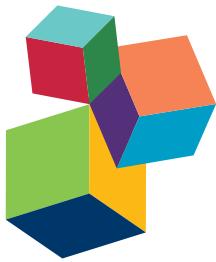


# MOLECULAR AND CELLULAR PLANT REPRODUCTION

EDITED BY: Dazhong Zhao, Kang Chong and Ravishankar Palanivelu  
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# MOLECULAR AND CELLULAR PLANT REPRODUCTION

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The diverse variations of fruit morphology in the Solanaceae family.

Image by Chaoying He

editorial office for their excellent contributions that made the publication of this e-book possible.

Plant reproduction is essential not only for producing offspring but also for increasing crop quality and yield. Moreover, plant reproduction entails complex growth and developmental processes, which provide a variety of opportunities for elucidating fundamental principles in biology. The combinational employment of molecular genetic approaches and emerging technologies, such as fluorescence-based imaging techniques and next generation sequencing, has led to important progresses in plant reproduction using model plants, crops, and trees. This e-book compiles 31 articles, including 1 hypothesis and theory, 4 perspectives, 12 reviews, and 14 original research papers. We hope that this E-book will draw attention of all plant biologists to exciting advances in the field of plant reproduction and help solve remaining challenging questions in the future. We wish to express our appreciation to all the authors, reviewers, and the Frontiers editorial office for their excellent contributions that made the publication of this e-book possible.

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# Table of Contents

## Editorial

### 06 Editorial: Molecular and Cellular Plant Reproduction

Dazhong Zhao, Kang Chong and Ravishankar Palanivelu

## Hypothesis & Theory

### 09 The concept of the sexual reproduction cycle and its evolutionary significance

Shu-Nong Bai

## Perspective

### 17 A little bit of sex matters for genome evolution in asexual plants

Diego Hojsgaard and Elvira Hörandl

### 23 Unresolved issues in pre-meiotic anther development

Timothy Kelliher, Rachel L. Egger, Han Zhang and Virginia Walbot

### 32 Insight into S-RNase-based self-incompatibility in Petunia: recent findings and future directions

Justin S. Williams, Lihua Wu, Shu Li, Penglin Sun and Teh-Hui Kao

### 38 Evolution and function of epigenetic processes in the endosperm

Claudia Köhler and Clément Lafon-Placette

## Review

### 43 Regulation of inflorescence architecture by cytokinins

Yingying Han, Haibian Yang and Yuling Jiao

### 47 Regulation of floral stem cell termination in Arabidopsis

Bo Sun and Toshiro Ito

### 53 Epigenetic regulation of rice flowering and reproduction

Jinlei Shi, Aiwu Dong and Wen-Hui Shen

### 66 Interpreting lemma and palea homologies: a point of view from rice floral mutants

Fabien Lombardo and Hitoshi Yoshida

### 72 Sterility Caused by Floral Organ Degeneration and Abiotic Stresses in Arabidopsis and Cereal Grains

Ashley R. Smith and Dazhong Zhao

### 87 Reproduction and the pheromonal regulation of sex type in fern gametophytes

Nadia M. Atallah and Jo Ann Banks

### 93 The male germline of angiosperms: repertoire of an inconspicuous but important cell lineage

Scott D. Russell and Daniel S. Jones

- 103 Organization and regulation of the actin cytoskeleton in the pollen tube**  
Xiaolu Qu, Yuxiang Jiang, Ming Chang, Xiaonan Liu, Ruihui Zhang and Shanjin Huang
- 116 The expression and roles of parent-of-origin genes in early embryogenesis of angiosperms**  
An Luo, Ce Shi, Liyao Zhang and Meng-Xiang Sun
- 126 Possible roles for polycomb repressive complex 2 in cereal endosperm**  
Kaoru Tonosaki and Tetsu Kinoshita
- 131 Using giant scarlet runner bean embryos to uncover regulatory networks controlling suspensor gene activity**  
Kelli F. Henry and Robert B. Goldberg
- 137 Evolutionary developmental genetics of fruit morphological variation within the Solanaceae**  
Li Wang, Jing Li, Jing Zhao and Chaoying He

### Research Article

- 147 Analysis of Arabidopsis floral transcriptome: detection of new florally expressed genes and expansion of Brassicaceae-specific gene families**  
Liangsheng Zhang, Lei Wang, Yulin Yang, Jie Cui, Fang Chang, Yingxiang Wang and Hong Ma
- 158 Comparative transcriptomic analysis of male and female flowers of monoecious Quercus suber**  
Margarida Rocheta, Rómulo Sobral, Joana Magalhães, Maria I. Amorim, Teresa Ribeiro, Miguel Pinheiro, Conceição Egas, Leonor Morais-Cecílio and Maria M. R. Costa
- 174 Transcriptomic insights into antagonistic effects of gibberellin and abscisic acid on petal growth in Gerbera hybrida**  
Lingfei Li, Wenbin Zhang, Lili Zhang, Na Li, Jianzong Peng, Yaqin Wang, Chunmei Zhong, Yuping Yang, Shulan Sun, Shan Liang and Xiaojing Wang
- 187 Molecular phenotypes associated with anomalous stamen development in Alternanthera philoxeroides**  
Zhu Zhu, Chengchuan Zhou and Ji Yang
- 197 TCP24 modulates secondary cell wall thickening and anther endothecium development**  
Han Wang, Yanfei Mao, Jun Yang and Yuke He
- 207 OsSDS is essential for DSB formation in rice meiosis**  
Zhigang Wu, Jianhui Ji, Ding Tang, Hongjun Wang, Yi Shen, Wenqing Shi, Yafei Li, Xuelin Tan, Zhukuan Cheng and Qiong Luo
- 217 MeioBase: a comprehensive database for meiosis**  
Hao Li, Fanrui Meng, Chunce Guo, Yingxiang Wang, Xiaojing Xie, Tiansheng Zhu, Shuigeng Zhou, Hong Ma, Hongyan Shan and Hongzhi Kong
- 223 Pollination triggers female gametophyte development in immature Nicotiana tabacum flowers**  
Michael S. Brito, Lígia T. Bertolino, Viviane Cossalter, Andréa C. Quiapim, Henrique C. DePaoli, Gustavo H. Goldman, Simone P. Teixeira and Maria H. S. Goldman
- 233 Loss of the Arabidopsis thaliana P4-ATPases ALA6 and ALA7 impairs pollen fitness and alters the pollen tube plasma membrane**  
Stephen C. McDowell, Rosa L. López-Marqués, Taylor Cohen, Elizabeth Brown, Alexa Rosenberg, Michael G. Palmgren and Jeffrey F. Harper

- 245 Knockin' on pollen's door: live cell imaging of early polarization events in germinating *Arabidopsis* pollen**  
Frank Vogler, Sebastian S. A. Konrad and Stefanie Sprunck
- 262 Epigenetic landscape of germline specific genes in the sporophyte cells of *Arabidopsis thaliana***  
Chol Hee Jung, Martin O'Brien, Mohan B. Singh and Prem L. Bhalla
- 274 Large genetic screens for gynogenesis and androgenesis haploid inducers in *Arabidopsis thaliana* failed to identify mutants**  
Virginie Portemer, Charlotte Renne, Alexia Guillebaux and Raphael Mercier
- 280 Correlation between a loss of auxin signaling and a loss of proliferation in maize antipodal cells**  
Antony M. Chettoor and Matthew M. S. Evans
- 294 Establishment of embryonic shoot-root axis is involved in auxin and cytokinin response during *Arabidopsis* somatic embryogenesis**  
Ying Hua Su, Yu Bo Liu, Bo Bai and Xian Sheng Zhang



# Editorial: Molecular and Cellular Plant Reproduction

Dazhong Zhao<sup>1\*</sup>, Kang Chong<sup>2</sup> and Ravishankar Palanivelu<sup>3</sup>

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**Keywords:** sexual and asexual reproduction, flowering time and flower development, embryo and fruit development, meiosis, sex determination, pollen tube growth, self-incompatibility and pollination, sterility and floral organ degeneration, epigenetics, gene regulatory networks and live-cell imaging

## Editorial on the Research Topic

### Molecular and Cellular Plant Reproduction

The world is now facing many severe problems due to increasing global demands for already limiting food and energy resources. Plant reproduction is crucial not only for producing offspring but also for improving crop quality and yield. Moreover, plant reproduction entails complex growth and developmental processes, which offer various opportunities for elucidating fundamental principles in biology. To provide a comprehensive update on the myriad of topics in the field of plant reproduction, we invited contributions for a Research Topic entitled “Molecular and Cellular Plant Reproduction”. After an overwhelming response, reflecting the active research being undertaken in plant reproduction, we published a total of 31 papers, including 1 hypothesis and theory, 4 perspectives, 12 reviews, and 14 original research articles. As a result, this special issue covers the broad scope and dynamics of studies on plant reproduction, ranging from a hypothesis of the sexual reproduction cycle, asexual reproduction, flowering time control, regulation of inflorescence architecture, floral stem cell termination, flower, lemma, palea, petal and anther development, anther dehiscence, sex determination, meiosis, pollen tube growth, self-incompatibility, pollination, embryogenesis, endosperm development, germline, antipodal and suspensor cell differentiation, fruit development, gynogenesis and androgenesis, to sterility caused by floral organ degeneration and abiotic stresses.

The life cycle of flowering plants features the alternation of diploid sporophyte and haploid gametophyte generations, in which a crucial step is to specify diploid reproductive cells from somatic cells. Animals generate diploid germlines during early embryonic development, while flowering plants determine reproductive cell lines post-embryonically. Bai proposes a sexual reproduction cycle (SRC) hypothesis, which generalizes sexual reproduction into an alternated three-step cycle consisting of meiosis, sex differentiation, and fertilization. In his hypothesis, Bai makes a remarkable attempt to address evolution as well as differences and similarities in sexual reproduction among various organisms, including fungi, plants, and animals. Sexual reproduction bestows selective advantages during evolution. Interestingly, Hojsgaard and Hörlndl argue that “a little bit of sex” is helpful for apomictic plants to avoid genomic decay and extinction. Zhu et al. show that asexually propagated *Alternanthera philoxeroides* produces dysfunctional stamens due to decreased expression of B-class genes, suggesting that loss of sexual reproductive capacity might help evolution of asexual reproduction in clonal plants or *vice versa*.

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Flower development is a long and complex process, which mainly includes four stages, i.e., vegetative to reproductive phase transition, specification of floral meristem identity, establishment of floral organ identity, and floral organ morphogenesis. In flowering plants, environmental and developmental cues trigger the conversion of the shoot apical meristem in adult plants to an inflorescence meristem, which subsequently gives rise to floral meristems. Reproductive cells that are specified from somatic cells in flowers initiate a germline lineage and then culminate with the production of gametes. Han et al. discuss recent findings about the role of cytokinin in inflorescence branching, which may contribute to the complexity of inflorescence architecture. Initiation and termination of floral stem cells in the right time and place are critical for flower formation. Sun and Ito review how transcriptional regulation and epigenetic machinery coordinately control floral stem cell termination during flower development. Using RNA-seq analysis, Zhang et al. identify ~24,000 flower-expressed genes at three stages, implying the need of complex gene networks for flower development and providing a valuable dataset for further studying genes important for flower development in *Arabidopsis* and other species. Flowers in monocots and dicots are structurally different in terms of their perianths. Lombardo and Yoshida discuss an interesting question about the origin of lemma and palea as well as regulation of their development by transcription factors in rice. Using *Gerbera hybrida*, which produces numerous petals, Li et al. profile differentially expressed genes in petals treated with gibberellin (GA) and abscisic acid (ABA), which provides a useful genomic resource for studying petal morphogenesis.

Flowering phase transition, floral meristem identity, and floral organ identity have been extensively studied. However, what is less clear and what has received less attention is how floral organs are established, especially the formation of anther and ovule, where male (pollen) and female gametophytes are differentiated from somatic cells, respectively. The anther includes reproductive microsporocytes (male meiocytes) and somatic anther wall cells. Kelliher et al. compare anther cell differentiation among *Arabidopsis*, rice, and maize and also discuss potential functions of small RNAs in anther patterning. Atallah and Banks review recent genetic and biochemical studies on how the pheromone antheridiogen (gibberellins) determines the sex of gametophytes in ferns via gibberellin signaling and biosynthetic pathways. Moreover, transcriptome analysis reveals differentially expressed genes in male and female flowers of the monoecious species *Quercus suber*, which may shed light on gene networks regulating sex specification Rocheta et al. Wang et al. show that the miR319a-targeted *TCP24* controls anther dehiscence by negatively regulating secondary cell wall thickening of the endothecium.

Meiosis is an essential process for sexual reproduction, leading to the formation of male and female gametophytes. Li et al. report their development of MeioBase, which provides a valuable database and various tools for meiosis research. Wu et al. show that the meiosis-specific cyclin-like protein SDS (SOLO DANCERS) is required for double-strand break (DSB) formation in rice, rather than DSB repair found in *Arabidopsis*, revealing distinct functions of this conserved protein in plants. Qu et al.

highlight the role of the actin cytoskeleton in pollen tube growth from several different angles, including the spatial distribution of actin filaments in the pollen tube, use of actin markers for dissecting organization and dynamics of actin filaments in growing pollen tubes, actin filament dynamics in the pollen tube, as well as functions and mechanisms of actin-binding proteins (ABPs) in actin filament generation and dynamics. McDowell et al. show that disruption of *AMINOPHOSPHOLIPID ATPASE 6* (*ALA6*) and *ALA7* genes causes changes in the properties of the pollen tube plasma membrane, demonstrating a role of *ALA* genes in pollen fitness under normal and temperature-stress conditions. In addition, Vogler et al. describe a live-cell imaging method for characterizing distinctive vesicle and F-actin polarization during pollen activation, germination, and the establishment of pollen tube tip growth in *Arabidopsis*.

Williams et al. review the molecular mechanism underlying S-RNase-based self-incompatibility by discussing uptake of S-RNase into pollen tubes, formation of SLF (S-locus F-box)-containing SCF complexes, the nature of SLF proteins, and the fate of non-self S-RNases in pollen tube. Brito et al. found that pollination promotes female gametophyte development in immature tobacco flowers; however, the nature of these induction signals and the sensing mechanisms in the female gametophyte remain mysterious.

Five reviews and 3 research papers cover embryogenesis, highlighting the importance of parent-of-origin genes, epigenetic control, and hormone signaling during embryo development. Luo et al. discuss the requirement of gamete-delivered transcripts and imprinted genes for zygotic division and cell fate determination during embryogenesis. They also briefly discuss the regulation of imprinted genes in embryos. In particular, Russell and Jones anticipate more complex functions of male gamete-expressed genes during embryogenesis after discussing initiation and origin of the male germ lineage in flowering plants, features of sperm cells, expression of male germ-derived transcripts, common but divergent profiles of transcriptomes and proteomes in egg and sperm, transposable elements in pollen and male germline, contribution of sperm cells to fertilization, as well as male expression and evolutionary selection.

Epigenetic control is critical for plant growth and development, including sexual reproduction. Shi et al. review how DNA methylation, histone methylation, polycomb silencing, histone acetylation, as well as small and long non-coding RNAs are involved in flowering time regulation, floral organogenesis, meiosis, embryogenesis, and the endosperm development in rice. Using the whole genome analysis, Jung et al. detected repression-related epigenetic histone modifications in a large number of germline genes, suggesting that epigenetic control is a main mechanism for repressing germline genes during sexual reproduction. Tonosaki and Kinoshita summarize the roles of various polycomb repressive complex 2 (PRC2) proteins in the cereal endosperm development and suggest that monocots and eudicots may use different programs to control endosperm development. Moreover, Köhler and Lafon-Placette propose that the active PRC2 in the central cell and endosperm contributes to the transposon silencing in the egg cell and embryo by maintaining DNA hypomethylation activities.

Localization and mutant studies on PIN and auxin signaling discover a correlation between auxin signaling and antipodal cell proliferation in maize, which may provide a clue about the function of antipodal cells in embryo development Chettoor and Evans; this is particularly important given that the function of antipodal cells has not been assigned even though their presence in angiosperms is near universal and has been known for a long time. Scarlet runner bean (SRB) has been used as a model for studying embryo development for over 40 years due to its giant embryo. Henry and Goldberg review recent progress in identifying gene regulatory networks controlling suspensor differentiation and development using comparative genomic tools. Su et al. show that auxin and cytokinin signaling are engaged in establishment of the shoot-root axis during somatic embryogenesis in *Arabidopsis*, which provides insight into apical-basal patterning in embryo development.

As the final product of reproduction, fruit is vitally important for seed development in plants and serves as an indispensable source of food for animals, including humans. Wang et al. review progress on dissecting developmental and genetic mechanisms regarding the evolution and development of fruit size, shape, color, and morphological novelties in *Solanaceae*. Portemer et al. report that mutants in gynogenesis or androgenesis cannot be readily found in mutant screens; they demonstrate this outcome by screening for mutants in an EMS-mutagenized *Arabidopsis* population. Flower development is highly sensitive to environmental factors. Floral organ degeneration or abortion occurs naturally, leading to unisexual or fully sterile flowers. Abiotic stresses cause abnormal flower development and consequently affect crop yield and quality. Smith and Zhao review physiological and molecular genetic mechanisms underlying floral organ degeneration and fertility. In addition, they discuss how abiotic stresses, including heat, cold, and drought, affect sexual reproduction at morphological and genetic levels. Further understanding of sterility caused by floral organ degeneration and abiotic stresses will help plant scientists use both traditional and molecular breeding approaches to develop crops with high yield in stress environments.

This special research topic covers a variety of important issues, providing evidence of the explosive interest and considerable

progress that has been made recently in plant reproduction. We envision that the articles compiled in this E-book will draw attention of all plant biologists to this research topic and help solve challenging and unresolved questions. Employment of combinatorial approaches involving molecular genetic tools and emerging technologies, such as fluorescence-based imaging techniques and next generation sequencing, will advance future studies on all reproductive processes in model plants, crops, and trees.

Finally, we would like to thank all the authors, reviewers, and the Frontiers editorial office for their contributions and hard work that made this interesting and special issue a reality.

## AUTHOR CONTRIBUTIONS

DZ wrote the editorial. KC and RP revised it.

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# The concept of the sexual reproduction cycle and its evolutionary significance

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The concept of a “sexual reproduction cycle (SRC)” was first proposed by Bai and Xu (2013) to describe the integration of meiosis, sex differentiation, and fertilization. This review discusses the evolutionary and scientific implications of considering these three events as part of a single process. Viewed in this way, the SRC is revealed to be a mechanism for efficiently increasing genetic variation, facilitating adaptation to environmental challenges. It also becomes clear that, in terms of cell proliferation, it is appropriate to contrast mitosis with the entire SRC, rather than with meiosis alone. Evolutionarily, it appears that the SRC was first established in unicellular eukaryotes and that all multicellular organisms evolved within that framework. This concept provides a new perspective into how sexual reproduction evolved, how generations should be defined, and how developmental processes of various multicellular organisms should properly be compared.

**Keywords:** sexual reproduction cycle, meiosis, heterogametogenesis, fertilization, generation

Sex is always a hot topic in human society and is an intensively investigated phenomenon in biology. Studies on sex determination in plants have focused on regulation of unisexual flower development (Ainsworth, 1999; Bai and Xu, 2013) since plant sex was defined based on unisexual flowers by Robbins and Pearson (1933), i.e., “a flower or plant is male if it bears only stamen and that is female if it bears only pistils.” However, plants with unisex-ual flowers account for only a small percentage of angiosperms, and angiosperms themselves are only some of the species in the plant kingdom. This raises the question of what sex is in angiosperms with perfect flowers (with both pistils and stamens) or in plants without flowers. Even further, do sex and sexual differentiation share any features in common in the plant and animal kingdoms?

To understand the regulatory mechanism of sex differentiation, following the concept originated from Robbins and Pearson (1933), we have investigated the regulation of unisexual flower development using cucumber for more than a decade. Cucumber is monoecious, and naturally bears both male and female flowers and, rarely, even hermaphrodite flowers, on the same plant. The ratio of male and female flowers can be affected by application of phytohormones such as ethylene (increasing the proportion of female flowers) or GA (increasing the proportion of male flowers). As hormones play key roles in mammalian sex expression, this phytohormonal regulation of the ratio of male and female flowers led to the use of cucumber a model system for research into plant sex determination starting in the 1960s (see Bai and Xu, 2013 and references there in). However, following the discovery that both male and female flowers contain initiated stamen and carpel primordia, we found that flowers become female because stamen development is inhibited early at stage 6, and that ethylene is involved in this inhibition (for detailed review, see Bai and Xu,

2013). These findings indicated that analysis of regulatory mechanisms in unisexual flowers will allow us to understand only how the inappropriate organs are inhibited, not how the appropriate organs are differentiated. We therefore referred to this situation as a “bird-nest puzzle,” meaning that it is not adequate to understand how a bird lays and hatches eggs through investigating how the nest was ruined (Bai and Xu, 2012).

Unisexual cucumber flowers are not the only example of this type of puzzle. All unisexual flowers in monoecious plants and many in dioecious plants for which the developmental mechanisms are known result from inhibition of one type of sexual organs (Bai and Xu, 2013; Akagi et al., 2014). To solve the “bird-nest puzzle,” we proposed a new way to define sex as a “heterogamete-centered dimorphic phenomenon,” sex differentiation as “the key divergence point(s) leading to the heterogamete differentiation” (Bai and Xu, 2013), which is in line with a definition previously suggested (Juraze and Banks, 1998), and generally applicable not only to all species in plant kingdom, but also in other kingdoms. We also hypothesized that before multicellular organisms emerged, a process called the sexual reproduction cycle (SRC) evolved based on the existing mitotic cell cycle in unicellular eukaryotes. This SRC starts from one diploid zygote, goes through meiosis, gametogenesis, and fertilization, and ends with two diploid zygotes. This review addresses the question of whether adopting the SRC concept can facilitate our understanding of sex.

