sigma-Aldrich). Temperature was 22/18°C (light/dark), photoperiod was 16 h, and light intensity was 90 μmol·m⁻²·s⁻¹. The 3 to 4-weeks-old *in vitro* grown transgenic trees (height, 10–12 cm) were transferred in 2.5 l pots with the commercial soil-sand-fertilizer mixture (Yrkes Plantjord Kronmull; Weibulls Horto, Hammenhög, Sweden) and grew in glasshouse under 18-h photoperiod at 20°C : 15°C (light : dark). Trees were fertilized with 150 ml of 1% Rika-S (N/P/K 7:1:5; Weibulls Horto) once a week (Vahala et al., 2013). The *GH3::GUS* lines are in the *Populus × canescens* background, a hybrid between *P. alba* L. × *P. tremula* L. (Teichmann et al., 2008). These lines were used for the exogenous auxin and auxin transport inhibitor applications (Figures 2 and 4). All the other experiments were conducted in the T89 background.

Dark Induction and Treatments with Auxin and Auxin Inhibitors

For dark-induction experiments, fully expanded leaves with a petiolar angle of 75 to 90° from 1.5 to 2 m tall trees were selected. Leaf blades were covered with aluminum bags under standard greenhouse conditions. Control samples were bagged in transparent plastic bags of the same weight. Each bag was labeled with a unique code referring to the genotype, tree replicate, leaf number, and treatment. Trees were gently shaken once per day, the dropped bags collected and the identifiers recorded.

Lanolin paste (Sigma-Aldrich) containing either 100 μM IAA, 2, 4-D, or morphactin (CF, Sigma-Aldrich) were placed with the help of a disposable 100 μl plastic tip at the junction between stem and petiole. For distal IAA application the lanolin paste containing 500 μM IAA was applied to the junction between the petiole and leaf blade.

For auxin induction experiment (Supplementary Figure S1), *in vitro* grown young shoots with 5–6 leaves of the respective GUS lines were treated with auxin [1 μM 1-naphthaleneacetic acid (1-NAA, Sigma-Aldrich) in 1/2 MS-medium (pH 5.8, Duchefa)] for 3 h in darkness and washed with 1/2 MS-medium and assayed for GUS.

Cloning of Promoter Sequences and Plant Transformation

Nine hundred to thousand bp long promoter fragments of *PttPIN1b*, *PttPIN5b*, and *PttWAT1* were PCR amplified from T89 using the primer combinations, PromPttPIN1b for/PromPttPIN1b rev, PromPttPIN5b for/PromPttPIN5b rev, or PromPttWAT1 for/PromPttWAT1 rev, respectively (Supplementary Table S3). The PCR products representing

promoter regions of *PttPIN1b*, *PttPIN5b*, or *PttWAT1* were subcloned into pGemTeasy and moved directionally as *PstI-SalI*, *SalI-EcoRI*, or *HindIII-BamHI* fragments into the vector pCambia1391Z (<http://www.cambia.org>), respectively. The resulting plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90, pSoup) and used for stable transformations of the hybrid aspen clone T89 according to Nilsson et al. (1992). For all constructs at least five lines were selected and tested for GUS expression. Three lines each were analyzed in detail and representative GUS expression patterns are shown in Figure 5.

Gene Expression Analyses

Total RNA was extracted from 3-mm-long petioles near the junction to the stem using RNeasy Plant Mini Kit (Qiagen). The extracted total RNA was quantified with a ND-1000 NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Two micrograms of total RNA was used as a template for reverse transcription with the QuantiTect Reverse Transcription Kit (Qiagen). Equal amounts of first-strand cDNAs were used as templates for real-time PCR amplification using the following primer combinations: *qPttPIN1bforw/qPttPIN1brev*, *qPttPIN5bforw/qPttPIN5brev*, and *qPttWAT1forw/qPttWAT1rev*. The T89 actin gene, *Actin1*, was amplified using primer combination *qPttActin1forw/qPttActin1rev* (Supplementary Table S3). Quantitative real-time PCR was performed using LightCycler® 480 SYBR Green I Master (Roche Diagnostics GmbH, Mannheim, Germany) with a LC4800 (Roche Diagnostics) qPCR machine. *PttPIN1b*, *PttPIN5b* and *PttWAT1* transcript levels were quantified in relation to *Actin1* levels. Microarrays (61 k Affymetrix Poplar array) employing four biological replicates were performed by MFTServices (Tuebingen, Germany). Data analysis has been performed with the help of the Robin software package (Lohse et al., 2010).

Histochemical Staining for GUS Activity

GUS expression patterns were determined in 3-mm-thick longitudinal median sections of leaf axils. Samples were infiltrated employing vacuum for 30 min with GUS buffer (50 mM sodium phosphate buffer (pH 7.2), 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 0.1% Triton X-100) containing 2 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (Duchefa Biochemie bv, The Netherlands) and then incubated in the dark at 37°C for 3–18 h. Pigments were removed in 70% (v/v) ethanol with gentle shaking. GUS expression patterns were examined with a bright-field microscope (Zeiss, Auxioplan) at low magnification ($\times 2.5$, $\times 10$) or a scanner (Epson Perfection V600 Photo).