## PREMISES FOR PROPOSAL OF THE SRC

We begin with the basic facts based on which the SRC was hypothesized.

Firstly, although DNA transmission in prokaryotes is sometimes referred to as “recombination” (Cavalier-Smith, 2002 and

references therein), it is widely accepted that sex and sex differentiation are phenomena occurring in eukaryotes, which contain chromosomes, nuclei, cell skeletons, and the mitotic cell cycle, regardless of the obscurity of their evolutionary origins (Cavalier-Smith, 2002; Kirschner and Gerhart, 2005; Knoll, 2014). We would restrict our discussion in eukaryotes.

Secondly, meiosis is highly conserved in almost all known eukaryotes, including animals, plants, fungi, and protists (Logsdon, 2007; Schurko and Logsdon, 2008). It is also widely accepted that meiosis may originate from mitosis, probably via occasional mistakes in cohesin binding and/or digestion on chromosomes (Cavalier-Smith, 2002; Marston and Amon, 2004).

Thirdly, despite the extreme diversity of morphology and recognition mechanisms of gametes, cell fusion of two haploid gametes into a new diploid zygote is conserved in all eukaryotes.

Fourthly, while dimorphism of gametes is common in the organisms with which people are familiar, the smaller gametes being referred to as sperm and the bigger ones as eggs, there are also heterogametes that can be distinguished only at the molecular level, with different mating types such as *MATa/α* in yeast and *MTA/MTD* in *Chlamydomonas* (Goodenough et al., 2007). Furthermore, heterogamy is not restricted to dimorphism, but rather can include multiple mating types, e.g., three in *Dictyostelium* and seven in *Tetrahymena* (Nanney and Caughey, 1953; Bloomfield et al., 2010; Cervantes et al., 2013). It is worth noting that such multiple mating types are mainly found in protists and fungi, but not in plant and animal kingdoms.

Fifthly, regardless of the presence or absence of germlines, e.g., in animals or plants, respectively, (Evens and Walbot, 2003), new generations in all multicellular organisms are generated through sexual reproduction consisting of the three key events: meiosis, sex differentiation, and fertilization. With this view, sexual reproduction is predicted to be more ancient than multicellular structures as sexual reproduction already existed in unicellular eukaryotic organisms.

There has been much debate about how sexual reproduction evolved (e.g., <http://en.wikipedia.org/wiki/Sex>; <http://www.britannica.com/EBchecked/topic/536936/sex>). It seems nearly impossible to obtain direct evidence regarding what happened during the period when sexual reproduction emerged, unless someday people can artificially “recapitulate” the evolutionary process. Nonetheless, we can try to explore the events involved in such evolution by analyzing the benefits for which the ancient evolutionary innovations could have been selected.

## MEIOSIS: A LUCKY MISTAKE?

The first indispensable event in sexual reproduction is considered to be meiosis. It is currently agreed that the most important benefit of meiosis is increasing genetic variation through recombination. However, by definition, meiosis is characterized by a reduction of chromosome numbers from diploid to haploid. How, then, did meiosis emerge and become selected?

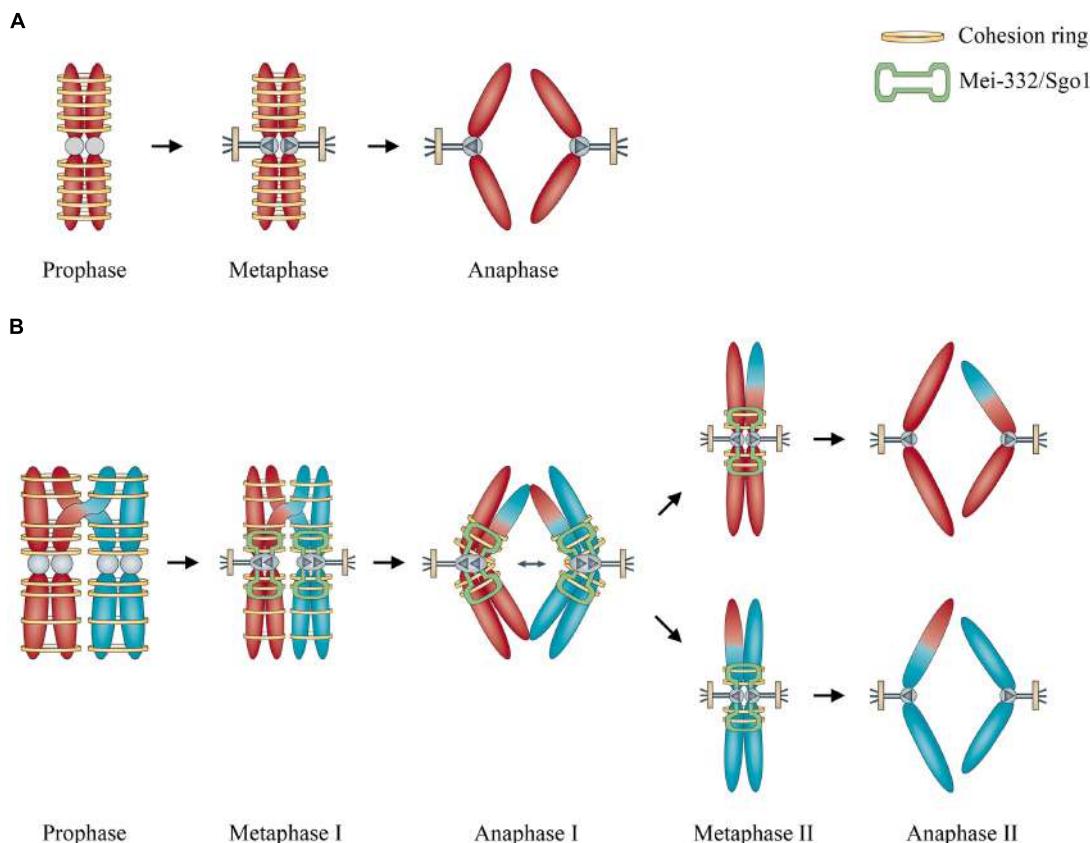
It is known that haploid cells, like diploid cells, can undergo mitosis, such as in budding yeast. Considering the complexity of chromosome organization, it would be reasonable to speculate that the earliest eukaryotic cells were haploid. If that were the case, meiosis would be predicted to have evolved not only after

the emergence of mitosis, but also after the emergence of diploid cells, which may have arisen from cell fusion or chromosome duplication in haploid cells.

While many organisms in the protist and fungus kingdoms live mainly in a haploid state (Campbell and Reece, 2005), almost all multicellular organisms in the animal and plant kingdoms use diploid cells as their building blocks. The prevalence of diploidy in the latter kingdoms suggests that diploidy must confer some advantages. If we naively believe that diploidy can doubly secure the genome stability of eukaryotic cells, then it follows that haploidy provides little leeway for mistakes. From this perspective, reduction of chromosome number would not be a good reason for meiosis to be selected. Instead, meiosis must occur and be selected for other reasons.

Based on Marston and Amon's (2004) comparison of mitosis and meiosis, cohesins play important roles in both processes. In mitosis, cohesins like Scc3, Smc1, and Smc3 facilitate the cohesion of the two sister chromosomes, whereas in meiosis, the cohesins can hold together non-sister chromosomes from two different chromosomes (Figure 1). This might be analogous to playing ringtoss: the ring is thrown to capture a target, but sometimes the ring mistakenly captures something else together with the target. If cohesion is required for mitosis, mistaken association of non-sister but homologous chromosomes by cohesins may possibly occur like an off-target ring toss, and this may result in meiosis, facilitated by an ultimately meiosis-specific cohesin Rec8 and a kinetochore-associated protein MEI-S332/Sgo1 (Marston and Amon, 2004) and with abnormal degradation of cohesins afterward (Cavalier-Smith, 2002). Recently Ross et al. (2013) reported an evolutionary analysis on how a neogene acquired an essential function for chromosome segregation in *Drosophila melanogaster*, opening up a new perspective for investigating how the molecular mechanism of fundamental events like meiosis was evolved.

Why would a mistakenly occurring, “unnecessary” cell division be selected evolutionarily? Probably because of meiotic recombination. Although DNA transmission from cell to cell already existed in prokaryotes, meiotic recombination is considered to be the first efficient mechanism evolved for autonomously increasing genetic variation. This begs the question of why genetic variation would be so important for a cell that meiosis conferred an advantage during evolution. Regardless of how the first cell arose from an RNA world or pre-cellular biosystem, afterward the cells were relatively isolated from the environment from which they emerged. Although the advent of the cell granted the biosystem tremendous independence and the ability to proliferate itself through cell division, it created a problem of adapting to the unpredictable changes in its environment. Spontaneous DNA mutation is the original way to adapt, but with low efficiency. By contrast, meiotic recombination can generate numerous genetic variations more efficiently. Among the variations randomly generated during meiosis could be those that are adaptive to the prevailing environmental conditions and enable cell survival in a changed environment. Therefore, increasing genetic variation for adaptation might be a primary reason for meiosis to be selected. The stress-induced meiosis observed in protists is consistent with this speculation.



**FIGURE 1 | Illustration of the role of cohesins in the origin of meiosis.** (A) Cohesins hold sister chromosome together during mitosis. (B) Cohesins mistakenly hold non-sister but homologous chromosomes together, enhanced by kinetochore-associated protein Mei-332/Sgo1 during meiosis. Modified from Marston and Amon (2004), by permission of Nature Publishing Group.

## DID FERTILIZATION EMERGE PRIOR TO MEIOSIS?

It is conventionally understood that the sexual reproduction starts from meiosis and ends at fertilization. However, as we speculated previously, it is likely that eukaryotes first emerged as haploid. If that is the case, cell fusion, rather than cell division, should be an ancient event in the emergence of diploid cells. Regardless of whether the first eukaryotic cell emerged when one prokaryotic organism engulfed another as Margulis and Sagan (1991) summarized and of how chromosome duplication originated, without diploid cells there would be no meiosis. Since fertilization is essentially a cell fusion process, we can predict that it is derived from the ancient cell fusion mechanism.

If indeed cell fusion arose prior to meiosis, once meiosis emerged in the resulting diploid cells, fertilization could be readily used as a mechanism to restore genome diploidy. Furthermore, if we think about the genetic variation randomly increased by meiosis, fertilization actually retains the variation derived from both cells that are the products of meiosis. In addition, considering the interaction between the meiotically produced cells and their environment, fertilization between the surviving haploid cells would actually execute a selective function in maintaining variations adaptive to that environment, as cells carrying non-adaptive variations would have not survived to participate in fertilization.

Therefore, the advantages of fertilization include not only restoration of genome diploidy, but more significantly, double retention of the selected genetic variations generated through meiosis.

However, each meiotic cell would generate four types of resulting cells after random recombination. If those four cells randomly paired and fused, too much variation would rapidly diversify the characteristic genome structure of the species along with the increase of round from one zygote to zygotes of next generations. This does not even take into account the genetic complexity from a population perspective, in which the meiotically produced cells could pair and fuse with those arising from other meiotic cells. Is there any way to solve that problem?

## A BENEFIT OF HETEROGAMY: LABELING MEIOTICALLY PRODUCED CELLS TO HARNESS VARIATION WHILE ENHANCING HETEROGENEITY

Heterogametes in animals and plants generally display morphological differences, i.e., small sperm and large egg cells in comparison with their progenitor meiotic cells. However, as mentioned above, heterogamy in unicellular eukaryotic organisms is frequently determined at the molecular level by a single genetic locus, and can include more than two mating types. If we use the assumption that unicellular eukaryotes evolved prior

to multicellular organisms, we may infer that the morphological and/or physiological differentiation of heterogametes in multicellular organisms should be considered elaborations of the very simple differences, such as at a single genetic locus, observed in gametes of unicellular eukaryotes. This inference is consistent with the recent finding that in *Volvox*, expansion of a mating locus causes heterogametes to change from being equal in size to being dimorphic (Ferris et al., 2010).

What is the advantage of heterogamy that enables the mating loci to be selected among the enormous genetic variation? If we remember the problems mentioned above regarding random pairing and fusion of meiotically produced cells in fertilization, we can speculate that heterogamy would significantly restrict diversity. With heterogamy, meiotically produced cells are classified into different groups that prevent pairing and fusion of those among the same group. In other words, heterogamy is essentially a mechanism of labeling meiotically produced cells to prevent “self-mating,” therefore “harnessing,” while enhancing, heterogeneity generated from meiosis and fertilization. As it allows pairing and fusion only between haploid cells from different groups, this harnessing mechanism creates a relatively stable interval during which the adaptive cells can be selected.

If the essential function of heterogamy is the labeling of meiotically produced cells and thereby harnessing variation while enhancing heterogeneity, there should be a multitude of ways to achieve this. Genetic loci for mating types probably represent the most ancient and simple way, but there could be many other modifications to enhance the differentiation for higher efficiency.

In majority of animals familiar to human experience, heterogametes are differentiated from germlines that migrate into and complete the differentiation in dimorphic gonads during embryogenesis. Heterogamy is determined mainly in gonad differentiation prior to germ cells undergoing meiosis. One may therefore believe that sex determination or differentiation is a precondition of the occurrence of meiosis. The situation appears similar in plants if we examine only angiosperms. However, if we take ferns and mosses in consideration, it is easily found that dimorphism of multicellular structures is not necessarily required for meiosis and that heterogamy in multicellular organisms can be achieved after meiosis (Figure 2), similar to what takes place in *Chlamydomonas* and *Volvox* (Goodenough et al., 2007; Ferris et al., 2010). If we compare the divergence points in green algae and the four groups of land plants, we see a trend in which the divergence point(s) that leads to the heterogamete differentiation shifted from gametophytes after meiosis to sporophytes before meiosis in green algae and angiosperms, respectively. Little is known regarding how this shift evolved. However, efficiency in gamete distribution and meeting might contribute to the shift: in *Chlamydomonas*, the two types of gametes differentiate in water and their pairing and fusion occurs randomly. In mosses and ferns, sperm cells are shed into water as well and swim to archegonia and eggs with water as the medium. In gymnosperms and angiosperms, in which the divergence points are shifted to sporophytes, the delivery of sperm is no longer restricted to water. This may allow these two groups of plants to increase their spatial distribution.

Similarly, if we examine mechanisms of animal sex differentiation with a broader view, there are also diversifications worth noting: although gametogenesis is carried out in the germlines, sex differentiation mainly occurs at the gonads. While mammalian gonad differentiation from bipotential to unisexual is triggered by sex-determining genes, a similar gonad differentiation is induced by environmental temperature in some reptiles (Ramsey and Crews, 2009). This implies that over the course of evolution there might be a trend in which determination of heterogametogenesis shifted from germ cells in *cis* to somatic gonads in *trans*, and further that the trigger(s) for gonad differentiation shifted from environmental signals to genetic factors encoded in chromosomes, and even further that the chromosomes bearing genetic factors determining sex evolved into sex chromosomes, as suggested by Charlesworth et al. (2005).

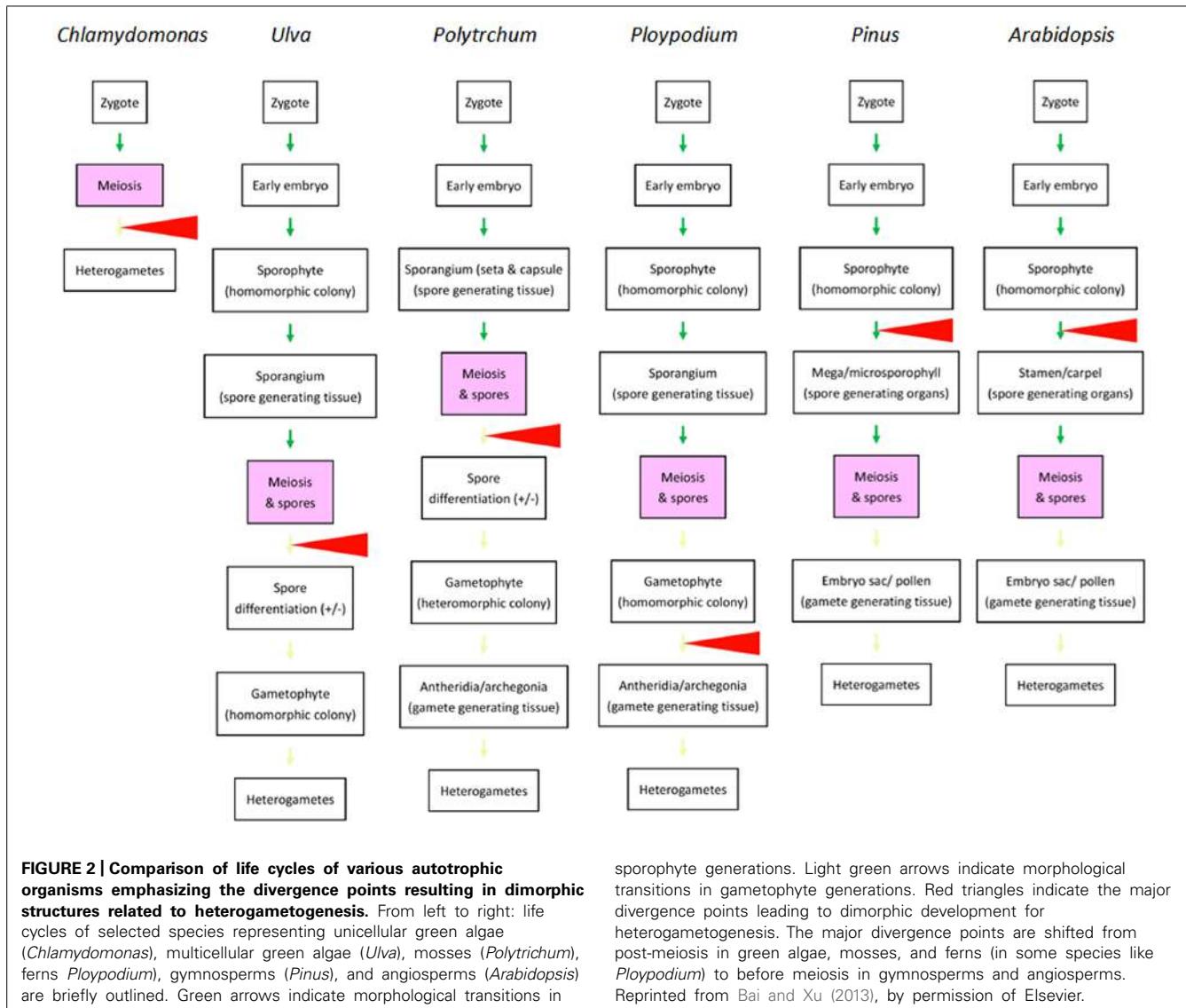
If the above speculation is accurate, sex differentiation indeed can be considered essentially a labeling mechanism for heterogamy, regardless of how diversified in form and complicated in regulation, in a wide spectrum of organisms from unicellular eukaryotes to multicellular animals, plants, and fungi.

## SEQUENTIAL DIFFERENTIATION OR INTEGRATION OF INDEPENDENTLY EVOLVED EVENTS?

In animals, meiosis and gametogenesis occur sequentially in germlines and sex differentiation occurs in somatic gonads into which the germline migrated. In plants, meiosis and gametogenesis occur separately from somatic cells of the sporophyte and gametophyte, while sex differentiation could occur either in sporophyte or gametophyte. How were meiosis, gametogenesis, and sex differentiation originally integrated? Considering that all three processes exist in protists, one possible scenario is that each evolved independently, and they were integrated together as a coordinated process by chance and thereafter genetically fixed as a program in protists. This scenario is possible because all three processes, meiosis, heterogametogenesis (including sex differentiation and gametogenesis), and fertilization, occur at the cellular level. Protists are unicellular eukaryotes and live in a population. These two characteristics provide the required conditions for SRC emergence: on one hand each cell can behave independently for emergence of meiosis and gametogenesis, and on the other, all cells live together closely enough to make both cell fusion and cell–cell recognition possible. Integration of the three events would have brought all of their selective advantages together and such integration, now referred as SRC, would be therefore selected during evolution.

## MODIFIED CELL DIVISION: ORIGIN OF GENERATIONS

In nearly all biology textbooks, meiosis is introduced in comparison with mitosis, whereas fertilization and sex determination or differentiation are introduced elsewhere. However, if we view meiosis and fertilization together, we find that one cell becomes four (except in some particular cases, such as angiosperms and mammalian, only one female meiotically produced cell remaining alive to differentiate into female gamete) through meiosis and two cells become one through fertilization. Thus, the net result of the entire SRC is that one cell becomes two, just like one round of mitosis. The fundamental difference between the SRC and the mitotic cell cycle is that the genetic compositions of the two cells



resulting from the SRC are different from that of the progenitor while the products of the mitotic cell cycle remain similar to that of their progenitor (Figure 3). It needs to be emphasized that what can appropriately be compared with the mitotic cell cycle is not meiosis alone, but rather the entire process of SRC, including meiosis, heterogametogenesis, and fertilization.

Cell division occurred well before eukaryotes evolved. Despite the difference in complexity between mitotic cell division in eukaryotes and cell fission in prokaryotes, the two processes are similar in that the two resulting cells retain the same genome structure as the starting cell. By contrast, the genome structures of the two cells resulting from the SRC are no longer the same as that of the original cell, as described above. They are genetically a new “generation.” From this perspective, although the terms “mother cells” and “daughter cells” are often used in describing the beginning and resulting cells in mitosis, these cells do not truly represent two generations. They are actually clones, the same as in the cell division observed in *Escherichia coli* leading to a proliferation of

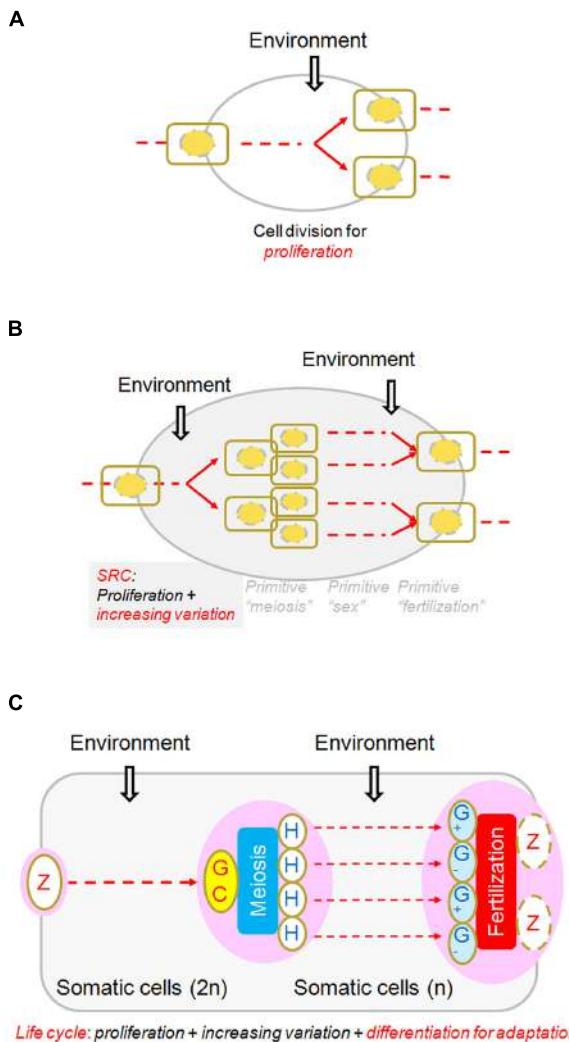
sporophyte generations. Light green arrows indicate morphological transitions in sporophyte generations. Red triangles indicate the major divergence points leading to dimorphic development for heterogametogenesis. The major divergence points are shifted from post-meiosis in green algae, mosses, and ferns (in some species like *Ploypodium*) to before meiosis in gymnosperms and angiosperms. Reprinted from Bai and Xu (2013), by permission of Elsevier.

the same generation. Only through the SRC is a new generation created.

According to Chen et al. (2013), genetic variations are generated in several ways, such as mutation and new gene origination, in addition to meiotic recombination. However, only variations retained through the SRC can be maintained from one generation to the next, rather than being diluted and ultimately disappearing through continuous cell divisions. In that sense, mainly because it was integrated as part of the SRC, meiotic recombination took a prominent position among the various ways of creating genetic variations.

### THE SRC: AN APPENDIX OF A MULTICELLULAR ORGANISM OR A FRAMEWORK ONTO WHICH MULTICELLULAR STRUCTURES ARE INTERPOLATED?

When animal development is discussed, an organism and embryogenesis takes center stage. Germline initiation is an appendant event during embryogenesis, while meiosis and gametogenesis are



only two events of germline differentiation. A similar situation occurs in our understanding of plant development. The focus is mainly on the morphogenesis of multicellular structures. However, if we take the standpoint that the SRC emerged in protists, an obvious inference is that multicellular structures all emerged within the framework of the SRC. Is this possible?

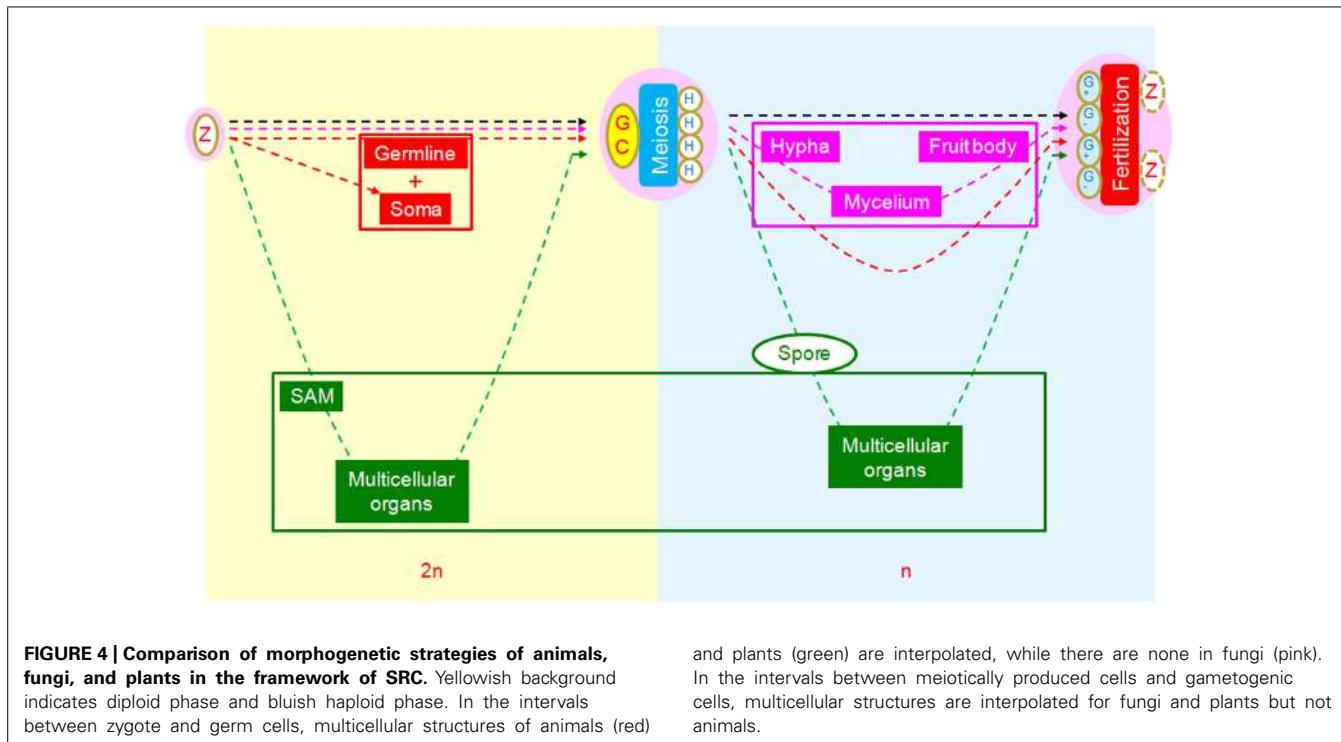
If we accept the argument that cell growth and division are coupled with optimal cell volume (Buchanan et al., 2000), and

the inference that meiosis is a stress-induced specific cell division for adapting to unpredicted environmental challenges, we can imagine that under optimal environmental conditions, a cell would keep dividing in order to maintain optimal cell volume. That means that between a zygote and a meiotic cell, and between meiotically produced cells and gametes, there would be two intervals during the process completing the SRC (Figure 3C). In unicellular protists, cells in these two intervals would be mainly freely living in a population. However, under certain conditions, for example nutritional shortages, the free-living cells might be aggregated or organized, such as in *Dictyostelium* (Dormann et al., 2002) and *Volvox* (Kirk, 2005). What would result if the conditional aggregations or organizations were genetically fixed? Multicellular organisms! If this speculation is valid, what is the relationship between the core cells involved in the SRC, such as the zygote, meiotic cells and gametes, and those involved in multicellular structure morphogenesis? A reasonable answer is that the SRC serves as a framework or backbone, within or on which multicellular structures consisting of "somatic cells" could interpolated into the two intervals when the environment was optimal. Different from simply maintaining cell volume through cell division, organized multicellular structures ultimately facilitate energy acquisition and environmental adaptation. All elaborated sex differentiation and mating behaviors can be viewed as nothing more than modifications of multicellular structures evolved afterward to facilitate meeting, recognition, and proper fusion of the two gametes.

From this perspective, we can also see a relatively novel scenario in which to compare the core developmental processes of animals, plants, and fungi using the SRC as a common reference framework (Figure 4). From this comparison, we observe three different strategies of morphogenesis. In animals, the multicellular structures (soma) are interpolated at the first interval between zygote and meiotic cells as rest of embryos in addition to germlines. In fungi, the multicellular structures are interpolated at the second interval between meiotically produced cells and gametogenic cells, with unknown mechanisms of cell aggregation (Meskauskas et al., 2004; Lee et al., 2010). In plants, multicellular structures are interpolated at both intervals, with an additive strategy (Bai and Xu, 2013).

## CONCLUSION: THE SRC AS A KEY EVOLUTIONARY INNOVATION

Taking the above analysis together, several speculations can be made: first, meiosis is the most effective mechanism evolved to autonomously increasing genetic variation; second, the SRC is the first known genetic program of cell differentiation and coordination, integrated with the three independently evolved events, i.e., meiosis, sex differentiation, and fertilization, among cell populations; third, with the SRC, eukaryotes were first equipped with an internal mechanism to adapt effectively to unpredictable environmental challenges. From this perspective, the SRC deserves to be recognized as a key evolutionary innovation. This innovation first arose in unicellular eukaryotes, and then, because of its advantages in adaptation to environmental challenges, was adopted by subsequent multicellular organisms as a conserved program. Only based on the SRC can generations be properly defined, thereby



allowing genetic analysis of biological processes. In addition, SRC establishes a reasonable framework in which to understand how multicellular organisms evolved in various kingdoms, including animals, plants and fungi.

I used to be highly curious about why the developmental processes of multicellular organisms are unidirectional, i.e., from zygotes to gametes. After I became aware of the existence of the SRC, this puzzle seemed easy enough to be solved because the SRC is a program that originally emerged and was selected as a response to environmental stresses. As environmental stresses occur ultimately independent of organisms and irreversible, the SRC process is irreversible and therefore unidirectional. Although the three core events in the SRC of many multicellular organisms have been genetically encoded during evolution and may not directly respond to environmental stresses, the unidirectionality was inherited from their unicellular eukaryotic ancestors.

Another issue confusing for a long time is how to understand the role of “asexual reproduction” in biological processes, such as speciation (Coney and Orr, 2004). With the concept of the SRC, it is clear that only the organism multiplication that is coupled with the SRC should be regarded as “reproduction,” as new generations are produced. Multiplication of organisms not coupled with SRC should not be regarded as “reproduction,” as no new generations are produced. Instead, the products of the latter are properly referred to as clones, equivalent to mitotic cell division, regardless of how complicated and similar the process is in comparison to the process starting from a zygote. In that sense, the past comparisons of “sexual reproduction” vs. “asexual reproduction” should more correctly be of “reproduction” vs. “proliferation.” The former refers to a multiplication of organisms based on the SRC, from one generation to the next in a lineage, while the latter

refers to a multiplication of organisms remaining within the same generation.

One more issue worth noting here is that the SRC is essentially a mechanism allowing diploid cells to adapt to unpredictable environmental challenges. If an organism adapts to its environment in the haploid form, there would be no selective pressure for it to adopt SRC. This can explain why there are numerous examples of meiosis and fertilization occurring only rarely in many haploid-dominant protists (Logsdon, 2007), and no stable SRC identified in many haploid-dominant fungi (Heitman et al., 2013).

In his book “Primitive Land Plants” first published in 1935, Bower (1935) had advised that in understanding plant morphology, we should adopt “upward comparative analysis,” i.e., use forms of organisms that emerged early as a reference framework, and treat the new traits in organisms that emerged later as modifications upon the earlier forms. Similar principles can also be applied to understanding sex and sexual reproduction.

Lastly, I would like to emphasize that without the investigation of unisexual flowers, which brought about the “bird-nest puzzle,” and subsequent historical retrospection of the definition of sex in plants, I would not have this chance to think about the essential role of sex in living systems. In addition, without analysis of highly diversified sex differentiation, i.e., divergence points leading to heterogametes in various plant groups, it would be impossible to disconnect the sex or sex differentiation from meiosis, as in all animal systems, sex differentiation always occurs prior to meiosis, and therefore meiosis appears undeniably linked to sex differentiation. In many aspects, our views of biological processes in plants have been borrowed from those developed in animal research, into which vastly more resources have been invested since we, as humans, belong to the animal kingdom. Sometimes, however,

plants provide an invaluable alternative due to their different morphogenetic strategies. Ultimately, how we look at biological events depends on what questions we address. If we want to understand fundamental principles of living systems, we need to properly compare animals and plants at least, as each can be used as a control for the other. Alternatively, if one only wants to know the mechanism of animal disease or crop yield for instance, that comparison might be not necessary. For questions about sex *per se*, proper comparisons among animals and plants, plus fungi, and more importantly protists, seem indispensable!

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# A little bit of sex matters for genome evolution in asexual plants

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Genome evolution in asexual organisms is theoretically expected to be shaped by various factors: first, hybrid origin, and polyploidy confer a genomic constitution of highly heterozygous genotypes with multiple copies of genes; second, asexuality confers a lack of recombination and variation in populations, which reduces the efficiency of selection against deleterious mutations; hence, the accumulation of mutations and a gradual increase in mutational load (Muller's ratchet) would lead to rapid extinction of asexual lineages; third, allelic sequence divergence is expected to result in rapid divergence of lineages (Meselson effect). Recent transcriptome studies on the asexual polyploid complex *Ranunculus auricomus* using single-nucleotide polymorphisms confirmed neutral allelic sequence divergence within a short time frame, but rejected a hypothesis of a genome-wide accumulation of mutations in asexuals compared to sexuals, except for a few genes related to reproductive development. We discuss a general model that the observed incidence of facultative sexuality in plants may unmask deleterious mutations with partial dominance and expose them efficiently to purging selection. A little bit of sex may help to avoid genomic decay and extinction.

**Keywords:** apomixis, Muller's ratchet, Meselson effect, polyploidy, heterozygosity

## INTRODUCTION

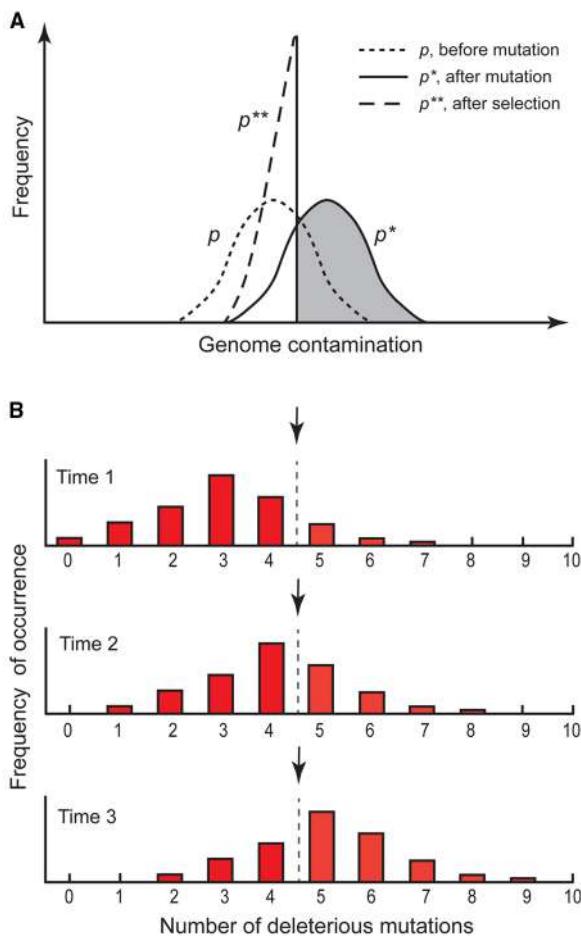
Currently, the understanding of evolution patterns of genomes on different phylogenetic groups is a hot topic in evolutionary biology. The arrival of next generation sequencing (NGS) technologies and the generation of huge amounts of genomic data is allowing researchers to dig in the past and better resolve organisms' natural history as well as evolutionary enigmas. One of such enigmas is the predominance of sex in nature (Otto, 2009). One of the most prominent theories explaining the benefits of sex (for broad analyses see, e.g., Bell, 1982; Birdsell and Wills, 2003) proposes that sexuality protects the genome from the accumulation of deleterious mutations (Muller, 1964; Kondrashov, 1988; Hörndl, 2009; Figure 1). Here we will discuss theoretical assumptions and empirical possibilities of presence/absence of meiosis for asexual plant genome evolution in the light of unexpected recent findings on sexual/asexual taxa of *Ranunculus*.

Sexuality is a crucial factor molding the genomic features of eukaryotes. In plants, the formation of a new individual through sexuality involves an alternation between the sporophytic ( $2n$ ) and the gametophytic ( $n$ ) generations via meiosis and gamete fusion, the two mechanisms that create new genetic combinations. Additionally, outcrossing further potentiates genetic variation in populations. Thus, with few exceptions, every single sexual organism has a distinctive genotype that differentiates it from parents and siblings. Therefore, meiosis is the main source of genetic recombination and mixis, and segregates genetic factors in the offspring creating genetic variation. By doing so, meiosis and

sexuality allows natural selection purging a lineage from harmful mutations. Because plant meiosis produces spores (mega- and microspores) and these spores develop into female and male gametophytes, in which considerable percentages of genes are being expressed (Joseph and Kirkpatrick, 2004), selection in a sexual plant will act at two developmental stages: during gametophyte development (haploid gametophytic selection) and after the formation of the zygote (sporophytic selection) Hörndl (2013).

In contrast, by circumventing or suppressing meiosis and syngamy, asexual organisms skip the alternation of generation cycle and hence elude the ploidy-phase change step. In angiosperms, asexually-derived individuals can be formed either as consequence of vegetative propagation, or of asexual seed formation (apomixis), a trait that is taxonomically widespread in plants (Hojsgaard et al., 2014a). While the first involves extra vegetative growth and fragmentation without undergoing the single-cell stage and embryogenesis, the latter comprises the development of a new organism out of an unreduced, unfertilized egg cell, embryogenesis and seed formation (Mogie, 1992). In apomictic plants, a combination of complex developmental features avoid recombination and reductional steps present in the normal sexual reproductive process, thus developing a seed carrying a clonal embryo (Asker and Jerling, 1992).

A central fact for genome evolution, however, is that apomixis in angiosperms is rarely obligate. Apomictic plants produce asexual and sexual progeny within the same offspring generation, i.e., from different ovules and seeds in the same mother plant,



**FIGURE 1 | Principles of Muller's ratchet.** (A) Scheme of distributions ( $p$ ) of mutations in a sexual population. Before mutation, distribution in the population is ( $p$ ), after mutation, distribution shifts upward to  $p^*$ . After recombination and selection against mutants, individuals in the gray part remain sterile and die, and the distribution goes backward to  $p^{**}$ . At equilibrium the means of  $p$  and  $p^{**}$  are equal (redrawn after Kondrashov, 1988). (B) Scheme of mutational load distributions in an asexual population. Initially, genotypes with zero mutations exist in the population, but are lost over time by drift. Without recombination, the class with zero or few mutations cannot be restored, and consequently mutations accumulate until a threshold level of extinction (arrow) is reached (redrawn after Maynard Smith, 1988).

and therefore asexuality is facultative. Consequently, a proportion of the offspring represents recombinants, but frequencies of sexuality vary a lot among genera, species and different modes of apomixis (e.g., Aliyu et al., 2010; Sartor et al., 2011; Šarhanová et al., 2012; Noyes and Givens, 2013; Hojsgaard et al., 2013, 2014a). The role that facultative sexuality and genetically highly diverse apomictic populations play in the evolution of angiosperms is still unclear.

## THEORETICAL SIGNIFICANCE OF APOMIXIS FOR THE EVOLUTION OF THE PLANT GENOME

Sexuality has the effect that deleterious mutations appear in various genotypic configurations in the offspring. Thus, harmful

mutations expressed under different states (e.g., homozygous, dominant heterozygous, etc.) will negatively affect the genotype's fitness and natural selection will remove such genotypes and purge the lineage from an increase in the mutational load. Evolutionary benefits of purging a lineage from mutation accumulation have long been seen as a major advantage of sexuality (Muller, 1964; Kondrashov, 1988; **Figure 1A**). Apomictic plants, by circumventing genetic reshuffling mechanisms, inherit the same genomic features of their female parental genome. Without recombination, once a genotype acquire a spontaneous mutation cannot reconstitute a non-mutated genotypic state (**Figure 1B and 2**). Thus, a deleterious mutation in any asexual individual will be transmitted to all the offspring and loaded onto the gene pool of that clonal lineage. In any segment of a genome with absence of recombination, the number of random independent mutations is expected to increase because a mutational load smaller than the least-loaded lineage can never be generated (the ratchet mechanism, Muller, 1964; the hatchet mechanism, Kondrashov, 1988). Over time, drift will ultimately lead to a loss of genotypes with a lower mutational load and, once a threshold on mutational load is reached, to extinction of the asexual lineage (**Figure 1B**; Kimura et al., 1963; Muller, 1964; Felsenstein, 1974). Moreover, genetic interference effects between loci (Hill-Robertson effect, Hill and Robertson, 1966) may increase effects of mutations (Kondrashov, 1988). The genetic load of clonal lineages will reduce their fitness and obstruct further adaptation, driving those lineages to an early extinction (Maynard Smith, 1978; Bell, 1982).

Apomixis in plants is nearly exclusive associated polyploids, and often it is a result of hybridity (e.g., Koch et al., 2003; Paun et al., 2006a; Pellino et al., 2013). Polyploidy can accelerate mutation accumulation since additional gene copies represent additional mutational sites. Effects of deleterious mutations will reduce the mean fitness of any individual (and ultimately of the population) with a rate  $cU$ , where  $c$  stand for the ploidy level and  $U$  for the mutation rate per haploid genome (Gerstein and Otto, 2009). However, the effects of single deleterious recessive mutations in heterozygous states can be masked by a functional gene copy of the wild or dominant allele in a diploid organism (Crow and Kimura, 1970; Kondrashov and Crow, 1991; Otto and Whitton, 2000). After a prolonged diploid stage, the return to haploidy leads to the expression of accumulated, but previously masked deleterious recessive alleles, and selection against mutations (Crow and Kimura, 1970; Hörandl, 2009). However, in a polyploid plant with more than two allele copies per locus, accumulated recessive mutations in heterozygous states may still be masked after a return to haploidy. Thus, masking effects will be stronger and recessive mutations may not be effective unless they show certain level of dominance (i.e., partial dominance). So far few genomic studies are available to understand the forces underlying mutation dynamics in polyploid plants. In theory, absence of haploid gamophytic selection plus masking of recessive alleles in polyploid condition should increase the mutational load compared to sexuals.