Results

An Auxin Response Maximum Precedes the Formation of an Abscission Zone

In *Populus*, gradual reduction of day length and temperature induces leaf abscission; whereas over-expression of phytochrome

A is sufficient to prevent abscission under these conditions (Olsen et al., 1997). However, it remains unclear if shorter day length is sensed by individual leaf blades, apices or if it is rather a response of the whole plant to reduced photoperiod. Therefore, we tested if shading of the leaf blade is sufficient to induce abscission in greenhouse-grown *Populus* trees. To this end we covered blades but not petioles of fully expanded leaves with aluminum foil bags. Shading induced cell divisions in the leaf axil and the formation of typical abscission zones 6 and 9 days, respectively, after the treatment started. Although the petioles were not shaded strong de-greening of the petiole was observed (Figure 1). By contrast, leaves in transparent bags of the same tree did not develop an abscission zone, were not shed and their petioles did not de-greened during the same period of observation (Figures 1A,E). Taken together, these results suggest that reduced photoperiod is sensed in the leaf blade and a mobile signal transports this information from the blade to the abscission zone, where it induces the development of an abscission zone.

Previous work in explants of annual species indicated that auxin could contribute to a leaf blade-derived abscission inhibiting signal (Louie and Addicott, 1970). We therefore

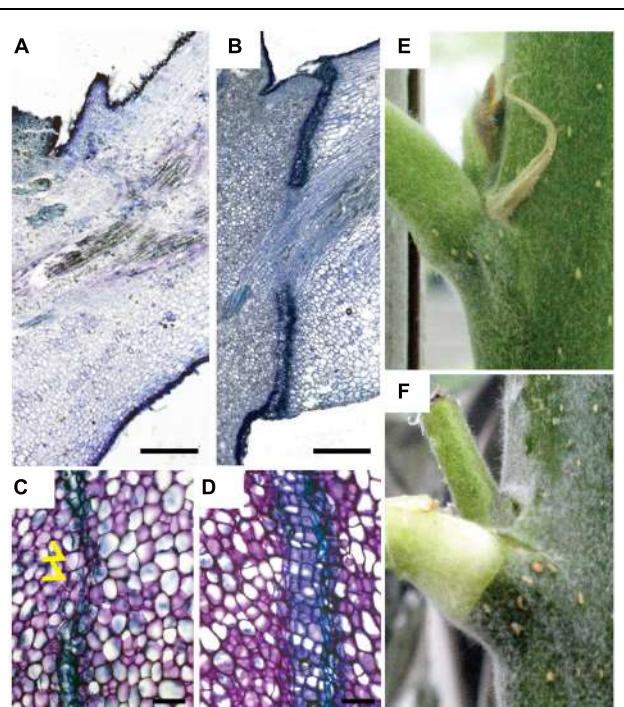


FIGURE 1 | A mobile signal from the leaf blade induces leaf abscission.

(A–D) Toluidine blue stained longitudinal median section of leaf axils. (A) No abscission zone visible in axil of non-shaded leaf blade. Blade was bagged in a transparent plastic bag for 9 days. (B) Mature abscission zone in an axil of a shaded leaf blade. Blade was covered for 9 days in aluminum foil. (C,D) Abscission zones 6 and 9 days after shading started, respectively. Yellow arrowheads point to dividing cells. (E) Leaf axil of a non-shaded blade. (F) Leaf axil of a blade shaded for 12 days. Chlorophyll distal to the abscission zone is degraded although leaf petiole was not shaded. Scale bars, 500 μm (A,B); 100 μm (C,D).

examined the expression patterns of the auxin response reporter *GH3::GUS* (Teichmann et al., 2008). In control petioles, *GH3::GUS* was strongest in the vascular tissues (**Figure 2A**). After 6 days of shading, this activity became weaker but at the lower (abaxial) side of the petiole a new auxin response maximum emerged, which gradually expanded to the upper (adaxial, after 9 days) side of the petiole preceding the formation and maturation of the abscission zone (**Figures 2B–E**).

We then applied auxin (indole-3-acetic acid, IAA) in lanoline paste directly to the axils of intact leaves in order to test if auxin can delay abscission of shaded leaves (**Figure 3**). Auxin application to the very proximal end of the petiole delayed dark-induced leaf abscission highly significantly by approximately 1 day. In order to examine if auxin can also work as a long-distance signal, which is transported from the leaf blade to the axil, we applied auxin (IAA) to the most distal end of the petiole. Also in this case, abscission was significantly delayed supporting the idea that auxin not only acts locally but has the potential to be a long-distance signal in leaf abscission.