Allelic sequence divergence, the so-called Meselson effect, is another consequence of long-term asexuality (Mark Welch and Meselson, 2000). Asexual seed reproduction, by suppress-

ing meiosis promotes the divergence between allelic sequences by neutral mutations (Kimura and Crow, 1964). Due to the loss of sexuality, alleles within lineages will gain neutral differences at a much higher rate than the normal substitution rates observed between alleles in sexual populations (Birky, 1996). Until now, the presence of genome-wide allele sequence divergence significantly larger than those in inter-lineages have been hard to prove. Processes such as gene conversion, mitotic recombination, efficient DNA repair, meiotic parthenogenesis in animals (automixis), occasional sex, ploidy reduction, and hybridization can moderate or remove sequence divergence (e.g., Schön and Martens, 1998; Schaefer et al., 2006; Liu et al., 2007; Mark Welch et al., 2008; Flot et al., 2013). Effects of facultative sexuality on allelic sequence divergence in plants remain unknown.

## IS A LITTLE BIT OF SEX SUFFICIENT TO AVOID MUTATION ACCUMULATION?

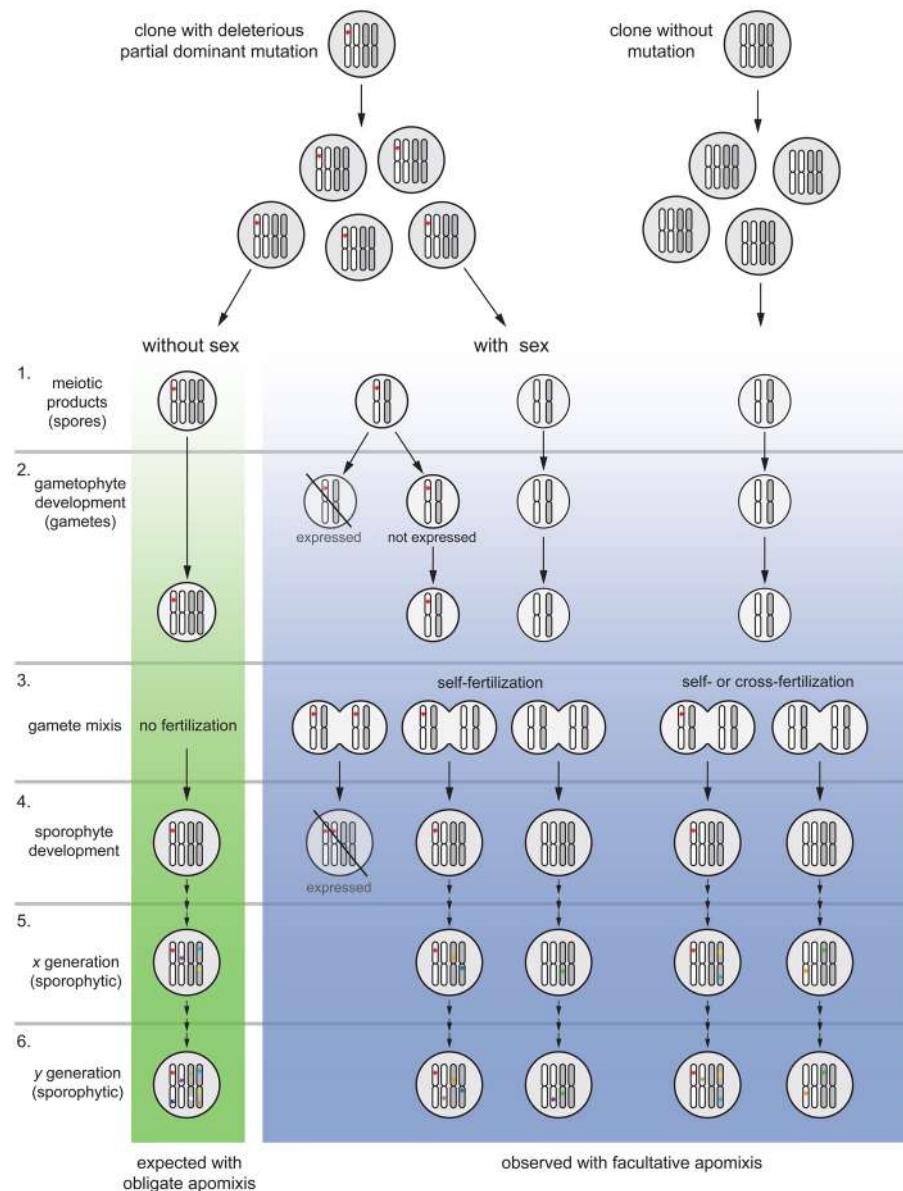
A recent transcriptome study of the *Ranunculus auricomus* complex, a system of diploid sexual species and hexaploid apomictic hybrids, in fact showed Meselson-like sequence divergence effects, but data did not support the idea of mutation accumulation (Pellino et al., 2013). Analyses of Muller's ratchet on high-quality single nucleotide polymorphisms (SNPs) and indels obtained from RNA-seq revealed for 1231 annotated genes that ratios of non-synonymous vs. synonymous substitutions (dN/dS) were mostly clearly below one, and did not differ significantly between apomictic-apomictic, apomictic-sexual and sexual-sexual comparisons (Pellino et al., 2013). A number of all annotated genes showed high dN/dS ratios (outlier values; see Figure 3 in Pellino et al., 2013), and hence appeared to be under divergent selection. A gene ontology analysis of outliers showed that a small proportion of those genes ( $n = 62$ ; 6.7%; Table 5 in Pellino et al., 2013) were associated with processes involved in meiosis and gametogenesis. Strikingly, most such outlier genes ( $n = 41$ ; 66.1%) were found in the apomictic-sexual comparison and thus indicated a significant enrichment of genes associated to reproductive shifts during ovule development when compared to those outliers in sexual (or apomictic) genomes. Whether these mutations have positive or negative effects, needs further investigations. However, most mutations that are under selection do have strongly deleterious effects (e.g., Loewe and Hill, 2010). Since we analyzed only RNA sequences we assume that the observed non-synonymous substitutions mostly have negative effects (dominant or partial dominant). If deleterious mutations would have accumulated genome-wide in the apomicts—a situation expected following the nature of the genetic code and transition/transversion rates (e.g., Yang and Bielawski, 2000)—they would drive the dN/dS ratios over 1. One interpretation of such results is that apomicts in *Ranunculus* are evolutionarily too young (c. 70,000 years) to have accumulated significant mutations and hence dN/dS ratios are still similar to those in sexual putative parentals.

The alternative explanation, however, assumes that apomicts do not accumulate genome-wide deleterious mutations because facultative sexuality purges deleterious mutations (Figure 2). Detailed developmental studies revealed that apomictic hexaploid

*Ranunculus* hybrids show varying proportions of sexually formed seed in all genotypes, with a grand mean of 29.1% (Hojsgaard et al., 2014b). Reduced seed set and lower pollen quality of apomicts compared to sexuals (Hörandl, 2008; Hojsgaard et al., 2014b) indicate negative effects of apomixis on fitness parameters. Population genetic studies data indicate considerable genetic diversity within and among populations (Paun et al., 2006a). Considering the expected turn-over of recombinant individuals in natural populations, regular sexuality in facultative apomicts can purge mutations via two mechanisms: First, ploidy reduction in a diploid plant can already unmask recessive deleterious mutations in the gametophyte and expose it to purging selection (Hörandl, 2009), while in a polyploid mutations showing partial dominance would be exposed to selection during haploidy (see Figure 2, stages 1 and 2); since the gametophytes represent few-celled mini-organisms, a high proportion of the genome is expressed and exposed to selection at this stage. In fact, proportions of sexual development decrease during haploid gametophyte development (Hojsgaard et al., 2013, 2014a). Second, via recombination, mutations will segregate and offspring with variable mutational load will be formed. Additionally, self-fertilization will generate zygotes with higher doses (e.g., hemizygous) of partially dominant mutated alleles, and consequently mutations will become “unmasked” and fully exposed to purging selection in the offspring (see Figure 2, stage 3). Thus, those genotypes where deleterious mutations are being expressed will be eliminated upon dosage level and only individuals carrying mutated alleles at low dosages will persist in the population (see Figure 2, stage 4). The lineage will consequently be regularly purged by eliminating genotypes carrying these mutations. Hence, novel mutations in a facultative will add up slower than in obligate apomicts in which each mutation is added to the mutational load (Figure 2, stages 5 and 6).

The ultimate efficacy will certainly depend on the level of functional meiosis and sexuality occurring in the population. Besides this, purging is expected to be even more efficient under diverse conditions. For example, depending on the level of penetrance and dominance of the mutation, purging would be faster as phenotypic effects would become exposed to natural selection at different dosages. A higher purging efficacy is expected with inbreeding (e.g., Agrawal and Chasnov, 2001). In fact, in a clonal population established from an apomictic mother, all neighboring individuals will carry the same deleterious mutation in their gametes. Occasional facultative sex among individuals will occur among the same genotypes, which is possible because of self-compatibility of apomictic plants (including hexaploid *Ranunculus*; Hörandl, 2008, 2010). Hence, plants effectively conduct self-fertilization, even if cross-pollination takes place among individuals (clone-mates); thus, alleles carrying deleterious mutations may rapidly increase in their dosage, and consequently their effects will be exposed to selection (Figure 2).

Another mechanism to increase efficacy of selection can be assumed from epistasis. If additional (recessive or non-recessive) deleterious mutations lead to a larger decrease of fitness because of negative interactions of these genes, then even truncating selec-



**FIGURE 2 | Model of purging mutations in a tetraploid, facultative apomictic plant lineage (blue column) compared to an obligate apomict (without meiosis; green column).** For simplicity, the model is presented for a new self-fertile allotetraploid lineage with regularly reduced male gametes; and partial dominant mutations are considered to be deleterious and expressed a 50% penetrance. Moreover, preferred homolog pairing is assumed during meiosis I (e.g., Comai, 2005) and considers only the perspective of a mutated deleterious allele, all other alleles pondered to be functionally equivalent. The effects of absence or presence of meiosis on mutation accumulation are illustrated after one generation following the occurrence of mutation (stages 1–4), and after several generations of obligate (without sex) or facultative apomixis (with residual levels of sex; stages 5–6).

- Once a deleterious mutation (red star) with a 50% penetrance is loaded onto the clonal offspring, without sex only unreduced female gametes rise (clonal) progeny. With sex recombinant spores are formed. 2. Expression of mutated alleles and deleterious effects would appear only in those gametophytes with a ploidy-phase change; thus, 50% of haploid gametes would be eliminated, biasing expected progeny proportions (but not progeny types). 3. During gamete mixis, parthenogenetic embryo development avoids

egg-cell fertilization in apomictic female gametophytes while meiotic ones can produce an array of progeny types upon self- or cross-fertilization syndromes. 4. A dosage increase (to duplex condition) and full expression of deleterious effects is expected in some recombinant offspring during sporophyte development, but not in non-recombinant ones. Only individuals carrying a low allele dosage (simplex condition) will remain in the population together with those without the mutation. 5–6. After a number of generations, mutations will gradually appear and added up to the genetic load in the obligate apomictic lineage. In the facultative apomictic lineage, occasional sex will segregate mutated alleles and purging selection will eliminate gametophytes and sporophytes with certain allelic dosages (as in stages 1–4). On the long run, an obligate apomictic genotype (left) will become sooner extinct compared to a facultative apomictic lineage which is continuously purged. The model does not yet consider possible purging effects via conversion during meiosis, and does not quantify facultative sexuality and actual frequencies of spore formation. The model fits to higher ploidy levels if the same penetrance level is assumed in mutated alleles. Assorted colored mutations represent independent events arisen randomly in the genome at different times.

tion can act and rapidly eliminate this genotype (Kondrashov, 1988). Hence, despite nearly-obligate apomictic clones within a population could accumulate mutations for some generations, fitter recombinant genotypes with a lower mutational load will continuously replace them (see Figure 2). Population genetic data on *Ranunculus* strongly support this hypothesis of clonal turnover (Paun et al., 2006b). Consequently, Muller's ratchet is halted or at least slowed down for the lineage as whole. A fourth possibility to avoid mutation accumulation is a large effective population size (Kondrashov, 1988) and/or high migration rates amid populations (Whitton et al., 2008). Hence, within populations genetic variability will be kept at high levels and selection will act on different genotypes. Large geographical distribution and clonal diversity of the hexaploid hybrids (see Paun et al., 2006a,b) suggest that also this factor contribute to genome evolution in *Ranunculus*.

The evolutionary consequences of facultative apomixis in plants have so far received little attention. Mendelian genetic studies on control mechanism of apomixis in angiosperms suggest that the apomixis-controlling genomic regions occur -in general- in a heterozygous state (Ozias-Akins and van Dijk, 2007). In *Ranunculus auricomus*, quantitative expression of apomixis is dosage-dependent on the apospory factor (A), which is a dominant Mendelian factor with variable penetrance in the sporophyte, but with lethal effects in haploid or homozygous states (Nogler, 1984). Consequently, (A) appears always heterozygous with the wild type allele a in various allelic configurations, which means that apomixis cannot become completely obligate (Nogler, 1984). The long term effects of facultative sexuality remain to be studied. Overall, data (Pellino et al., 2013) suggest that apomictic polyploid lineages on the one hand accumulate Meselson-effect-like neutral substitutions in divergent gene copies, and on the other hand, mask partially dominant deleterious alleles in clones, which may become exposed to purifying selection via facultative sexuality. Comprehensive empirical studies will be needed to further test theoretical models and answer the question of how much and to what extend "a little bit of sex" protects apomictic plants from genomic decay and extinction.

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# Unresolved issues in pre-meiotic anther development

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Compared to the diversity of other floral organs, the steps in anther ontogeny, final cell types, and overall organ shape are remarkably conserved among Angiosperms. Defects in pre-meiotic anthers that alter cellular composition or function typically result in male-sterility. Given the ease of identifying male-sterile mutants, dozens of genes with key roles in early anther development have been identified and cloned in model species, ordered by time of action and spatiotemporal expression, and used to propose explanatory models for critical steps in cell fate specification. Despite rapid progress, fundamental issues in anther development remain unresolved, and it is unclear if insights from one species can be applied to others. Here we construct a comparison of *Arabidopsis*, rice, and maize immature anthers to pinpoint distinctions in developmental pace. We analyze the mechanisms by which archesporial (pre-meiotic) cells are specified distinct from the soma, discuss what constitutes meiotic preparation, and review what is known about the secondary parietal layer and its terminal periclinal division that generates the tapetal and middle layers. Finally, roles for small RNAs are examined, focusing on the grass-specific phasiRNAs.

**Keywords:** *arabidopsis*, *rice*, *maize*, *cell fate specification*, *tapetum*, *meiosis*, *phased small RNA*

## INTRODUCTION

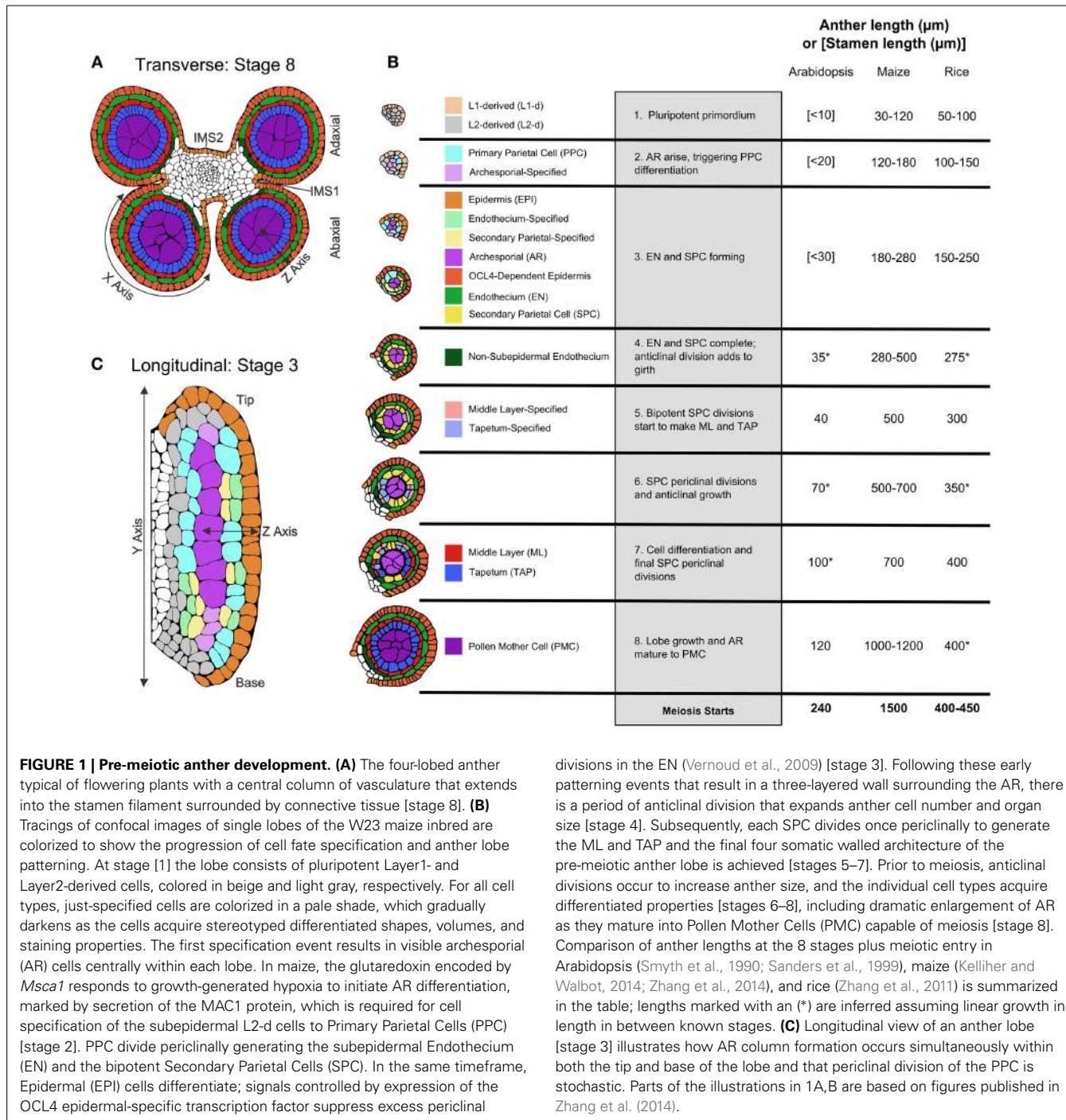
Successful anther development results in pollen dispersal. Steps required to achieve this are conveniently divided into three phases: organ patterning and initial cell differentiation, meiosis, and post-meiotic gametophyte development. Historically most studies have focused on meiosis and pollen biogenesis, with the assumption that a simple lineage model explained how the typical four somatic wall layers with central archesporial (AR) cells arose from a stamen primordium (**Figure 1A**). Starting with recovery and analysis of mutants defective in cell fate specification about 20 years ago (Sheridan et al., 1996; Canales et al., 2002; Zhao et al., 2002; reviewed by Ma, 2005), new theories and molecular insights into the first phase of anther development were proposed, disputed, and continue to be revised and elaborated.

## EARLY STEPS IN ANTER ONTOGENY

The most detailed description of cellular numbers, shapes, and volumes during fate acquisition is available for maize utilizing 3-D reconstruction from confocal microscopy (Kelliher and Walbot, 2011) rather than transverse sectioning. Patterning to achieve the four somatic wall layers and central pre-meiotic cells typical of anther lobes is summarized in **Figure 1B**, employing pale coloration to indicate initial specification, with cell types darkening as differentiated features emerge. First, note that these stages, numbered one through eight, are processes, not discrete events. For example, starting from a primordium full of pluripotent cells, AR cells are the first to differentiate in anther lobes. In maize, discrete differentiation events from different precursors generate a column of ~10 AR cells over the course of 1 day. The first molecular marker of differentiating AR

cells is MAC1 secretion; this ligand triggers pluripotent subepidermal cells to become bipotent Primary Parietal Cells (PPC) (**Figure 1B**, stage 2). The PPC then divide once periclinally to generate Endothecium (EN) and Secondary Parietal Layer (SPL) cells (**Figure 1B**, stage 3). In a given transverse section these steps occur successively, but viewing the anther longitudinally it is clear that AR differentiation in the anther base and tip occurs simultaneously with PPC periclinal divisions in the middle of the lobe (**Figure 1C**). Although it was long assumed that L1 presumptive epidermal cells and L2 internal cells have distinct fates locked in by their positions within an apical meristem, maize AR cells can differentiate from L1 cells during stage 2 low oxygen treatments: thus it appears that every maize anther primordium cell is pluripotent (Kelliher and Walbot, 2012). These observations lay to rest the lineage model where germinal and somatic cell fates diverge from a single “hypodermal cell” division event within each lobe.

Periclinal divisions generate new anther cell types and add cell layers to the anther wall, but most anther cell division is anticlinal (within layers). In maize a rapid elongation phase featuring exclusively anticlinal divisions prolongs stage 4 (**Figure 1B**), prior to SPL periclinal division into the Middle Layer (ML) and Tapetum (TAP) (**Figure 1B**, stages 5–7). This is followed by a second phase of rapid anther growth, prior to differentiation of post-mitotic AR cells into meiotically competent Pollen Mother Cells (PMC) (**Figure 1B**, stage 8). Although rice anthers are similar in size to maize during AR specification, both periods of rapid anticlinal cell division and expansion are absent (**Figure 1B**). As a result rice anthers starting meiosis are about one-third the length of maize; in rice, there is substantial anticlinal division during and just after



meiosis. Arabidopsis anther primordia are considerably smaller than either grass—a combination of fewer, smaller cells—and like rice there is only modest pre-meiotic growth.