Inhibition of Polar Auxin Transport Delays Formation of Abscission Zones

The polar auxin transport inhibitor morphactin (9-hydroxyfluorene-9-carboxylic acid, CF) has previously been shown to delay leaf abscission in citrus (Goren et al., 1986). Local application of morphactin to the axil of shaded leaves

delayed separation by approximately 7 days, to an even stronger extent as observed for the application of the auxin influx carrier substrate 2,4-D (**Figure 4A**). We then tested whether morphactin modulates auxin response in leaf axils. In mock-treated axils, 6 days after shading started, a new local *GH3::GUS* maximum appeared at the lower side of the petiole. After 9 days, in partly separated petioles, most of the auxin response reporter activity occurred on the proximal side of the abscission zone (**Figures 4B–D**). By contrast, in presence of morphactin formation of the new auxin response maximum as well as of the abscission zone were delayed and only became visible 18 days after shading started (**Figures 4E–G**). Hence, morphactin likely acts on the formation of the abscission zone by preventing the establishment of a new auxin response maximum.

Auxin Transporters are Down-Regulated during Abscission

In order to identify auxin transporters, which could be involved in the formation of a new auxin response maximum during abscission we performed a microarray experiment. Total RNA was isolated from dissected leaf axils 9 days after shading started and from axils covered with transparent plastic bags. Among the approximately 2400 differentially expressed genes auxin related transcripts were strongly overrepresented (Supplemental Dataset). Within the group of the 200 most strongly down-regulated genes several *AUX/IAAs*, *SAURs* but also the putative

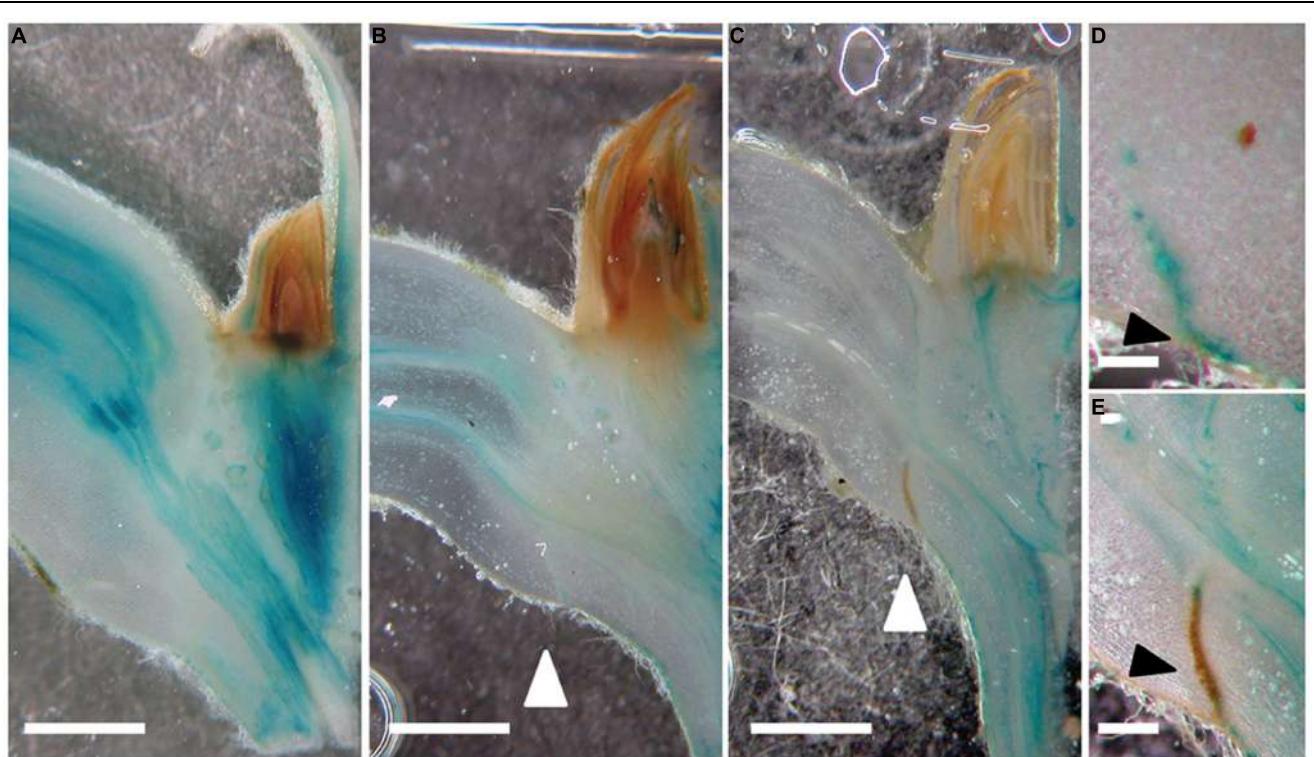


FIGURE 2 | A new auxin response maximum is established prior to the formation of an abscission zone. **(A–E)** *GH3::GUS*; 0 (**A**), 6 (**B,D**), and 9 days (**C,E**) after shading started. White arrow heads point to the abscission

zones. **(E)** Mature abscission zone appears in brown; GUS precipitate in blue. Scale bars correspond to approximately 1 mm (**A–C**); 0,5 mm (**D,E**). Black arrowheads point to the forming (**D**) and mature abscission zone (**E**).

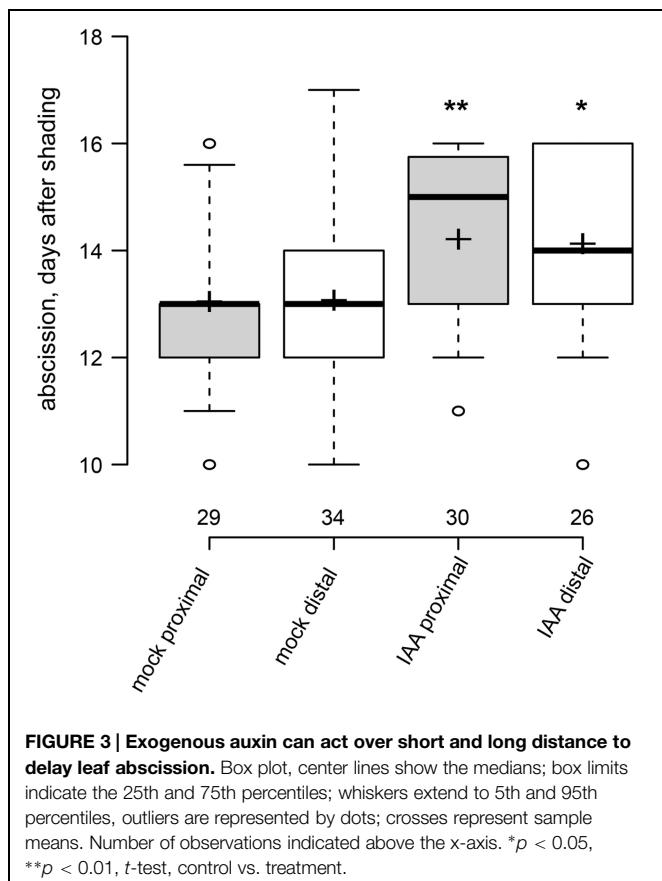


FIGURE 3 | Exogenous auxin can act over short and long distance to delay leaf abscission. Box plot, center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 5th and 95th percentiles, outliers are represented by dots; crosses represent sample means. Number of observations indicated above the x-axis. * $p < 0.05$, ** $p < 0.01$, t-test, control vs. treatment.

auxin transporters *PtrPIN1b*, *PtrPIN5b*, and *PtrWAT1* were present (Table 1; data available at NCBI GEO, GSE69277). By contrast, none of the known putative auxin transporters were up-regulated. As its closest *Arabidopsis* homolog PIN1, *PtrPIN1b* localizes to the plasma membrane (Liu et al., 2014) and is most likely involved in intercellular auxin transport. On the other hand, the closest homologs of *PtrPIN5b* (PIN5) and *PtrWAT1* (WAT1) localize in *Arabidopsis* to the endoplasmic reticulum and the tonoplast, respectively (Mravec et al., 2009; Ranocha et al., 2013), and are therefore expected to be involved in intracellular auxin transport and homeostasis. We isolated promoter fragments of *PttPIN1b*, *PttPIN5b*, *PttWAT1*, and used them to drive the GUS reporter gene. Expression of all reporter gene constructs was inducible by exogenous application of auxin (Supplementary Figure S1). In axils from non-shaded leaf blades, *pPttPIN1b::GUS* reporter activity was mainly associated with the vascular system across the entire axil (Figure 5A). Nine days after shading started additional *pPttPIN1b::GUS* activity was observed along the differentiating abscission zone (Figure 5D). Similarly, expression of the intracellular auxin transporters was most prominent in vascular tissues of axils from non-shaded leaf blades (Figures 5B,C). Shading led to strong, patchy expression of *PtrPIN5b::GUS* and *PtrWAT1::GUS* on the proximal side of the emerging abscission zone, whereas these genes were hardly expressed on the distal side (Figures 5E,F). These findings underline the importance of auxin transport in the formation