Given conserved internal anther anatomy, we expect similar regulatory processes among different flowering plants. Highlights of these common themes include: (1) many anther-specific mutants (Ma, 2005; Timofejeva et al., 2013); (2) a complex and dynamic transcriptome (Zhang et al., 2014); (3) communication between cell layers using secreted proteins (Wang et al., 2012); (4) presumptively locally produced hormones (Zhang et al., 2014),

and (5) developmentally regulated small RNAs (Johnson et al., 2009). It is difficult to propose imposition of hormone or other gradients from materials delivered through the central vasculature (Figure 1A). Thus, unanswered questions include how the pace of anticlinal cell division is regulated autonomously within an anther lobe and what specific cues (activators and repressors) regulate pericinal divisions. It is striking that AR and later PMC development does not require normal somatic layers, as documented for the maize *mac1* mutant (Wang et al., 2012). Indeed, once specified, AR cells express a unique transcriptome,

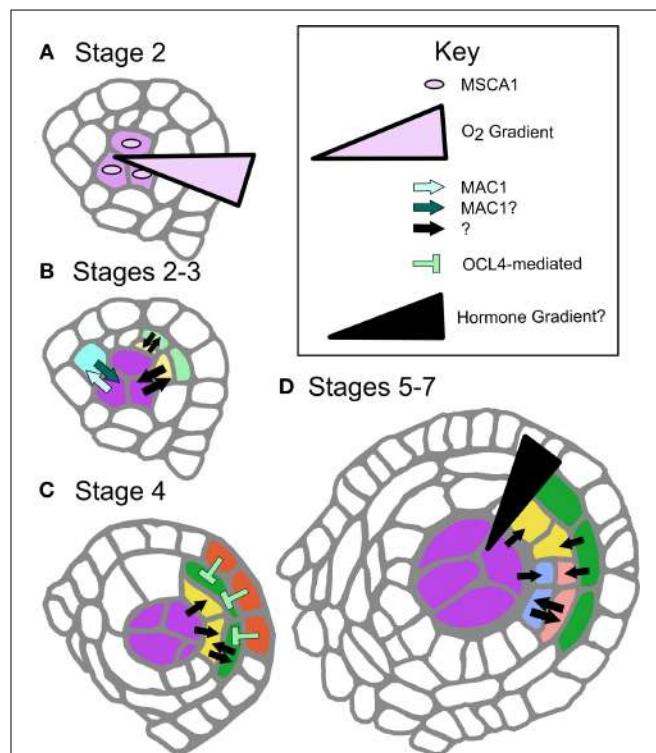
including precocious synthesis of transcripts for meiotic proteins, and robust production of transcripts for ribosomes and RNA binding proteins (Kelliher and Walbot, 2014). A major unanswered question is whether anther cell types express but then sequester mRNAs for use at later stages, a developmental mechanism widely employed in animal germlines (Zhang et al., 2014).

### INITIAL EVENTS RESULTING IN AR COLUMN FORMATION

A major breakthrough in plant reproductive genetics was identification of the *Arabidopsis* transcription factor *SPOROCYTELESS* (*SPL*) which was shown to be essential to AR cell differentiation and meiotic entry in both anthers and ovules (Yang et al., 1999). The late onset of AR-specific expression (~stage 4), however, suggested that *SPL* is only involved after initial fate acquisition, which at the time was thought to depend upon the inheritance of reproductive determinants via asymmetric periclinal division of a single founding hypodermal cell in each lobe. This lineage model ruled the field for decades, largely because early-acting mutants were challenging to identify and characterize, especially in *Arabidopsis* and rice. Then, in 2003 Chaubal et al. reported on a maize mutant, *male sterile converted anther1* (*msc1*), in which AR cells failed to form and were replaced by vascular bundles. When *MSCA1* was identified as a glutaredoxin (Albertson et al., 2009), and homologs were shown to affect AR fate acquisition in rice (Hong et al., 2012) and *Arabidopsis* (Xing and Zachgo, 2008), the first connection between protein redox status and plant reproductive cell fate was made.

The application of confocal microscopy enabled a detailed morphometric analysis of AR column formation, which established that instead of just one founding germinal cell per lobe determined by inheritance, there were many initial AR cells specified from multiple progenitors. The lineage model was incorrect, and it was proposed that the internal position of AR cells within lobes determined their ultimate fate (Kelliher and Walbot, 2012). It was reasoned that such internal cells should be more hypoxic than their neighbors because of the high metabolic demand of rapid proliferation and the lack of air space in the tightly packed tissue (Figure 2A). To test the idea, hypoxia treatments were applied and rescued AR fate acquisition in *msc1*, dramatically increased AR cell counts in fertile anthers, and stimulated ectopic AR formation in both fertile and mutant anthers (Kelliher and Walbot, 2012). While redox manipulations were not attempted in other species, the mutant phenotypes of *Msc1* homologs in rice (*MIL1*) (Hong et al., 2012) and *Arabidopsis* (*ROXY1/ROXY2*) (Xing and Zachgo, 2008) indicate that glutaredoxin-based control of AR fate might be a conserved feature across flowering plants.

A major unanswered question is whether *MSCA1* is expressed and active in the pluripotent L2-d cells surrounding the AR column, which experience mild hypoxia but will acquire a somatic fate. Glutaredoxins such as *MSCA1* are known to bind and activate bZIP-type TGA transcription factors (Murmu et al., 2010); we hypothesize that among the *MSCA1* targets are transcription factors that promote *Mac1* expression, the first molecular marker of AR fate acquisition. A secreted peptide, MAC1 signals pluripotent cells to differentiate as PPC (somatic) and divide periclinal forming the EN and *SPL* (Figure 2B). If *MSCA1* is active



**FIGURE 2 |** Defined and proposed signaling networks in pre-meiotic maize development. **(A)** Specification of AR cells is dependent on oxygen status interaction with *MSCA1*; hypoxic conditions stimulate AR cell differentiation. **(B)** As AR cells differentiate, they secrete the ligand MAC1, which is putatively perceived by LRR-RLK-type receptors on the L2-d cells, specifying these cells as soma and likely stimulating them to become PPC. MAC1 is also inferred to negatively regulate proliferation of AR cells until an entire column is formed in W23 (Wang et al., 2012). At these and later stages there are likely other cell-to-cell communication networks, indicated as black arrows with question marks. These signals may be other ligand-receptor pairs, siRNAs, or other as yet undiscovered factors. **(C)** The differentiated EPI expresses transcription factor OCL4, which indirectly represses periclinal division in the neighboring EN, possibly by assisting with EN differentiation (Vernoud et al., 2009). **(D)** The trigger for periclinal division of the *SPL*, differentiation of the ML and TAP cell fates and maintenance of these fates is completely unexplored. A variety of cell-to-cell communication pathways might be in use (black arrows), or there may be a locally produced hormone gradient(s) along the Z-axis.

in these presumptive PPCs, how does MAC1 signaling counteract or attenuate *MSCA1* activity, overcoming mild hypoxia to enforce a somatic destiny? Likewise, does low oxygen tip the balance toward *MSCA1* in the growing AR column, where newborn AR cells differentiate despite MAC1 secretion from existing AR cells (Figure 1C)? Ectopic AR cells arising from redox manipulation have column-forming abilities, even in *mac1*; therefore, column formation may be an emergent property of AR cells that is neither inhibited nor promoted by MAC1. Future work will continue to examine potential interactions between *MSCA1* and MAC1 with respect to column formation.

Reproductive cell fate acquisition has put glutaredoxins at the forefront of the study of the genetic-environmental interface. These proteins appear to provide flexibility and responsiveness to hard-wired developmental programs and continue to be

implicated in diverse plant developmental processes. In rice, the *mil1* glutaredoxin affects AR and SPL cell fate following AR differentiation (Hong et al., 2012) (stages 3–4), while the Arabidopsis glutaredoxin double mutant *roxy1/2* differentially affects ab- and ad-axial AR development (Xing and Zachgo, 2008), causing defects in stage 2 adaxial lobes and stage 7 abaxial lobes, implying a continued requirement for redox management during anther development and the involvement of multiple glutaredoxins in the differentiation of both germinal and somatic anther tissues. Mis-expression of *MSCA1* in the shoot apical meristem gives opposite phyllotaxy in *Abph2* (Jackson et al., 2013), demonstrating the power of this protein to influence body plan elaboration. While genetic programming has a major role in early anther development—initial AR cell counts vary among inbred lines from ~10 in W23 to ~25 in A619—hypoxic treatments can double or triple initial AR counts without forcing a trade off in somatic cell populations. Therefore, the initial size of the AR population is controlled by both genetic factors and environmental conditions, and pollen production can be manipulated via genetic or extrinsic, redox-based treatments.

### PATTERNING THE ANTER SOMATIC NICHE

The somatic niche surrounding the developing AR cells is equally important for anther fertility. Contingent on MAC1-mediated signaling, L2-d cells are specified as somatic niche cells. The first morphological marker of somatic fate is PPC periclinal division that results in two somatic daughter cells (stage 3), that through subsequent periclinal divisions will differentiate as EN, ML, and TAP (Kelliher and Walbot, 2011; Timofejeva et al., 2013). Although EN, SPL, ML, and TAP cells are typically represented as differentiating immediately after a periclinal division, this is an over-simplification. Indeed, careful analysis of cell shape in maize indicates that these cells must expand in preferential dimensions before gradually achieving mature cell morphology (Kelliher and Walbot, 2011). Borrowing terminology from animal development, we consider somatic cells as passing through multiple steps, starting with cell fate specification, commitment, and finally differentiation. Periclinal division is the first marker of specification, and differentiation has occurred by the morphological end-point, but without deeper knowledge of cellular and molecular details, determining commitment timing is impossible. Does specification occur before a periclinal division, or after? When is commitment reached, and how are we to interpret the numerous mutants that persist in making periclinal divisions, i.e., *ocl4*, *ms23*, and *ms32* in maize (Chaubal et al., 2000; Vernoud et al., 2009; Moon et al., 2013, **Figure 2C**), and *tdf1* in Arabidopsis (Zhu et al., 2008)?

A second issue is within-layer stochasticity in periclinal division initiation. AR cells initiate meiosis synchronously, yet the periclinal divisions resulting in the EN/SPL and later the ML/TAP are both asynchronous and exhibit no discernible spatial pattern (Kelliher and Walbot, 2011; Zhang and Li, 2014). Each cell appears to divide periclinal after a variable number of anticlinal divisions. Could signals directing periclinal divisions be perceived during a restricted portion of the cell cycle, resulting in a large population of cells deaf to the signal when first available?

Asynchrony of anticlinal somatic division would then result in asynchrony of periclinal divisions.

The complexity and lack of information on signaling within the soma makes investigations into the regulation of somatic cell patterning and specification in the anther ripe for pursuit. Despite the relative dearth of information, there are some hints that allow predictions (**Figure 2**). First, the secreted peptide and receptor model may be a common theme. Mutants in Arabidopsis and rice homologs *AtTPD1* and *OsTDL1A* have excess AR cells but develop a normal EN and SPL (stage 5, **Figure 1B**) (Feng and Dickinson, 2010). Clues from these mutants suggest the MAC1 peptide family binds LRR-RLK receptors (*AtEXS/EMS1* and *OsMSP1*, respectively). As LRR-RLKs are known to dimerize, hetero-dimerization utilizing different isoforms or family members may contribute to differential signal interpretation in peripheral L2-d and presumptive AR cells during column formation, or between other cell types at later stages (Wang et al., 2012). Elucidating the suite of LRR-RLKs could uncover a distinct “combinatorial code” of dimeric receptors on different cell types. This idea is consistent with the presence of many putatively secreted peptides (Wang et al., 2012) and the expression of numerous LRR-RLKs in anthers (Kelliher and Walbot, 2014; Zhang et al., 2014). The anther is one of many organs where peptide signaling has emerged as a major developmental paradigm. In particular, tissues that lack direct access to vasculature, such as stem cells in the shoot apical meristem and meristemoid cells of the stomatal lineage (Li and Torii, 2012), often employ this mode of cell-cell communication.

The role of hormones or other chemical gradients within anther lobes is another area yet to be explored on a cell type-specific level. In a commonly cited example, periclinal division in Arabidopsis roots involves an auxin gradient delivered by polarized auxin flow (Cruz-Ramirez et al., 2012). Given the anatomy of the anther, it is unlikely that a similar flow exists because cell types are not a uniform distance from the vasculature. Local hormone production and perception would more likely be a feature of anther development (**Figure 2D**). Transcriptome analysis indicates that both gibberellin and brassinolide associated genes are differentially regulated in stage 7 pre-meiotic anther development; these would make strong initial candidates for further exploration as regulators of anther cell fates (Zhang et al., 2014).

Despite identification of many maize male-sterile mutants disrupting pre-meiotic somatic patterning (**Table 1**), this phase of early anther development is underrepresented in the rice and Arabidopsis literature. The small size of rice and Arabidopsis anthers at these stages (20–70 and 100–350  $\mu\text{m}$ , respectively, compared to 120–700  $\mu\text{m}$  for maize) along with the rapidity of development (in maize, events span nearly 3 days, roughly twice as long as Arabidopsis) makes isolation and precise analysis of discrete stages difficult (**Figure 1B**). Many mutants that fail to complete meiosis have been categorized as meiotic mutants, even when the defect is earlier and the primary defect is in the soma. There is no consensus concerning the specific roles of each somatic cell type. The tapetum has been characterized as a highly transcriptionally active, secretory cell type required for exine formation. But the ML can be present as either a

**Table 1 | A comprehensive list of anther mutants sequentially organized from organ specification through meiosis.**

| <b>Stage affected</b> | <b><i>A. thaliana</i></b>                           | <b>Rice</b>             | <b>Maize</b>                    | <b>Annotation</b>              | <b>Phenotype</b>   | <b>doi</b>                       |
|-----------------------|---|-------------------------|---------------------------------|--------------------------------|--|----------------------------------|
| 1                     | <i>agamous</i>                                      | <i>mads3, mads58</i>    | <i>Zmm2, Mads2</i>              | MADS-box transcription factor  | stamens converted to petals (Arabidopsis) or lodicules (grasses)                     | 10.1105/tpc.3.8.749              |
| 1                     |   |                         | <i>ems71924, ems72032</i>       | <i>not cloned</i>              | stamen adaxialization  | 10.1534/g3.112.004465            |
| 1                     |   |                         | <i>ems71990, ms-si*355</i>      | <i>not cloned</i>              | absence of anthers in some florets   | 10.1534/g3.112.004465            |
| 1                     | <i>rdr6</i>   | <i>rol (SHOOTLESS2)</i> | <i>rdr6</i>                     | RNA-directed RNA polymerase    | stamen abaxialization (defect in tasi-ARF biosynthesis)                              | 10.1105/tpc.110.075291           |
| 2                     | <i>bam1 bam2 double mutant</i>                      |                         |                                 | LRR receptor-like kinases      | all internal lobe cells become AR; no somatic cells                                  | 10.1105/tpc.105.036871           |
| 2                     |   | <i>mil1</i>             | <i>msca1</i>                    | glutaredoxin                   | AR fail to differentiate ( <i>Os</i> ) or differentiate as vasculature ( <i>Zm</i> ) | 10.1007/s00425-002-0929-8        |
| 2 (ad), 8 (ab)        | <i>roxy1 roxy2 double mutant</i>                    |                         |                                 | glutaredoxin (thioreductase)   | adaxial lobes: AR specification failure; abaxial: PMC degrade                        | 10.1111/j.1365-313X.2007.03375.x |
| 3                     | <i>nzz = spl</i>                                    | no homology in grasses  |                                 | MADS-box transcription factor  | AR differentiation failure; somatic cell layer defects                               | 10.1104/pp.109.145896            |
| 3                     | <i>tpd1</i><br>( <i>Zm/Os</i> ,<br>5 ( <i>At</i> )) | <i>tdl1a = mil2</i>     | <i>mac1</i>                     | small secreted protein ligand  | somatic cell specification failure; overproliferation of AR                          | 10.1105/tpc.016618               |
| 3                     | <i>exs = ems1</i>                                   | <i>msp1</i>             |                                 | LRR receptor-like kinase       | somatic cell specification failure; overproliferation of AR                          | 10.1016/S0960-9822(02)01151-x    |
| 4                     |   | <i>ocl4</i>             |                                 | HD-ZIP IV transcription factor | additional periclinal divisions in subepidermal cell layer                           | 10.1111/j.1365-313X.2009.03916.x |
| 4                     |   |                         | <i>ems63089, tcl1, mtm00-06</i> | <i>not cloned</i>              | undifferentiated somatic cell layers   | 10.1534/g3.112.004465            |
| 5                     |   | <i>tip2</i>             |                                 | bHLH transcription factor      | all three anther wall layers fail to differentiate properly                          | 10.1105/tpc.114.123745           |
| 5                     | <i>er erl1 erl2 triple mutant</i>                   |                         |                                 | LRR receptor-like kinases      | missing anthers and somatic cell differentiation defects                             | 10.1093/mp/ssn029                |
| 6                     | <i>tdf1</i>   |                         |                                 | R2R3 Myb transcription factor  | early vacuolization in epidermis and endothecium, tapetal failure                    | 10.1111/j.1365-313X.2008.03500.x |
| 6                     | <i>serk1 serk2 double mutant</i>                    |                         |                                 | LRR receptor-like kinases      | SPL periclinal division failure  | 10.1105/tpc.105.036731           |
| 7                     |   |                         | <i>ms23, ms*6015</i>            | <i>not cloned</i>              | additional periclinal divisions in the tapetal layer                                 | 10.1534/g3.112.004465            |

(Continued)

**Table 1 | Continued**

| <b>Stage affected</b> | <i>A. thaliana</i>               | Rice                         | Maize           | <b>Annotation</b>               | <b>Phenotype</b>  | <b>doi</b>                       |
|-----------------------|----------------------------------|------------------------------|-----------------|---------------------------------|---|----------------------------------|
| 7                     |                                  |                              | <i>ems72063</i> | <i>not cloned</i>               | undifferentiated soma; excess periclinal divisions in tapetum     | 10.1534/g3.112.004465            |
| 7                     |                                  |                              | <i>ems72091</i> | <i>not cloned</i>               | additional periclinal divisions in the middle layer               | 10.1534/g3.112.004465            |
| 7                     | <i>mpk3 and mpk6</i>             |                              |                 | MAP kinases                     | somatic cell specification failure; overproliferation of AR       | 10.1093/mp/ssp029                |
| 8                     |                                  | <i>dtm1</i>                  |                 | ER membrane protein             | tapetal differentiation failure                                   | 10.1111/j.1365-313X.2011.04864.x |
| 8                     | <i>dyt1</i>                      |                              |                 | bHLH transcription factor       | tapetal differentiation failure                                   | 10.1111/j.1365-313X.2012.05104.x |
| 8                     | <i>myb33 myb65 double mutant</i> |                              |                 | GAMYB-like transcription factor | tapetal differentiation failure                                   | 10.1105/tpc.104.027920           |
| 8                     |                                  | <i>ms9, ms11, ms13, ms14</i> |                 | <i>not cloned</i>               | tapetal differentiation failure                                   | 10.1534/g3.112.004465            |
| 8                     |                                  | <i>ms32</i>                  |                 | bHLH transcription factor       | excess periclinal divisions in tapetum after normal wall is built | 10.1111/tpj.12318                |
| 8                     |                                  | <i>csmid1</i>                |                 | <i>not cloned</i>               | excess pre-meiotic callose and slow dissolution of the tetrad     | 10.1007/s00497-011-0167-y        |
| 8                     |                                  | <i>ms8</i>                   |                 | beta-1,3-galactosyl transferase | cell growth defects in epidermis and tapetum, meiotic arrest      | 10.1007/s00497-013-0230-y        |
| 8                     |                                  | <i>gamyb-4</i>               |                 | GAMYB transcription factor      | tapetal differentiation failure; meiotic arrest                   | 10.1111/j.1744-7909.2010.00959.x |
| meiosis               |                                  | <i>udt1</i>                  |                 | bHLH transcription factor       | tapetal differentiation failure; meiotic arrest                   | 10.1105/tpc.105.034090           |
| meiosis               |                                  | <i>mel1</i>                  |                 | Argonaute                       | tapetal differentiation failure; meiotic arrest                   | 10.1105/tpc.107.053199           |

The left hand column indicates the first developmental stage at which a mutant phenotype is observed, using the staging rubric outlined in **Figure 1**. In cases where the onset of the phenotype differs among species or tissues, abbreviations are used ("Zm" = maize, "Os" = rice, "At" = *Arabidopsis*, "ab" = abaxial anther lobes, and "ad" = adaxial anther lobes). If a given mutant is phenocopied by homologs from the other two species, the gene names are given in the corresponding species' column. An exception to this rule was made for the first maize entry, "Zmm2, Mads2," because while mutants in these genes have not been found, the genes are clearly agamous orthologs by sequence comparison and expression pattern in the third floral whorl, which is characteristic of C class genes. The next two columns contain the phenotypic description of the mutant and protein annotation if a causative gene has been cloned. Uncloned mutants are indicated as "not cloned," and these are clustered in a single row in cases where they roughly phenocopy each other (for example, maize *ems71924* and *ems72032* have nearly identical anther polarity phenotypes, and may be allelic). In the final column, a doi is provided for the founding mutant of each class. The high number of blank spaces in the species' columns reflects the challenge of comparisons between model species. The bHLH, MYB, and LRR-RLK genes are all found in large families making identification of orthologs between species problematic. Furthermore, mutations in a single gene that cause a clear phenotype in one plant species may not be available in others because of functional gene redundancy from lineage specific gene duplication. And there is already evidence that orthologs can regulate different steps reflecting evolutionary diversification of developmental pathways. For these reasons we do not anticipate a high degree of correspondence between *Arabidopsis*, rice, and maize.

single layer (as in rice, maize, and *Arabidopsis*), or several layers, but is always consistent within a species (Esau, 1965; D'Arcy and Keating, 1996). Middle-layer like tissue and dartboard lobe architecture are relatively ancient, dating to the gymnosperm microsporangia (Esau, 1965; D'Arcy and Keating, 1996), but as of yet no specific function has been proposed for the ML. Secondary wall thickening of the EN is involved in anthesis at the end of anther development, but no attention has been given to earlier roles. Germ cell establishment and the subset of tapetal mutants that result in meiotic arrest have received the vast majority of attention.

## ROLE OF SMALL RNAs IN ANTER PATTERNING

Developing anthers depend on small RNAs for gene regulation, cell-to-cell communication, and epigenetic reprogramming. For example, in rice, anther adaxial-abaxial polarity is regulated by trans-acting siRNAs (tasiRNAs), which are secondary siRNAs derived from *TAS* transcripts (Toriba et al., 2010). These transcripts are first processed by miRNA-guided cleavage and then converted to double-stranded RNAs by RNA-dependent RNA polymerase6 (RDR6), followed by Dicer4 (DCL4) cleavage yielding 21-nt tasiRNAs (Allen et al., 2005). Mutants that disable components of the tasiRNA biogenesis pathway display severe defects in floral morphology and fertility. Rice *dcl4* exhibits a disruption in lemma abaxial-adaxial polarity and abnormal anther development (Liu et al., 2007). In the *rod-like lemma* (*rol/rdr6*) mutants, stamen, and lemma development are severely compromised (Toriba et al., 2010). A single nucleotide polymorphism in the same gene results in reduced fertility at high temperature (Song et al., 2012a). The phenotypes of these mutants are largely caused by loss of tasiRNAs, resulting in upregulation of target *Auxin Response Factor* genes required for abaxial identity (Pekker et al., 2005).

Recently two size classes of phased siRNAs (phasiRNAs) were found to be preferentially expressed in grass inflorescences, particularly in anthers (Johnson et al., 2009; Song et al., 2012b). Both phasiRNA types are derived from low copy intergenic regions, and each requires a specific miRNA trigger (miR2118 for the 21-nt class and miR2275 for 24-nt class) to initiate cleavage and RDR6-dependent second-strand synthesis. The resulting double-stranded RNAs are then cleaved by DCL4 to produce 21-nt phasiRNAs and by DCL5 for 24-nt phasiRNAs (Ariket et al., 2013). Despite similarities to tasiRNA biogenesis, the functions of these 21- and 24-nt phasiRNAs and their targets are largely unknown. The phasiRNAs lack complementarity to genes or transposons, suggesting novel roles rather than post-transcriptional gene silencing or RNA-directed DNA methylation.

Post-meiotically, small RNAs do provide germinal cell genome surveillance in microspores. Triggered by miRNAs and dependent on RDR6 and DCL4 (Creasey et al., 2014), the 21-nt epigenetically-activated siRNAs (easiRNAs) are derived from transposon transcripts exclusively expressed in the vegetative nucleus of *Arabidopsis* pollen grains; easiRNAs then move to sperm cells to direct transcriptional gene silencing (Slotkin et al., 2009). As a result, sperm cell chromatin is highly condensed and enriched in the repressive epigenetic modifications while

chromatin in the vegetative cell nucleus is largely decondensed (Slotkin et al., 2009).

Despite sharing biogenesis factors including DCL4 and RDR6, 21-nt phasiRNAs are different from 21-nt easiRNAs in three ways. First, although 21-nt phasiRNAs have been found in dicots, they are mostly derived from a single *NB-LRR* defense gene family (Zhai et al., 2011); in contrast hundreds of grass loci encode phasiRNAs. Second, phasiRNAs are produced prior to meiosis or gametogenesis (Komiya et al., 2014) while easiRNAs are found specifically in gametophytes. Third, easiRNAs are derived from repetitive regions, while phasiRNAs are produced from unique or low copy sequences. These distinctions between phasiRNAs and easiRNAs suggest their functional divergence and imply a sporophytic role for phasiRNAs in anther development.

*ARGONAUTE* proteins have diverged rapidly in plants, with 10 members in *Arabidopsis* (Chen, 2009), 19 in rice (Kapoor et al., 2008), and 17 in maize (Zhai et al., 2014); many are preferentially expressed in germinal cells (Zhai et al., 2014). The continued diversification of *ARGONAUTE*s suggests that many new functions for small RNAs during plant reproduction await discovery. *MEL1*, a rice homolog of *Arabidopsis AGO5*, mainly localizes to the cytoplasm of pre-meiotic sporocytes. *mel1* loss-of-function mutants exhibit abnormal tapetal formation and contain aberrant PMC that arrest in early meiosis (Nonomura et al., 2007). Recently *MEL1* was demonstrated to bind 21-nt phasiRNAs preferentially (Komiya et al., 2014). Histone methylation patterns are altered in *mel1* mutant meiocytes (Nonomura et al., 2007), hinting at a role for *MEL1* and its associated 21-nt phasiRNAs in chromatin modification. Because the tapetal somatic defect precedes the documented meiocyte phenotypes in *mel1* anthers, it is not yet clear if *MEL1*/21-nt phasiRNAs act directly or indirectly to disrupt meiosis.

Maize *AGO104* is a homolog of *Arabidopsis AGO9* and its transcripts accumulate specifically during pre-meiosis just following germinal differentiation in anthers (Kelliher and Walbot, 2014) and during meiosis (Singh et al., 2011). Maize *ago104* mutants show defects in meiotic chromatin condensation and subsequent failure to properly segregate chromosomes (Singh et al., 2011). Small RNAs interacting with *AGO104* are yet to be profiled, but the heterochromatic decondensation in maize *ago104* mutants suggests a role in germinal cell epigenetic reprogramming. With rapid progress in deep sequencing of small RNAs and RNA-IP-seq, we are likely to acquire a better understanding of the diverse ways small RNAs contribute to anther development and plant reproduction.

## AUTHOR CONTRIBUTIONS

Each author wrote one section of the manuscript, edited all sections, and approved final submission.

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**Conflict of Interest Statement:** Timothy Kelliher and Virginia Walbot are inventors on a patent application (61/598,544) filed by Stanford University entitled “Method for Modulating the Number of Archesporial Cells in a Developing Anther.” 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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# Insight into S-RNase-based self-incompatibility in *Petunia*: recent findings and future directions

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S-RNase-based self-incompatibility in *Petunia* is a self/non-self recognition system that allows the pistil to reject self-pollen to prevent inbreeding and to accept non-self pollen for outcrossing. Cloning of *S-RNase* in 1986 marked the beginning of nearly three decades of intensive research into the mechanism of this complex system. S-RNase was shown to be the sole female determinant in 1994, and the first male determinant, S-locus F-box protein1 (SLF1), was identified in 2004. It was discovered in 2010 that additional SLF proteins are involved in pollen specificity, and recently two *S*-haplotypes of *Petunia inflata* were found to possess 17 SLF genes based on pollen transcriptome analysis, further increasing the complexity of the system. Here, we first summarize the current understanding of how the interplay between SLF proteins and S-RNase in the pollen tube allows cross-compatible pollination, but results in self-incompatible pollination. We then discuss some of the aspects that are not yet elucidated, including uptake of S-RNase into the pollen tube, nature, and assembly of SLF-containing complexes, the biochemical basis for differential interactions between SLF proteins and S-RNase, and fate of non-self S-RNases in the pollen tube.

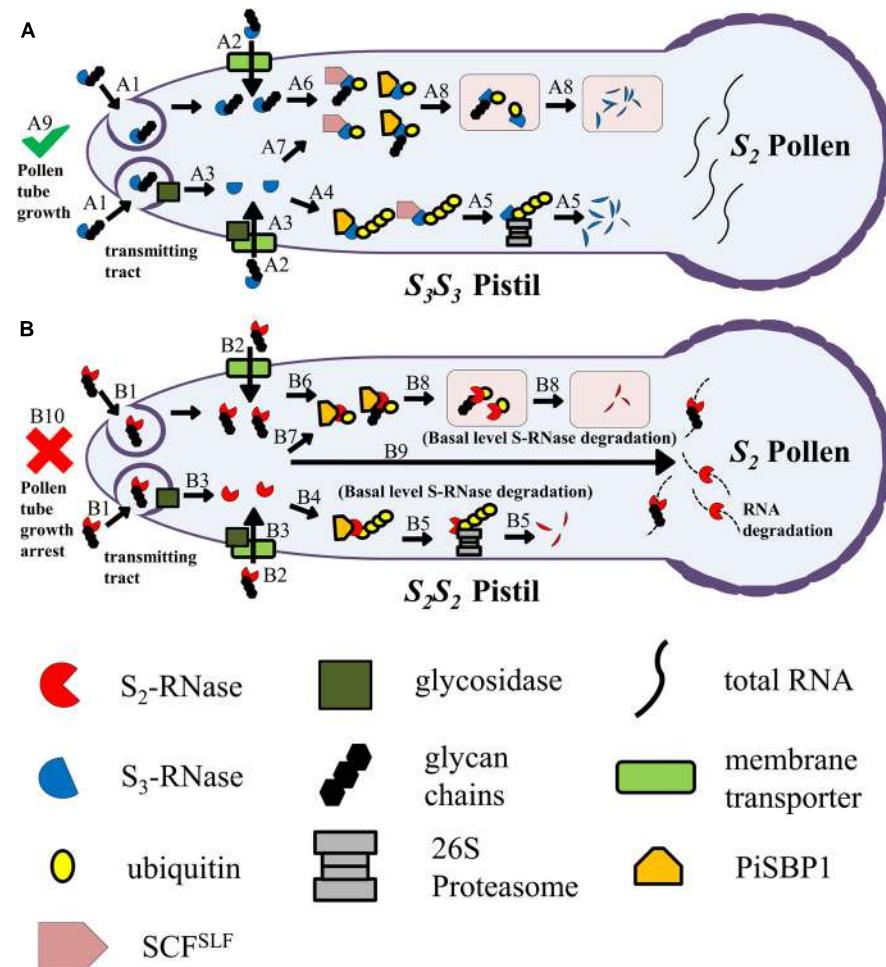
**Keywords:** *Petunia*, SCF<sup>SLF</sup> complex, self-incompatibility, S-locus F-box protein, Solanaceae, S-RNase

## INTRODUCTION

Self-incompatibility (SI) is a pre-zygotic reproductive barrier, which prevents inbreeding in many families of angiosperms (Takayama and Isogai, 2005; Franklin-Tong, 2008). *Petunia* possesses the Solanaceae type SI in which this reproductive barrier is regulated by the highly polymorphic *S*-locus. The *S*-locus houses the female determinant gene, *S-RNase* (Lee et al., 1994; Murfett et al., 1994), and multiple male determinant genes, *S-locus F-box* (SLF) genes (Sijacic et al., 2004; Kubo et al., 2010). In *Petunia*, 32 *S*-haplotypes have been reported (Sims and Robbins, 2009). A diploid pistil carries two different *S*-haplotypes, each producing an allelic variant of S-RNase. S-RNase is synthesized in the transmitting tissue of the style and secreted into the transmitting tract where pollen tubes grow from the stigma to the ovary. A pollen tube takes up both self S-RNase (product of the same *S*-haplotype as that carried by pollen) and non-self S-RNase (product of a different *S*-haplotype from that carried by pollen; Luu et al., 2000; Goldraij et al., 2006); however, only self S-RNase can inhibit the growth of the pollen tube (in the upper one-third of the style) through its RNase activity (Huang et al., 1994).

The understanding of how a pollen tube escapes the cytotoxic effect of non-self S-RNase has undergone several major developments and revisions in the past decade. SLF was first discovered in *Antirrhinum hispanicum* (Lai et al., 2002; Qiao et al., 2004b), which possesses the same type of SI. Subsequently, the first SLF in *Petunia*, now named SLF1, was confirmed as a male determinant via an *in vivo* functional assay (Sijacic et al.,

2004). The presence of an F-box domain in the N-terminal region of SLF led to the proposal that SLF, like conventional F-box proteins, is a component of a class of E3 ubiquitin ligase, the SCF (Skp1-Cullin1-F-box protein) complex, involved in ubiquitin-mediated protein degradation by the 26S proteasome (Lai et al., 2002; Qiao et al., 2004a; Hua and Kao, 2006). The substrate of an SCF<sup>SLF</sup> complex appears to be non-self S-RNase(s) for the specific allelic variant of SLF in the complex, as an *in vitro* protein pull-down assay showed that non-self interactions between allelic variants of SLF and S-RNase were stronger than self-interactions. This could explain why only self S-RNase can exert a cytotoxic effect on the pollen tube, as it is not ubiquitinated or degraded in the pollen tube. However, given that there are a large number of *S*-haplotypes in *Petunia* and given that allelic variants of S-RNase exhibit a high degree of sequence diversity, it is difficult to envision how an allelic variant of SLF could interact with so many non-self S-RNases, but not with a single self S-RNase. This conundrum was solved when it was discovered that at least two paralogous genes of SLF1 are also involved in pollen specificity (Kubo et al., 2010). A new model, “collaborative non-self recognition,” proposes that multiple SLF proteins produced by pollen of a given *S*-haplotype collaboratively recognize and detoxify all non-self S-RNases (i.e., each SLF is only capable of interacting with a subset of its non-self S-RNases), but none can interact with their self S-RNase (Kubo et al., 2010). To date, *S*<sub>2</sub>-haplotype and *S*<sub>3</sub>-haplotype of *Petunia inflata* have been shown to possess the same 17 SLF genes based on pollen



**FIGURE 1 | Model for uptake of S-RNase by the pollen tube in the transmitting tract of the pistil, and fates of self and non-self S-RNases after uptake. (A)** An  $S_2$  pollen tube, growing in an  $S_3S_3$  pistil, takes up  $S_3$ -RNase (a non-self S-RNase). Two possible types of uptake mechanisms are depicted: clathrin-dependent or clathrin-independent endocytosis (A1) and membrane-transporter mediated (A2). During uptake, the N-linked glycan chains of  $S_3$ -RNase may be removed by a membrane-associated glycosidase (A3). The deglycosylated  $S_3$ -RNase becomes poly-ubiquitinated (A4), mediated largely by the conventional  $SCF^{SLF}$  complex and to a much lesser extent by PiSBP1 and the PiSBP1-containing novel  $SCF^{SLF}$  complex (not shown). The poly-ubiquitinated  $S_3$ -RNase is destined for degradation by the 26S proteasome (A5).  $S_3$ -RNase may remain glycosylated and be mono-ubiquitinated (A6), again mediated largely by the conventional  $SCF^{SLF}$  complex and to some extent by PiSBP1 and the PiSBP1-containing novel  $SCF^{SLF}$  complex (not shown). The deglycosylated  $S_3$ -RNase may also be similarly mono-ubiquitinated (A7). The mono-ubiquitinated (deglycosylated)  $S_3$ -RNase is then targeted to vacuoles or vacuole-like organelles for degradation (A8). All the steps

depicted result in detoxification of the  $S_3$ -RNase molecules inside the  $S_2$  pollen tube, allowing it to reach the ovary to effect fertilization (A9). **(B)** An  $S_2$  pollen tube, growing in an  $S_2S_2$  pistil, takes up  $S_2$ -RNase (self S-RNase).  $S_2$ -RNase is taken up by the same mechanisms (B1 and B2) as those depicted for  $S_3$ -RNase in **(A)**, and may also be subjected to deglycosylation (B3). None of the SLF proteins in the  $SCF^{SLF}$  complexes are able to interact with their self S-RNase to mediate its degradation or compartmentalization. However, similar to the scenarios depicted in **(A)**, PiSBP1 may mediate poly-ubiquitination of  $S_2$ -RNase (B4) for basal-level degradation by the 26S proteasome (B5), and may mediate mono-ubiquitination of both  $S_2$ -RNase (B6) and deglycosylated  $S_2$ -RNase (B7). The mono-ubiquitinated (deglycosylated)  $S_2$ -RNase is then targeted to vacuoles or vacuole-like organelles for degradation (B8). However, the majority of  $S_2$ -RNase molecules remain intact and will degrade RNA (B9) to result in growth arrest of the pollen tube (B10). Note: To make it easier to follow the different fates of self and non-self S-RNase,  $S_2S_2$  and  $S_3S_3$  pistils are used in this figure; however, in nature, pistils of a self-incompatible species are normally heterozygous for the S-locus.

transcriptome analysis (Williams et al., 2014b). Moreover, eight other *Petunia* S-haplotypes have recently been shown to possess 16–20 SLF genes (Kubo et al., 2015). So far, eight of them (*SLF1*, *SLF2*, *SLF3*, *SLF4*, *SLF5*, *SLF6*, *SLF8*, and *SLF9*) have been confirmed by an *in vivo* functional assay to be involved in pollen specificity (Sijacic et al., 2004; Kubo et al., 2010, 2015; Williams et al., 2014a).

Despite the impressive progress made in understanding the complex non-self recognition between the female determinant and multiple male determinants, there are still many aspects of S-RNase-based SI that remain unknown. In this article, we discuss the current understanding of some of these aspects, summarize the key features of the discussion in the model shown in **Figure 1**, and list the known and putative proteins involved in **Table 1**.

**Table 1 | Known and putative proteins involved in S-RNase-based self-incompatibility.**

| Component of SI process                     | Known proteins                             | Putative proteins                                   |
|---|--|---|
| S-RNase uptake                              | S-RNase                                    | Membrane transporters, endocytosis-related proteins |
| SCF <sup>SLF</sup> complex                  | PiCUL1-P, PiSSK1, SLF proteins, PiRBX1     | PiSBP1  |
| Novel SCF <sup>SLF</sup> complex            |  | SLF1, PiCUL1-G, PiSBP1                              |
| S-locus F-box proteins                      | SLF1-SLF17                                 |   |
| Fate of non-self S-RNase in the pollen tube | SCF <sup>SLF</sup> complex, 26S proteasome | Glycosidase, PiSBP1, PiCUL1-G, vacuolar proteases   |

## UPTAKE OF S-RNASE INTO POLLEN TUBES

For S-RNase to exert its cytotoxic effect it must be taken up by the pollen tube in the transmitting tract; however, the mechanism of this critical step in SI is not yet known. S-RNase could be taken up by endocytosis and/or membrane transporters, both of which are used by the pollen tube to take up extracellular proteins (Moscatelli et al., 2007; Chen et al., 2010).

A delicate equilibrium of exocytosis and endocytosis is required for the rapid polarized growth of the pollen tube (Camacho and Malhó, 2003). Both clathrin-dependent and clathrin-independent endocytosis have been shown to be involved in recycling plasma membrane proteins/lipids and in regulating pollen tube growth (Onelli and Moscatelli, 2013). One notable example is the uptake of SCA (stigma/style cysteine-rich adhesin), a protein that guides the pollen tube toward the ovary; it is internalized in the tip region of a growing pollen tube through clathrin-dependent endocytosis and sorted to the multi-vesicular bodies and vacuoles (Kim et al., 2006; Chae et al., 2007).

ATP-binding cassette (ABC) transporters constitute a large protein family in plants (Geisler, 2014), and have been reported to be involved in various processes, e.g., pathogen attack response, deposition of plasma membrane lipids, nutrient accumulation in seeds, and transport of phytohormones (Kang et al., 2010; Tunc-Ozdemir et al., 2013). ABC transporters are active transporters, deriving energy from ATP hydrolysis, and they are either exporters or importers (Geisler, 2014). In plants, ABC transporters have been classified into 13 subfamilies (Rea, 2007). An ABC transporter of apple, MdABC1, has been implicated in the transport of S-RNase into the pollen tube (Meng et al., 2014). Analysis of the *S<sub>2</sub>*-pollen, *S<sub>3</sub>*-pollen, and *S<sub>3</sub>S<sub>3</sub>* leaf transcriptomes of *P. inflata* (Williams et al., 2014b) reveals the presence of 476, 334, and 851 BLAST annotated transporters, and interestingly, ~59% of the potential transporters in each pollen transcriptome are annotated as ABC transporters, whereas only ~25% of the potential transporters in the leaf transcriptome are annotated as ABC transporters (our unpublished data). Given that S-RNase is pistil-specific and is taken up only by the pollen tube, if ABC transporters are involved in the uptake of S-RNases, they would likely be among those that are pollen-specific.

Regardless of how S-RNase is taken up by the pollen tube, the uptake machinery must be able to interact with a large number of highly divergent allelic variants of S-RNase. Despite the sequence diversity, S-RNase contains five conserved regions (C1–C5; Ioerger et al., 1991). Alignment of the amino acid sequences of 20 allelic variants of S-RNase in *Petunia* available in the NCBI non-redundant nucleotide database reveals 21 perfectly conserved amino acid residues. As the crystal structure of *Nicotiana alata* S<sub>F11</sub>-RNase has already been determined (Ida et al., 2001), those conserved residues that are exposed on the outside surface would be good candidates to use for investigating their role in interaction with a transporter and/or a transmembrane receptor involved in uptake.

## THE SCF<sup>SLF</sup> COMPLEX

The SLF-containing SCF complex, SCF<sup>SLF</sup>, of *P. inflata* has been shown by co-immunoprecipitation (Co-IP) followed by mass-spectrometry (MS) to contain a conventional Rbx1 (PiRBX1; a RING-finger protein), a pollen-specific Cullin1 (PiCUL1-P), and a pollen-specific Skp1-like protein (PiSSK1; Li et al., 2014). Similar components have been identified in the SCF complex of *Petunia hybrida* (Entani et al., 2014; Liu et al., 2014) and *Pyrus bretschneideri* (Xu et al., 2013). Phylogenetic studies showed that SSK1 and Cullin1 proteins implicated in SI form their own monoclades (Xu et al., 2013; Yuan et al., 2014), and that the 17 SLF proteins of *P. inflata* form a monoclade. Also, SLF proteins were the only F-box proteins that co-immunoprecipitated with PiSSK1 (Li et al., 2014). Interestingly, tomato SpCUL1, sharing 91% sequence identity with PiCUL1-P, is involved in unilateral incompatibility between tomato species (Li and Chetelat, 2010) and is also required for compatible pollination in *Solanum arcanum* (Li and Chetelat, 2013). Thus, three of the four components of the SCF<sup>SLF</sup> complex (PiSSK1, PiCUL1-P, and SLF) appear to have evolved specifically to function in SI.