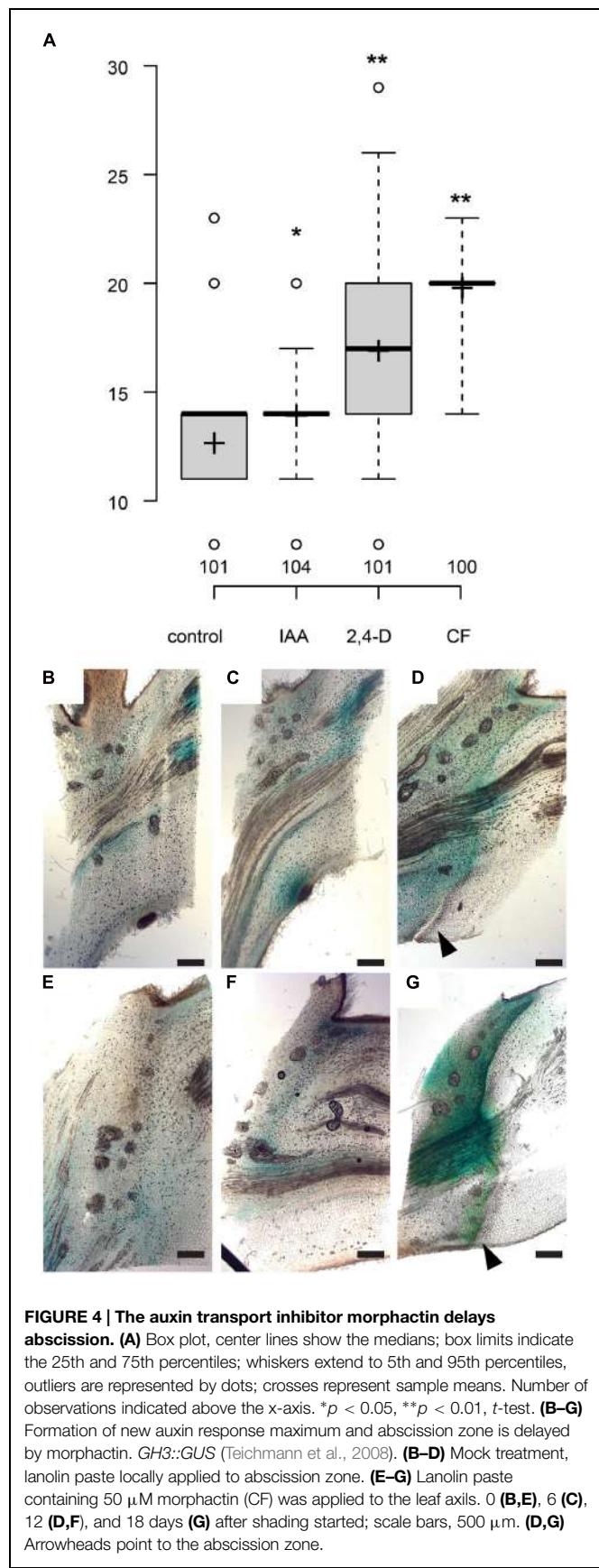


FIGURE 4 | The auxin transport inhibitor morphactin delays abscission. (A) Box plot, center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 5th and 95th percentiles, outliers are represented by dots; crosses represent sample means. Number of observations indicated above the x-axis. * $p < 0.05$, ** $p < 0.01$, t-test. (B–D) Mock treatment, lanolin paste locally applied to abscission zone. (E–G) Lanolin paste containing 50 μ M morphactin (CF) was applied to the leaf axils. 0 (B,E), 6 (C), 12 (D,F), and 18 days (G) after shading started; scale bars, 500 μ m. (D,G) Arrowheads point to the abscission zone.

TABLE 1 | Genes involved in auxin transport and response are down-regulated in axils of shaded leaves.

Rank	ID	logFC	P-value	adj.P-Value	Closest At homolog	Process
8	PtpAfx.16882.2.S1_at	-5,8	6,7E-10	4,6E-06	IAA19	AUX/IAA
19	PtpAfx.25068.1.A1_at	-4,7	1,5E-07	7,2E-05	IAA4	AUX/IAA
20	PtpAfx.7696.4.S1_a_at	-4,7	1,8E-08	2,7E-05	IAA4	AUX/IAA
21	Ptp.128.1.S1_at	-4,7	2,7E-09	1,3E-05	IAA14	AUX/IAA
30	Ptp.6148.1.S1_at	-4,4	1,4E-06	1,9E-04	PIN5	Efflux
31	PtpAfx.7696.2.A1_a_at	-4,4	4,4E-07	1,1E-04	IAA4	AUX/IAA
36	PtpAfx.213779.1.S1_at	-4,3	9,7E-08	5,9E-05	SAUR75	Response
40	PtpAfx.21075.1.S1_at	-4,2	6,8E-07	1,3E-04	SAUR14	Response
45	PtpAfx.73583.1.S1_at	-4,0	8,7E-08	5,7E-05	IAA4	AUX/IAA
47	PtpAfx.102281.1.A1_at	-4,0	2,7E-06	2,7E-04	SAUR75	Response
50	Ptp.127.1.S1_s_at	-4,0	7,6E-07	1,4E-04	IAA4	AUX/IAA
60	Ptp.1099.1.A1_at	-3,9	7,1E-07	1,4E-04	GH3-10	Homoestasis
74	PtpAfx.7696.4.S1_at	-3,6	1,7E-07	7,8E-05	IAA4	AUX/IAA
75	PtpAfx.204265.1.S1_at	-3,6	1,3E-04	3,0E-03	SAUR14	Response
84	Ptp.1274.1.S1_s_at	-3,5	8,2E-06	5,0E-04	PIN1	Efflux
94	PtpAfx.204337.1.S1_at	-3,3	1,5E-06	2,0E-04	SAUR63	Reponse
97	PtpAfx.123174.1.A1_at	-3,2	2,1E-06	2,3E-04	IAA4	AUX/IAA
103	PtpAfx.21896.2.S1_s_at	-3,2	3,7E-07	1,1E-04	IAA13	AUX/IAA
113	PtpAfx.249.95.S1_a_at	-3,0	1,2E-07	6,6E-05	WAT1	Homeostasis
161	Ptp.6738.1.S1_at	-2,7	2,2E-04	4,1E-03	SAUR51	Response
11	Ptp.6116.1.S1_at	4,5	1,1E-05	5,8E-04	SUR2	Homeostasis
13	PtpAfx.54125.1.A1_s_at	4,5	6,8E-06	4,5E-04	SUR2	Homeostasis
94	PtpAfx.210014.1.S1_at	3,1	1,8E-04	3,6E-03	GH3-6	Homeostasis
180	PtpAfx.221307.1.S1_at	2,6	4,0E-05	1,3E-03	SAUR17	Response