A *P. hybrida* RING-finger protein, termed PhSBP1, was found to interact with S-RNase by a yeast two-hybrid assay (Sims and Ordanic, 2001). Subsequently, the PhSBP1 homolog from *P. inflata*, termed PiSBP1, was found to interact with an SLF protein (*S<sub>2</sub>*-SLF1), a different Cullin1 (PiCUL1-G) and S-RNase by a yeast two-hybrid assay (Hua and Kao, 2006; Meng et al., 2011), leading to the suggestion that PiSBP1 plays the roles of both Skp1 and Rbx1, and forms a novel SCF complex with PiCUL1-G and SLF1 (Hua and Kao, 2006). In *Malus × domestica* (apple), both homologs of PiSBP1 and PiSSK1 were found to interact with an SLF (named SFB for S-Locus F-Box) and a Cullin1 (MdCUL1) by an *in vitro* protein binding assay (Minamikawa et al., 2014). MdSSK1 interacted with the SLF protein more strongly than did MdSBP1, and the transcript level of *MdSSK1* was >100 times higher than that of *MdSBP1*. Thus, the conventional SCF<sup>SLF</sup> complex is thought to play a major role in mediating ubiquitination and degradation of non-self S-RNases. This finding may explain why PiSBP1 was not identified from the Co-IP products using either *S<sub>2</sub>*-SLF1 or PiSSK1 as bait (Li et al., 2014). PiSBP1 may also function as a mono-subunit E3 ubiquitin ligase as it catalyzed ubiquitination of S<sub>3</sub>-RNase in the presence of E1, E2, and ubiquitin in an *in vitro* assay (Hua and Kao, 2008). However, the interaction of PiSBP1 with S-RNase does not show allele

specificity, so it may mediate basal level S-RNase degradation, perhaps as a safety mechanism to ensure that all non-self S-RNases are cleared from the pollen tube.

The approach of using Co-IP followed by MS has been successful in identifying three (SLF1, SLF4, and SLF13) of the 17 SLF proteins of *P. inflata* as the F-box component of the SCF<sup>SLF</sup> complexes (Li et al., 2014). Among these 17 SLF proteins, eight (including SLF1 and SLF4) have so far been confirmed to be involved in pollen specificity via an *in vivo* functional assay (Sijacic et al., 2004; Williams et al., 2014a; Kubo et al., 2015). This assay involves raising transgenic plants to examine the effect of expressing a particular allelic variant of an SLF on the SI behavior of the transgenic pollen. In cases when breakdown of SI is observed in the transgenic plants, progeny from crosses with wild-type plants of appropriate S-genotypes have to be examined as well. The Co-IP-MS results suggest that this approach may be a much less time-consuming and labor-intensive alternative to the *in vivo* functional assay for assessing the SI function of SLF proteins. If all SLF proteins are assembled into similar SCF complexes, this would raise the questions of how all these sequence-divergent SLF proteins (e.g., 45.3–87.7% sequence identity between the 17 SLF proteins of S<sub>2</sub>-haplotype; Williams et al., 2014b) are capable of being assembled into their respective SCF<sup>SLF</sup> complexes, and whether the allelic variants of S-RNase taken into a pollen tube may favor the “selection” of particular SLF proteins that can interact with and detoxify the non-self S-RNases, especially when the common components of the complex are limited. It would be interesting to study as well the dynamics of the SLF-containing SCF complexes during growth of compatible pollen tubes in the pistil.

### THE S-LOCUS F-BOX PROTEINS

For the SLF proteins of *Petunia* that have been studied so far, each interacts with only one, or a few, of the S-RNases examined (Sijacic et al., 2004; Kubo et al., 2010; Williams et al., 2014a). This pattern of interactions between SLF proteins and S-RNases is consistent with the prediction by the collaborative non-self recognition model (Kubo et al., 2010). How then does one SLF, but not other SLF proteins, interact with a certain S-RNase? How have all these multiple SLF proteins evolved so that pollen of a given S-haplotype has a complete arsenal to counter the cytotoxic effect of all non-self S-RNases, but avoid interacting with their self S-RNase?

Typically, F-box proteins contain two domains: the F-box domain in the N-terminal region and a protein–protein interaction domain located in the C-terminal region (C-terminal domain or CTD; Gagne et al., 2002; Cardozo and Pagano, 2004; Wang et al., 2004). Thus, it is reasonable to examine the CTD of an SLF to identify the amino acids that are involved in its interaction with a particular S-RNase. One approach to identify such amino acids would be to compare the amino acid sequences of SLF proteins that interact with the same S-RNase with the amino acid sequences of SLF proteins that do not. The amino acids in the CTD that are conserved among all the SLF proteins that interact with the same S-RNase, but are divergent among all those that do not, are likely important for the specific interaction with that S-RNase. This approach will benefit from knowing interaction

relationships between as many SLF proteins and S-RNases, as the information can then be used to design strategies to determine the biochemical basis for the differential interactions.

Among the interaction relationships established between SLF proteins and S-RNases of *P. inflata*, S<sub>2</sub>-SLF1 interacts with the largest number, four, of the S-RNases examined and all the other SLF proteins interact with none or at most one S-RNase (Sijacic et al., 2004; Kubo et al., 2010; Sun and Kao, 2013; Williams et al., 2014a). The SLF that interacts with more S-RNases than do all other SLF proteins might be the first to have come into existence during the evolution of the SI system. If the first SLF could interact with a number of non-self S-RNases, it would allow pollen to detoxify new non-self S-RNases as more S-haplotypes evolved, without having to generate a new SLF. However, there might be a practical limit as to the number of non-self S-RNases with which each SLF could interact, so that when the maximum capacity is reached, a new SLF would be needed to allow pollen to recognize and detoxify additional non-self S-RNases as more S-haplotypes continued to evolve.

If an SLF has evolved to interact with and detoxify a particular S-RNase, there would be no selective pressure to generate another SLF with the same function. However, from the standpoint of defense against the toxic effect of non-self S-RNases, it would be beneficial to pollen if more than one SLF were capable of detoxifying any particular non-self S-RNase, as this will minimize the deleterious effect caused by mutations that render an SLF incapable of interacting with and detoxifying a non-self S-RNase. In order to maintain an SI system over a long period of time, not only must self-pollen be rejected by the pistil, but also non-self pollen must be accepted by the pistil through the collective effort of all SLF proteins to detoxify all non-self S-RNases. It would be of interest to determine whether, during the evolution of the SLF genes, there has been indeed such a redundancy built in for pollen to deal with every non-self S-RNase. The results from studying the effect of silencing the expression of S<sub>2</sub>-SLF1 in pollen of S<sub>2</sub>S<sub>3</sub> transgenic plants are consistent with the presence of additional SLF proteins for detoxifying S<sub>3</sub>-RNase, S<sub>7</sub>-RNase, and S<sub>13</sub>-RNases, as transgenic pollen producing very low levels of, if any, S<sub>2</sub>-SLF1, remained compatible with S<sub>3</sub>-, S<sub>7</sub>-, and S<sub>13</sub>-carrying pistils (Sun and Kao, 2013). Moreover, Kubo et al. (2010) found that two SLF proteins produced by S<sub>5</sub> pollen of *P. hybrida* interacted with the same S-RNase, S<sub>9</sub>-RNase.

### THE FATE OF NON-SELF S-RNASES IN THE POLLEN TUBE

The current view about what happens to non-self S-RNases, after being taken up by the pollen tube, is that they become ubiquitinated in the cytosol through mediation by appropriate SCF<sup>SLF</sup> complexes, and subsequently degraded by the 26S proteasome. However, the fate of non-self S-RNases may need a closer examination, considering the following observations in *Petunia*. A cell-free protein degradation system showed that recombinant S<sub>1</sub>-, S<sub>2</sub>-, and S<sub>3</sub>-RNases (expressed in *E. coli* and thus non-glycosylated) were all degraded by the 26S proteasome in pollen tube extracts; however, native glycosylated S<sub>3</sub>-RNase purified from styles was not degraded to any significant extent (Hua and Kao, 2006). After purified native S<sub>3</sub>-RNase was deglycosylated, the deglycosylated protein was degraded as efficiently as recombinant

S-RNases (Hua and Kao, 2006). Moreover, native S<sub>9</sub>-RNase was ubiquitinated to various extents, with most being mono-ubiquitinated, by an SCF<sup>S7-SLF2</sup> complex in an *in vitro* ubiquitination assay, but the degradation of the mono-ubiquitinated S<sub>9</sub>-RNase in an *in vitro* protein degradation assay was not efficient (Entani et al., 2014).

It is possible that S-RNase is deglycosylated once taken into the pollen tube. Non-glycosylated S-RNase has been shown to function normally in rejecting self-pollen (Karunandaa et al., 1994), so deglycosylation should not affect the function of S-RNase. Deglycosylated non-self S-RNase then becomes poly-ubiquitinated and degraded in the cytosol. TTS, a tobacco transmitting tissue glycoprotein, has been shown to be incorporated into the pollen tube wall and deglycosylated (Wu et al., 1995), suggesting that deglycosylation of S-RNase by the pollen tube is possible. It is also possible that poly-ubiquitination and degradation by the 26S proteasome is not the only pathway for detoxification of S-RNase. S-RNase (and deglycosylated S-RNase) may also be mono-ubiquitinated and transported to a compartment for further degradation. Mono-ubiquitination has been shown to play an important role in fast labeling of proteins for bulk degradation by autophagy (Kim et al., 2008). Two plant plasma membrane proteins have been shown to be degraded in the vacuole (Cai et al., 2012). This possible fate of S-RNase can explain two contrasting findings about the destination of S-RNase after its uptake into the pollen tube. Luu et al. (2000) observed that S-RNase of *Solanum chacoense* was mainly localized in the cytosol of both compatible and incompatible pollen tubes, whereas Goldraij et al. (2006) observed that S-RNase of *N. alata* was sorted into vacuole-like organelles in both compatible and incompatible pollen tubes. S-RNase may be first taken up into the cytosol of the pollen tubes as observed by Luu et al. (2000), and after the interaction with appropriate SCF<sup>SLF</sup> complexes (and to a much lesser extent with PiSBP1 and the PiSBP1-containing SLF complex), those non-self S-RNases that are mono-ubiquitinated are sorted to vacuoles or vacuole-like organelles, as observed by Goldraij et al. (2006), for degradation. In the above-mentioned *in vitro* protein degradation assay of Entani et al. (2014), perhaps the compartments might be disrupted and the vacuolar proteases might not be active under the assay conditions, such that no significant degradation was observed for mono-ubiquitinated S<sub>9</sub>-RNase. Self S-RNase may follow similar pathways as non-self S-RNase, except that the bulk of its molecules remain stable due to the inability of any of the SLF proteins to interact with and detoxify it.

## CONCLUSION

In summary, there are several facets of S-RNase-based SI that require further investigation in order to obtain a comprehensive understanding of this complex self/non-self-recognition system. It is clear that S-RNase functions inside the pollen tube to exert its cytotoxicity, but how S-RNase is taken up into the pollen tube is completely unknown. The initial fates of both self and non-self S-RNase after entering the pollen tube also remain unclear. The model presented in **Figure 1** depicts several possible pathways for uptake and subsequent fates of self and non-self S-RNases. We believe that the difference in the ultimate fate of self and non-

self S-RNase is due in large part to non-self recognition between SLF proteins and S-RNases. A non-self S-RNase interacts with at least one of the SLF proteins and becomes ubiquitinated and suffers degradation, whereas a self S-RNase does not interact with any of the SLF proteins and thus remains stable. The existence of multiple SLF proteins in pollen of any S-haplotype and the highly polymorphic nature of the S-locus suggest that there are hundreds of SLF proteins (allelic variants of each of the multiple SLF proteins) with a wide range of sequence similarity available for studying interactions with S-RNases. However, so far, the interaction relationships have only been determined between a very small number of SLF proteins and S-RNases. A comprehensive study is necessary to understand the biochemical basis of differential interactions between SLF proteins and S-RNases, and the information obtained will not only further our understanding of S-RNase-based SI, but also lay the foundation for studying the structural basis of the interactions between F-box proteins and their substrates.

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# Evolution and function of epigenetic processes in the endosperm

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The endosperm is an ephemeral tissue surrounding the embryo that is essential for its development. Aside from the embryo nourishing function, the endosperm serves as a battlefield for epigenetic processes that have been hypothesized to reinforce transposable element silencing in the embryo. Specifically, global DNA demethylation in the central cell may serve to produce small RNAs that migrate to egg cell and embryo to induce de novo DNA methylation. The Polycomb Repressive Complex 2 (PRC2) is particularly targeted to DNA hypomethylated regions, possibly alleviating the negative effects associated with loss of DNA methylation in the endosperm. The functional requirement of the PRC2 in the endosperm can be bypassed by increasing the maternal genome dosage in the endosperm, suggesting a main functional role of the endosperm PRC2 in reducing sexual conflict. We therefore propose that the functional requirement of an endosperm PRC2 was coupled to the evolution of a sexual endosperm and mechanisms enforcing transposon silencing in the embryo. The evolutionary consequences of this scenario for genome expansion will be discussed.

**Keywords:** endosperm, evolution, epigenetics, imprinting, Polycomb Repressive Complex 2

## INTRODUCTION

The endosperm is a nutritive tissue formed in seeds of flowering plants that surrounds the embryo and is essential for its development. Embryo and endosperm are the products of two distinct fertilization events and enclosed by the maternally derived integuments that form the seed coat. While the embryo is derived from the fertilized egg cell, the endosperm is the descendent of the fertilized central cell (Li and Berger, 2012). The majority of angiosperms have an eight-nucleated, seven-celled Polygonum-type embryo sac that develops from the single surviving haploid megasporangium after three rounds of nuclei divisions, followed by nuclei migrations and cellularization. The female gametophyte consists of the egg cell flanked by two synergid cells, a diploid central cell derived from fusion of two polar nuclei and three antipodal cells that will degenerate shortly before or after fertilization (Sprunck and Gross-Hardt, 2011). As a consequence of the central cell being diploid, the endosperm of most species is triploid. Ancestral to the seven-celled female gametophyte is the four celled female gametophyte that contains a haploid central cell that will form a diploid endosperm after fertilization (Friedman and Williams, 2003; Segal et al., 2003). There are also angiosperms (e.g., Piperaceae) where all four megasporangia survive after meiosis and contribute to female gametophyte formation, giving rise to central cells with more than two nuclei that will form a high-ploidy ( $>3n$ ) endosperm after fertilization (Friedman et al., 2008).

## EVOLUTION OF PLOIDY SHIFTS IN THE ENDOSPERM

It has been hypothesized that the transition from a purely maternal embryo nourishing tissue to a biparental endosperm resulted

in two possible conflicts: (i) conflict of male and female parents over the allocation of nutrients to the developing progeny and (ii) conflict among the developing progeny for resources from the maternal sporophyte (Haig and Westoby, 1989a,b; Friedman, 1995). The kin-conflict theory provides a theoretical framework for both conflicts (Haig, 2013). This theory considers that the resources provided by the maternal sporophyte to provision the offspring are limited and that the relatedness of the endosperm to the maternal sporophyte is decisive for its ability to acquire nutrients for the developing embryo. In outcrossing species, the sexual endosperm containing maternal and paternal genomes is less related to the maternal sporophyte and sibling embryos compared to its own embryo (Charnov, 1979; Friedman et al., 2008). Therefore, paternally contributed alleles maximizing nutrient allocation to the endosperm will promote development of the embryo supported by this genetically related endosperm on the expense of the sibling embryos (Haig and Westoby, 1989a,b; Haig, 2013). Increasing the number of maternal genomes contributed to the central cell could have evolved as a mechanism to control resource provisioning to the developing progeny and to limit the selfish behavior of the endosperm. Therefore, transitions from the haploid central cell to the diploid and higher ploidy central cell can be viewed as evolutionary transitions to resolve conflict between maternal and paternal genomes on the provisioning of the progeny (Friedman et al., 2008).

## EPIGENETIC PROCESSES IN THE ENDOSPERM

In *Arabidopsis*, DNA methylation occurs in CG, CHG, and CHH (where H = A, T, or C) sequence contexts and is controlled

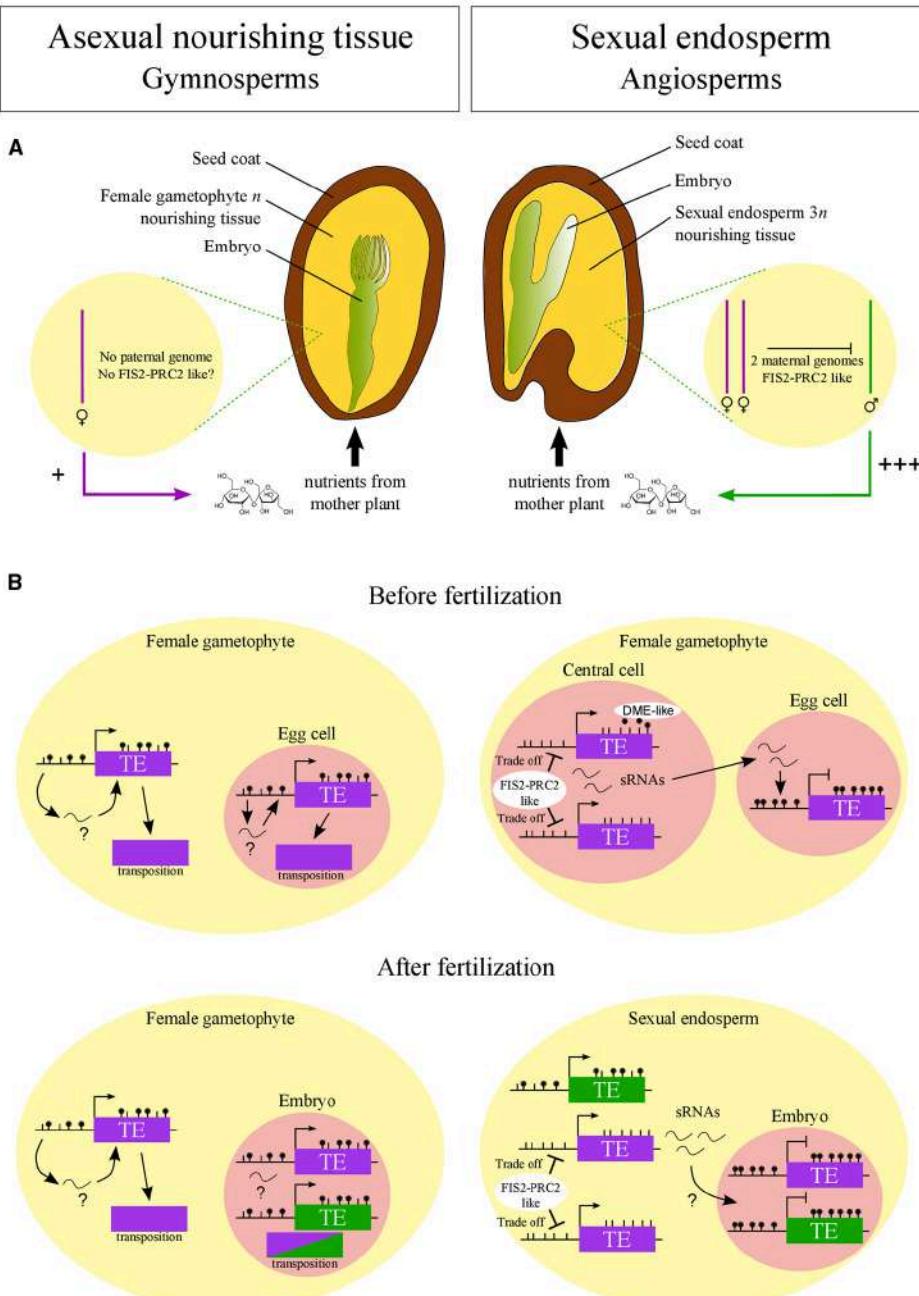
by three families of DNA methyltransferases that have different sequence preference. The DNA methyltransferase MET1 acts as a maintenance methyltransferase for symmetric CG residues, while non-CG methylation is maintained by the CHROMOMETHYLASE3 (in CHG context) and the DOMAINS REARRANGED METHYLASES 1/2 (DRM1/2) and CMT2 (in CHH context). The small RNA (sRNA) pathway targets DRM1/2-mediated *de novo* methylation in all sequence contexts and is required for the maintenance of CHH methylation (Kim and Zilberman, 2014). Genome-wide methylation studies of embryo and endosperm in *Arabidopsis* seeds revealed that the endosperm is globally hypomethylated compared to the embryo (Gehring et al., 2009; Hsieh et al., 2009), signifying substantial epigenome differences of the two fertilization products. Hypomethylation in the endosperm is restricted to the maternally inherited alleles, suggesting that the hypomethylated status is established in the central cell and inherited to the endosperm (Ibarra et al., 2012). In *Arabidopsis*, demethylation of the maternal genome requires the DNA glycosylase DEMETER (DME) that excises 5-methylcytosine preferably at small transposable elements (TEs) and is expressed in the central cell of the female gametophyte before fertilization (Hsieh et al., 2009). Maternal demethylation is nearly fully reversed in *dme* mutant endosperm (Ibarra et al., 2012), indicating that DME is likely the only enzyme accounting for global DNA methylation differences between the maternal and paternal endosperm genomes in *Arabidopsis*. The process of extensive endosperm demethylation is likely conserved between monocots and dicots, but involves specific differences caused by divergent evolution of the DME-like family (Zemach et al., 2010). Importantly, DME function is not restricted to the female central cell but DME also acts in the vegetative cell in pollen, the companion cell to the sperm cells. Similar to its role in the central cell, DME is causing hypomethylation of distinct regions in the vegetative cell. Almost half of those hypomethylated regions in the vegetative cell overlap with hypomethylated regions identified in the maternal genomes of the endosperm that likely descend from the central cell (Ibarra et al., 2012). While *de novo* methylation in CHH context is generally depleted in sperm (Calarco et al., 2012; Ibarra et al., 2012), regions that become hypomethylated by DME in the vegetative cell have increased levels of CHH methylation in sperm (Ibarra et al., 2012), suggesting communication between vegetative cells and sperm cells. Thus, it seems likely that sRNAs that are formed from demethylated regions in the vegetative cell migrate to sperm cells and reinforce methylation at distinct target sites, a hypothesis that remains to be experimentally tested. In agreement with this notion, 21 nt and 24 nt sRNAs corresponding to differentially methylated regions accumulate in sperm cells (Slotkin et al., 2009; Calarco et al., 2012) and prominently target regions of imprinted genes (discussed below) that maintain the paternal allele silenced after fertilization (Calarco et al., 2012). While traveling of a micro-RNA from the vegetative cell to sperm cells was proposed (Slotkin et al., 2009), this study may have suffered from an unspecific promoter (Grant-Downton et al., 2013), biasing the conclusions. Nevertheless, expression of a micro-RNA in the central cell can silence a reporter in the egg cell (Ibarra et al., 2012); revealing that 21 nt sRNAs (that are preferentially formed from microRNAs)

can indeed travel from the companion cells to the neighboring gametes. While previously only 24 nt sRNAs were known to establish *de novo* methylation by the sRNA-dependent DNA methylation pathway (RdDM; Cao and Jacobsen, 2002; Cao et al., 2003), recent data revealed a role of 21 nt sRNAs in silencing of transcriptionally active TEs via the RdDM pathway (McCue et al., 2015).