RNA was extracted 9 days after dark-induction and gene expression was compared to the control treatment (leaves covered with transparent plastic bags of the same weight). Four biological replicates per treatment. Poplar 61 k Affymetrix array. The 200 most strongly up- or down-regulated were ranked and genes associated with auxin transport, response, or homeostasis were extracted from this list. Rank 1 corresponds to the most strongly up- and down-regulated genes, respectively.

of a new auxin maximum and the formation of an abscission zone.

Regulation of Auxin Transport during Abscission is Independent of Ethylene Signaling

We then wondered if auxin acts through ethylene signaling on leaf abscission as it has been suggested previously (for review, Estornell et al., 2013). We first analyzed abscission in 35S::etr1-1 ethylene insensitive trees (Love et al., 2009). Expression of the dominant *Arabidopsis* etr1-1 allele under the control of the 35S promoter renders *Populus* trees insensitive to ethylene. 35S::etr1-1 trees formed anatomically normally looking abscission zones after shading of leaf blades indicating that early steps of abscission could take place in absence of ethylene signaling (Figures 6A,B). 35S::etr1-1 leaves, however, separated later or not at all from the plant body (Figure 6C). We then tested if the expression of auxin transporter genes is reduced after shading in 35S::etr1-1 trees to a similar extent than in wild type. In wild-type leaf axils, *PttPIN1b*, *PttPIN5b*, and *PttWAT1* were significantly lower 6 days after shading started compared to non-shaded leaf axils (Figure 6D). Similar levels of dark-induced reduction in expression of *PttPIN1b* and *PttPIN5b* were observed in the ethylene-insensitive 35S::etr1-1 trees (Figure 6D). By contrast,

close homologs of the pectin methylesterase QUARTET1 and the polygalacturonase ADPG1, which are required for cell separation during microgenesis and dehiscence (Francis et al., 2006; Ogawa et al., 2009), were among the most strongly up-regulated genes after leaf shading and their up-regulation was dependent on ethylene signaling (Figure 6E; Supplementary Table S2). Together these findings suggest that the regulation of auxin transporters and the formation of an abscission zone are independent of ethylene signaling. In order to test if auxin acts on leaf abscission solely upstream of ethylene or in an independent parallel pathway we tested if auxin has an effect on the timing of abscission in 35S::etr1-1 axils of shaded leaves (Figure 6C). Exogenous auxin further delayed abscission and the number of leaves, which did not abscise, in an additive manner, as compared to mock treated 35S::etr1-1 axils, suggesting that auxin acts in an independent pathway, parallel to ethylene signaling, on leaf abscission.

Discussion

Based on earlier work, an auxin gradient spanning the abscission zone has been proposed to regulate timing of organ separation (Addicott et al., 1955). Instead of an auxin response gradient we observed a discrete auxin response maximum, which precedes the

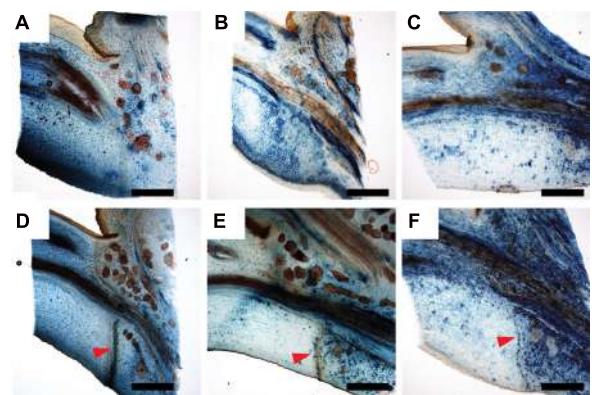


FIGURE 5 | Expression of *WAT1::GUS* and *PIN5b::GUS* is shifted to the proximal side of the abscission zone after shading of leaf blades. (A–C) *PtrPIN1b::GUS*; **(B,E)** *PtrWAT1::GUS*; **(C,F)** *PtrPIN5b::GUS*. **(A–C)** axes of non-shaded leaf blades; **(D–F)** 9 days after shading started. Red arrowheads point to mature abscission zone. Scale bars, 1 mm.

formation of an abscission zone, indicating that auxin provides positional cues for the specification and/or differentiation of an abscission zone. Similar spatially discrete auxin maxima or gradients have been proposed to regulate the positioning of leaf and lateral root primordia as well as the zonation patterning in the cambium (Dubrovsky et al., 2008; Kierzkowski et al., 2013; Bhalerao and Fischer, 2014). In the cambium high auxin concentrations correlate with meristematic activity and cell division of protoplasts requires the presence of auxin (Bhalerao and Fischer, 2014). Intriguingly, formation of the abscission zone not only involves specialization of cells but also in many cases cell proliferation. In contrast to an auxin response maximum, which precedes the formation of an abscission zone, Sorefan et al. (2009) reported that a local auxin response minimum is required for differentiation of the separation layer of the valve margin. At the site of reduced auxin response secondary walls are deposited, which are instrumental during the separation process. Interestingly, a strong auxin response is detected in the replum, the tissue between valve margins, coincidentally, overlapping with the region where cell divisions occur during the formation of an abscission zone (Wu et al., 2006; Sorefan et al., 2009). Thus, the mode of auxin action might be similar in abscission zones as suggested for the cambium, high concentrations permit cell division, below a certain threshold cell expansion and differentiation are favored.

Recently, an auxin response maximum has been observed in the abscission zone of *Arabidopsis* petals (Basu et al., 2013). In auxin influx carrier mutants, petal break strength is increased and therefore *AUX1* and its paralogs might be involved in the formation of an auxin concentration maximum in the abscission zone (Basu et al., 2013). In mutants of the auxin efflux carrier *PIN4*, additional root cap layers are observed (Frilm et al., 2002). In wild-type, the outermost tier of the root cap is continuously shed from the root. The *pin4* phenotype is either a consequence of over-proliferation of root cap cells or, more likely, of impaired cell separation. Our findings of delayed auxin maximum formation

and abscission upon inhibition of polar auxin transport and the strong reduction of auxin carriers in dark-induced leaf axils are in support with the idea that auxin transport is instrumental for the establishment of an instructive auxin response maximum during abscission.

Interestingly, expression of *iaaL*, which conjugates auxin, under the control of an abscission zone specific polygalacturonase promoter results in reduced auxin response and increased petal break strength (Basu et al., 2013). Polygalacturonases are integral parts of cell wall remodeling during the separation process (Roongsattham et al., 2012) and it is likely that the promoter used to drive *iaaL* is specifically active during later phases of abscission rather than during the formation of an abscission zone. Consequently, relatively low auxin concentrations during the separation phase are sufficient to hasten abscission. Hence, auxin is likely to have a dual function during abscission in first providing positional information for the formation of the abscission zone and secondly as a signal, which regulates temporal aspects of the separation. Such a mode-of-action ensures that under high auxin concentrations an abscission zone can differentiate but separation cannot be initiated. As a consequence premature abscission, which can be deleterious for plant performance, might be prevented.

Ethylene induced reduction of auxin transport capacity in the midrib of poplar leaves correlates with leaf abscission (Riov and Goren, 1979). Furthermore, it has been suggested that reduced uptake of auxin into the cells of the abscission zone increases their sensitivity toward ethylene (Taylor and Whitelaw, 2001). We observed that formation of an abscission zone and the regulation of auxin transport in petioles of dark-induced leaves are independent of ethylene signaling. Importantly, auxin could further delay abscission of ethylene insensitive petioles. Therefore, auxin may act in parallel and independently of ethylene on the hydrolysis of middle lamellae. The existence of such an ethylene-independent, additional signaling pathway has been suggested earlier, based on the observation that petal abscission in the ethylene insensitive mutants *ein2* and *etr1-1* is only incompletely inhibited (Bleeker and Patterson, 1997).

Evidence for a non-cell-autonomous signal, which provides positional information for the formation of an abscission zone, was first provided by the analyses of the MADS box transcription factor *JOINTLESS* in tomato. *jointless* mutants do not form abscission zones in pedicels (Mao et al., 2000). Interestingly, *JOINTLESS* function in the clonal layer L3 (vasculature) is sufficient to rescue the *jointless* phenotype. This finding strongly suggests that *JOINTLESS* can act through a mobile factor on cells in the cortex and epidermis of the pedicel to induce the formation of an abscission zone (Szymkowiak and Irish, 1999). The establishment of a new auxin maximum prior to the maturation of the abscission zone and regulation of auxin transporters in the abscission zone after dark induction are arguments in favor of auxin to provide cues to position the abscission zone, similar as for the formation of primordia in apical meristems and during differentiation of cambial derivatives. Intriguingly, exogenous auxin is sufficient to induce ectopic expression of the peptide hormone *INFLORESCENCE DEFICIENT IN ABSCISSION* (IDA), which controls cell separation during floral

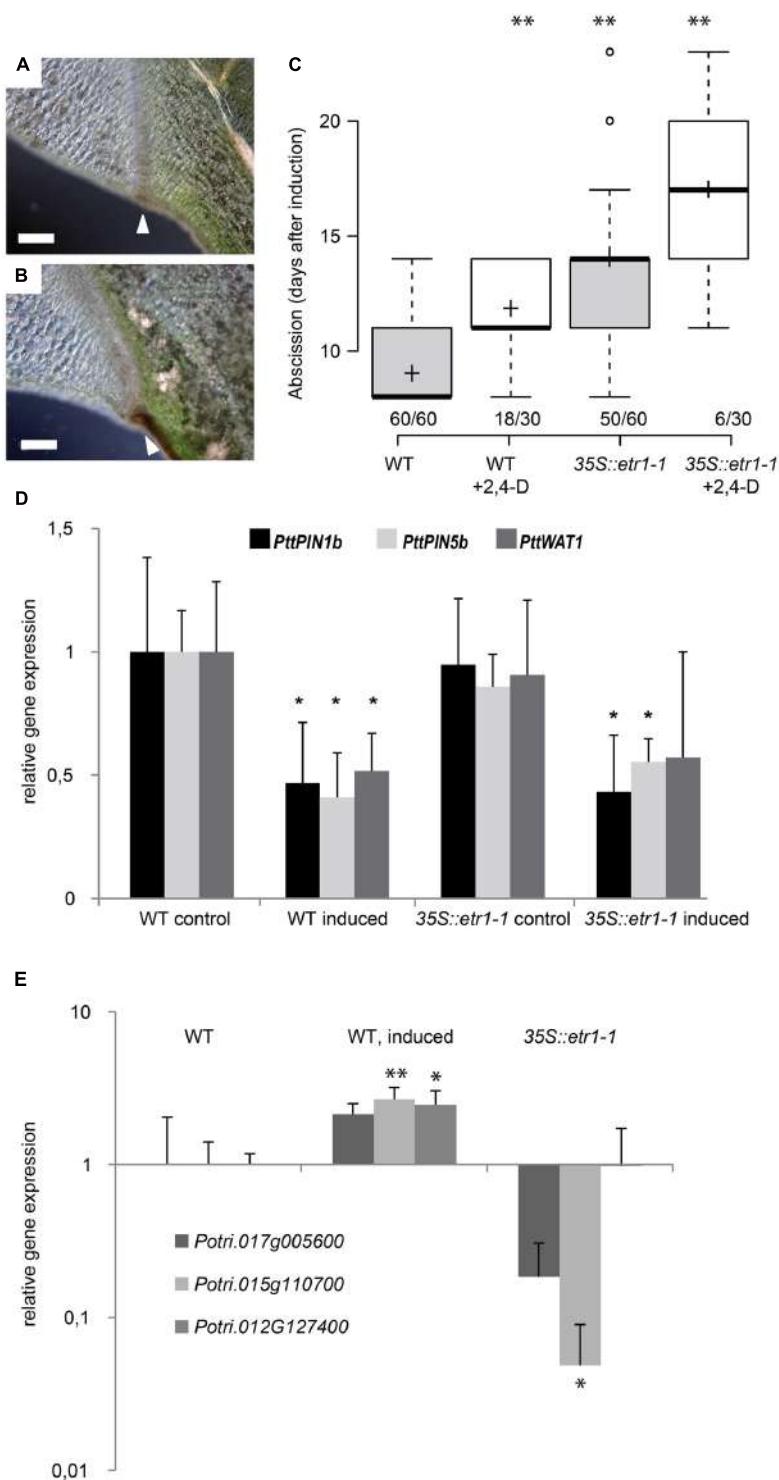


FIGURE 6 | Auxin acts independently of ethylene on leaf abscission.

(A,B) Abscission zone in wild-type **(A)** and 35S::etr1-1 **(B)** axils, 9 days after shading started. Scale bars, 200 μm. White arrowheads point to mature abscission zones. **(C)** Additive effects of 100 μM 2,4-D and inhibition of ethylene signaling (35S::etr1-1) on timing of leaf abscission. Box plot, center lines show the medians; box limits indicate the 25th and 75th percentiles; whiskers extend to 5th and 95th percentiles, outliers are represented by dots; crosses represent sample means. Number of observations indicated above the x-axis (number of

shed leaves/total number of dark-induced leaves). $**p < 0.01$, t -test. **(D,E)** qRT-PCR, averages, and SD of three biological replicates. Normalized to ACT1 expression. $*p < 0.05$, t -test, non-shaded versus shaded for 6 days. **(D)** Gene expression of auxin transporters. **(E)** Gene expression of pectin modifying enzymes. Potri.017g005600 (homologs to the polygalacturonase encoding gene ADPG1), Potri.015G110700 (homologs to the pectin methylesterase encoding gene QRT1) and Potri.012G127400 (homologs to the pectin methylesterase inhibitor encoding gene PME1).

organ abscission and lateral root primordia emergence (Aalen et al., 2013; Kumpf et al., 2013). Besides the functional analyses of the identified auxin transporters *PtrPIN1b*, *PtrPIN5b* and *PtrWAT1*, future directions of research should include the determination of subcellular localization of auxin efflux carriers in the petiole. Subsequent modeling of auxin distribution could reveal if a scenario of auxin providing positional information to the cells in the petiole is plausible.

Author contributions

XJ, JZ, and UF performed and analyzed the experiments. AP and UF designed the research and edited the manuscript. XJ and UF wrote the manuscript.

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Supplementary Material

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fpls.2015.00634>

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