*De novo* DNA methylation increases during embryo development, suggesting increased activity of the RdDM pathway during embryo development (Jullien et al., 2012). In agreement with this view, *de novo* DNA methylation in the embryo depends on the activity of DRM2 and in part as well on DRM1 (Jullien et al., 2012). After fertilization, increased production of sRNAs occurs in siliques, reaching maximum levels at 6 days after anthesis (Mosher et al., 2009). Increased sRNA production correlates with a steadily increasing *de novo* methylation in the embryo (Jullien et al., 2012), giving rise to the hypothesis that sRNAs migrate from the endosperm to the developing embryo and enforce TE silencing in the embryo by *de novo* DNA methylation. *De novo* DNA methyltransferases DRM1 and DRM2 seem not to be active in the early endosperm (Jullien et al., 2012) but are expressed around the time of endosperm cellularization (Belmonte et al., 2013), in agreement with the presence of substantial levels of CHH methylation levels in the cellularized endosperm (Hsieh et al., 2009; Ibarra et al., 2012).

## GENOMIC IMPRINTING IN THE ENDOSPERM

As a consequence of DNA hypomethylation in the central cell, the parental genomes are differentially methylated in the endosperm, which can cause genes to become preferentially expressed from either the maternally or paternally inherited alleles. Parent-of-origin dependent gene expression as a consequence of epigenetic modification of maternal and paternal alleles in the gametes is a well-known phenomenon termed genomic imprinting (Gehring, 2013). Hypomethylation of TEs can cause either activation or silencing of the neighboring genes. What determines whether a gene will become activated or silenced in response to hypomethylation remains to be resolved, however, it seems likely that the distance of the TE to the gene is decisive. While many maternally and paternally expressed imprinted genes (MEGs and PEGs, respectively) have TEs in the vicinity of the 5' region, many PEGs have TEs additionally in the coding and 3' region of the gene (Wolff et al., 2011; Ibarra et al., 2012). Hypomethylation might expose binding sites for the repressive FERTILIZATION INDEPENDENT SEED (FIS)-Polycomb Repressive Complex 2 (PRC2), as it has been proposed for the differentially methylated region downstream of the *PHERES1* gene (Villar et al., 2009). The PRC2 is an evolutionary conserved repressive complex that modifies histones by applying histone trimethylation marks on histone H3 at lysine 27 (H3K27me3; Simon and Kingston, 2013). In *Arabidopsis*, there are at least three PRC2 complexes with different functional roles during plant development. The FIS-PRC2 is specifically expressed in the central cell and in the endosperm and consists of the subunits MEDEA (MEA), FIS2, FERTILIZATION INDEPENDENT ENDOSPERM (FIE), and MSI1 (Hennig and Derkacheva, 2009). Genome-wide profiling of H3K27me3 occupancy in the endosperm revealed that several



**FIGURE 1 | Hypothesized emergence of FIS2-PRC2-like complex in angiosperms connected to its role in sexual endosperm. (A)** In angiosperms, FIS2-PRC2-like function may have arisen to repress the paternal genome in the endosperm as predicted by the kin-conflict theory. The selfish behavior of the paternal genome, promoting nutrient allocation to the progeny, is symbolized by “+++”. According to this hypothesis, in gymnosperms, where the nourishing tissue is purely maternal, FIS2-PRC2-like function is not required. **(B)** FIS2-PRC2-like function may have allowed the emergence of DNA hypomethylation via

DME-like activity and small RNA (sRNA) production in central cell and endosperm. FIS2-PRC2 acts on hypomethylated regions, limiting the deleterious activity of transposable elements (TE). This tradeoff between TE silencing and activity allows the production of sRNAs traveling to the egg cell and embryo, reinforcing TE silencing. Such a demethylation process may not have emerged in gymnosperms, leading to a limited silencing of TEs and explaining the genome expansion in this taxon. Purple and green colors symbolize maternal and paternal genomes, respectively.

DNA hypomethylated TEs were targeted by the FIS-PRC2, revealing a redistribution of the FIS-PRC2 dependent on the location of DNA methylation (Weinhofer et al., 2010). A similar redistribu-

tion of H3K27me3 to TEs occurs in mutants deficient for MET1 (Deleris et al., 2012), as well as in mammalian cells depleted for DNA methylation (Reddington et al., 2013; Saksouk et al., 2014)

revealing a general ability of PRC2 to target and possibly silence hypomethylated TEs.

## EVOLUTION OF EPIGENETIC PROCESSES IN THE ENDOSPERM AS A MECHANISM TO PREVENT GENOME EXPANSION

Thus far, we do not know when endosperm-expressed PRC2 genes have emerged during angiosperm evolution. While the FIS-PRC2 subunit encoding genes *FIS2* and *MEA* are specific for *Arabidopsis thaliana* (Spillane et al., 2007; Chen et al., 2009), functional homologs of both genes are expressed in the triploid endosperm of monocots and lower eudicots (Haun et al., 2007; Luo et al., 2009; Gleason and Kramer, 2012) suggesting that the evolution of FIS-PRC2-like complexes is connected with the evolution of a sexual endosperm. Nevertheless, homologs but not orthologs fulfill the FIS2-PRC2 functional role in dicots and monocots and different PRC2 genes are regulated by genomic imprinting in monocots (Danilevskaya et al., 2003; Luo et al., 2009) and dicots (Grossniklaus et al., 1998; Luo et al., 2000), raising the hypothesis that FIS-PRC2-like complexes have evolved independently in both plant groups. The functional requirement of the FIS-PRC2 can be bypassed by increasing the maternal genome dosage in the endosperm (Kradolfer et al., 2013), suggesting that the FIS-PRC2 serves to suppress expression of paternally contributed genes. Supporting this view, *Arabidopsis* mutants lacking a FIS-PRC2 can form a functional diploid endosperm (Nowack et al., 2007); revealing that by reducing paternal genome dosage the functional requirement of the FIS-PRC2 can be bypassed. We therefore hypothesize that in gymnosperms, where the female gametophyte forms an endosperm-like nourishing structure, a FIS-PRC2-like complex would not be required. This notion is supported by the fact that the FIS-PRC2 prevents autonomous endosperm formation and thus couples fertilization to endosperm development (Guitton and Berger, 2005), a function that is not required in gymnosperms.

As outlined above, the PRC2 is targeted to regions with reduced DNA methylation in the endosperm (Weinhofer et al., 2010; Zhang et al., 2014) and could, therefore, potentially repress activity of TEs upon loss of DNA methylation. It can thus be envisioned that DNA hypomethylation activities in the central cell evolved concomitantly with a central cell/endosperm-expressed PRC2 that alleviated the negative effects associated with loss of DNA methylation. Following this logic, the evolution of hypomethylation mechanisms in the central cell to enforce silencing of TEs in the egg cell and descendant embryo were closely coupled to the evolution of FIS-PRC2-like complexes in the central cell and endosperm to ensure TE silencing after DNA hypomethylation. This scenario would imply that TE transcription upon DNA hypomethylation and PRC2-mediated TE repression are balanced to ensure sufficient TE-derived sRNAs being made to enforce silencing in the embryo but TE transposition remains suppressed (**Figure 1**).

If indeed a FIS-PRC2-like complex as well as DME-like enzymes evolved together with the sexual endosperm, neither of both should be present in the large female gametophytes of gymnosperms. The female gametophyte in gymnosperms serves

an endosperm-like role in supporting embryo growth, but it is of pure maternal origin and forms a multicellular structure before fertilization. Consequently, the enormous genome expansion in gymnosperms may be a consequence of the lack of efficient TE silencing mechanisms that possibly have evolved after the female gametophyte became sexualized (**Figure 1**).

Testing this hypothesis requires to test whether orthologs of FIS-PRC2-like genes and DME-like genes are expressed in the female gametophyte of gymnosperms and basal angiosperms, which remains a challenge of future investigations.

Together, we propose that the evolution of mechanisms enforcing TE silencing in the embryo evolved concomitantly with the sexual endosperm. The evolution of PRC2 activity in the central cell and endosperm allowed DNA hypomethylation activities being active in the central cell to enforced TE silencing in the egg cell and embryo. Consequently, genome size restriction by efficient control of TE silencing may be directly coupled with the evolution of a sexual endosperm.

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# Regulation of inflorescence architecture by cytokinins

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In flowering plants, the arrangement of flowers on a stem becomes an inflorescence, and a huge variety of inflorescence architecture occurs in nature. Inflorescence architecture also affects crop yield. In simple inflorescences, flowers form on a main stem; by contrast, in compound inflorescences, flowers form on branched stems and the branching pattern defines the architecture of the inflorescence. In this review, we highlight recent findings on the regulation of inflorescence architecture by cytokinin plant hormones. Results in rice (*Oryza sativa*) and *Arabidopsis thaliana* show that although these two species have distinct inflorescence architectures, cytokinins have a common effect on inflorescence branching. Based on these studies, we discuss how cytokinins regulate distinct types of inflorescence architecture through their effect on meristem activities.

**Keywords:** branching, cytokinin, floral meristem, inflorescence, shoot apical meristem

## INTRODUCTION

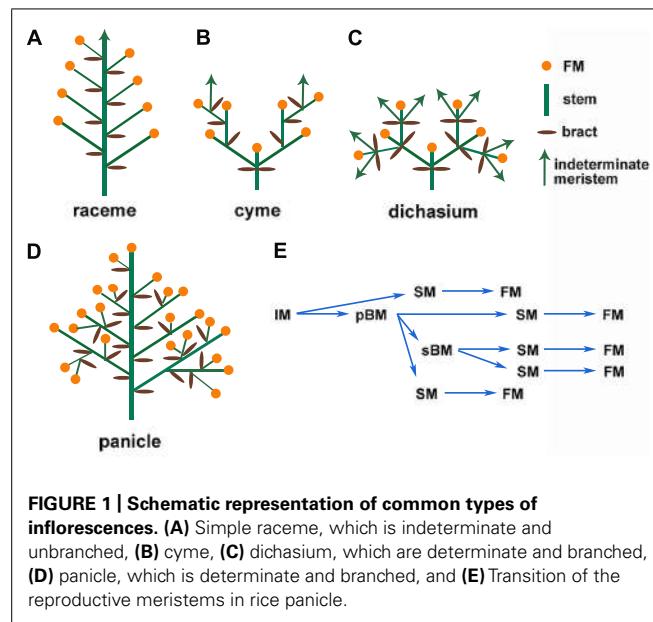
Plants have an enormous, striking diversity of forms, with varying numbers and arrangements of organs in different sizes and shapes; this diversity derives from regulation of meristem activity. The aerial organs of a plant come from the shoot apical meristem (SAM) which gives rise to leaves, stem, and axillary meristems during the vegetative stage and transforms into the inflorescence meristem (IM) after the floral transition. The various developmental patterns of the IM in different species produce diverse inflorescence architectures, which not only attract artists and plant scientists, but also draw the attention of plant breeders, because inflorescence traits directly affect crop yields. Branching hierarchy and complexity depend on the species, but are also affected by environmental factors, including nutrition, light, and temperature (Tanaka et al., 2013; Kyozuka et al., 2014; Teo et al., 2014).

The enormous diversity of inflorescence architecture also leads to difficulties in defining consensus criteria to classify these structures. Following Weberling's (1989) suggestions, inflorescence architectures can be broadly grouped into inflorescences without branching (simple) and inflorescences with branching (compound). Another key parameter is whether the IM ends in a terminal flower (determinate) or continues to produce structures, including branches and flowers (indeterminate). Following these key distinctions, at least three typical groups of inflorescence architectures are commonly seen, namely the raceme (simple, indeterminate, as in *Arabidopsis*), the cyme (complex, determinate, as in tomato), and the panicle [complex, determinate, as in wheat (*Triticum aestivum*); or complex, indeterminate, as in maize (*Zea mays*), especially tassel; **Figure 1**; Prusinkiewicz et al., 2007; Kellogg et al., 2013]. These distinct inflorescence architectures

result from different developmental programs that are elaborated below.

Development of the IM conditions the branching of the inflorescence. In *Arabidopsis*, the IM directly initiates floral meristems (FMs, which are determinate meristems) on its flanks; this forms a simple raceme (**Figure 1A**; Benlloch et al., 2007; Tanaka et al., 2013; Teo et al., 2014). The grasses have more diverse inflorescence architectures (Kellogg et al., 2013). In a generalized grass inflorescence, the IM gives rise to several branch meristems (BMs, which are usually indeterminate meristems). These BMs may initiate secondary BMs to form lateral branches and spikelet meristems (SMs) that then initiate FMs (**Figure 1E**). In maize and other Andropogoneae species, determinate spikelet-pair meristems (SPMs) are produced from the IM or BMs, and each SPM makes two SMs. The SM initiates one or more FMs (Kellogg et al., 2013; Kyozuka et al., 2014). These intermediate BMs cause secondary or higher-order branches, which form a compound inflorescence termed the panicle (Benlloch et al., 2007). Therefore, the branch structure determines the final inflorescence pattern, which contributes to the enormous diversity of inflorescence architectures. Specific genetic regulatory networks control every stage and transition of meristem activity, as described in several recent reviews (Tanaka et al., 2013; Kyozuka et al., 2014).

Meristem activity, especially determinacy, fundamentally affects inflorescence architecture. For example, in the raceme-type inflorescence of *Arabidopsis*, the IM continues to initiate FMs; by contrast, in the cyme-type inflorescence of tomato, the IM forms a terminal flower immediately after developing a new IM below it, which reiterates this pattern (**Figures 1A,B**). The panicle-type inflorescence is initially indeterminate and initiates BMs and FMs before it finally terminates in a FM in some species.



At least two groups of genes, relatives of *Arabidopsis LEAFY* (*LFY*) and *TERMINAL FLOWER1* (*TFL1*), play a central role in meristem determinacy. *LFY* promotes determinate FM identity and termination of IMs, and *TFL1* maintains the indeterminacy of IMs to prevent termination (Prusinkiewicz et al., 2007).

Recent work has identified cytokinins as key regulators of inflorescence architecture in plants with different inflorescence types, through regulation of meristem activity, which is often also associated with meristem identity. Cytokinins have profound effects on plant development and growth, including meristem activity (Kyozuka, 2007; Werner and Schmülling, 2009; Perilli et al., 2010). Accumulating data point to a role for cytokinins in influencing inflorescence complexity by fine-tuning IM and BM determinacy. Also, recent work reveals that cytokinins can regulate the initiation of meristems from floral organ axils (the junction where the floral organ meets the stem), and thus convert a determinate flower into an inflorescence (Han et al., 2014). Here we review these two mechanisms through which cytokinins regulate inflorescence architecture.

### CYTOKININS PROMOTE IM ACTIVITY

Increasing cytokinin concentrations and signaling activity increase meristem size and activity. Reduced meristem activity often leads to conversion of an IM or a BM into a terminal flower, which subsequently affects inflorescence architecture.

Work in rice and in *Arabidopsis* showed that cytokinin levels affects meristem activity and inflorescence complexity. The ATP/ADP isopentenyl transferases (IPTs) catalyze the first step of cytokinin biosynthesis (Miyawaki et al., 2006). *Arabidopsis atipt3 5 7* triple mutants and *atipt1 3 5 7* quadruple mutants have lower levels of cytokinins, which leads to reduced IM size, formation of a terminal flower, and conversion of an indeterminate inflorescence to a determinate inflorescence (Miyawaki et al., 2006). Rice *LONELY GUY* (*LOG*) encodes a cytokinin-activating enzyme catalyzing the final step of cytokinin biosynthesis and *LOG* is strongly

expressed in BMs and FMs of developing panicles. The absence of *LOG* results in early termination of IM and BMs, which reduces branching complexity (Kurakawa et al., 2007). *Arabidopsis* has nine *LOG* homologs and the triple *log3 log4 log7* and septuple *log1 log2 log3 log4 log5 log7 log8* mutants produce fewer FMs, suggesting reduced IM activity (Kuroha et al., 2009; Tokunaga et al., 2012).

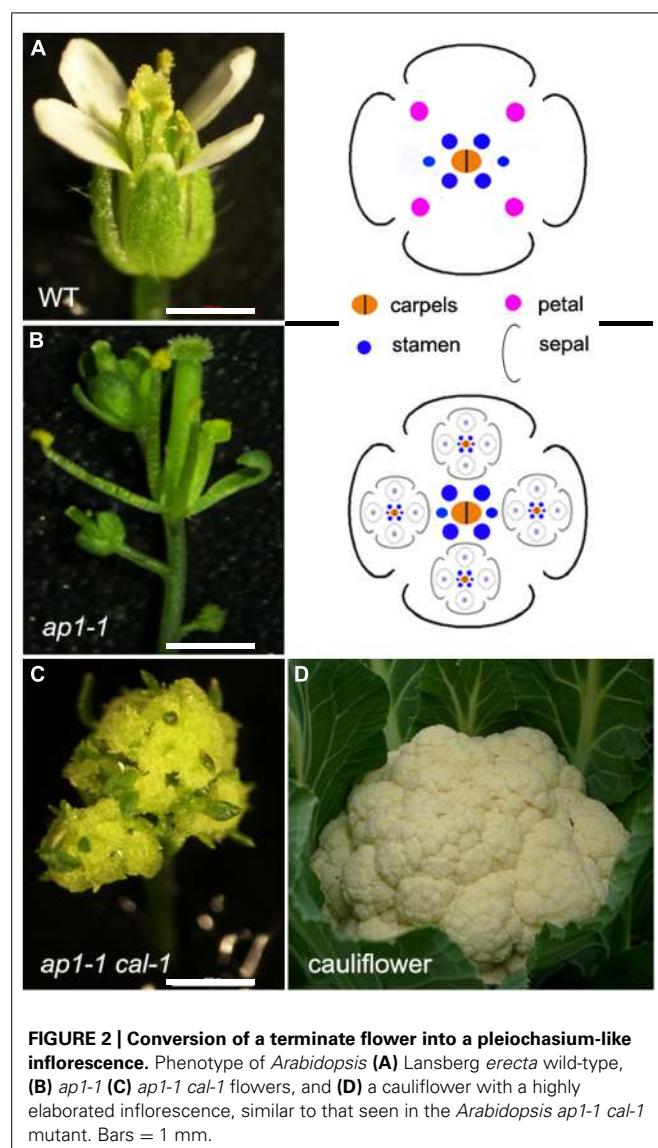
In addition to cytokinin homeostasis, defects in cytokinin signaling also leads to simplified inflorescence architecture. Cytokinins are perceived by transmembrane histidine kinase receptors, such as *Arabidopsis HISTIDINE KINASE 2* (AHK2), AHK3, and AHK4. The *ahk* triple mutants have a smaller IM that terminates early, resulting in a simplified inflorescence with only a few flowers (Nishimura et al., 2004).

Conversely, elevated cytokinin homeostasis results in increased inflorescence complexity. Cytokinin oxidase/dehydrogenase (CKX) plays a major role in the degradation of bioactive cytokinins (Mok and Mok, 2001). *Arabidopsis* plants overexpressing *CKX1* or *CKX3* have dramatically reduced cytokinins contents and IMs that produce very few flowers (Werner et al., 2003). CKX overexpression in tobacco plants also leads to fewer flowers and conversion of IMs from indeterminate to determinate (Werner et al., 2001). Similarly, rice varieties with lower *OsCKX2* expression have more elaborated and larger panicles with more primary and secondary branches and higher yield, and rice varieties with higher *OsCKX2* activity have the opposite phenotype, with fewer branches and lower yield (Ashikari et al., 2005; Li et al., 2013).

Cytokinins promote IM activity and affect inflorescence architecture by promoting expression of the meristematic gene *WUSCHEL* (*WUS*) and suppressing the meristem inhibitors *CLAVATA1* (*CLV1*) and *CLV3*. Plants ectopically treated with cytokinins show a *clv*-like phenotype with larger IMs and more floral organs (Venglat and Sawhney, 1996; Lindsay et al., 2006). Cytokinins suppress the expression of *CLV1*; this suppression results in upregulation of *WUS* expression (Brand et al., 2000; Schoof et al., 2000; Lindsay et al., 2006; Gordon et al., 2009). In addition, cytokinins directly induce *WUS* expression, independent of *CLV1*, and *WUS* enhances cytokinin signaling, forming a positive feedback loop (Leibfried et al., 2005; Gordon et al., 2009). Computational modeling shows that a combination of the negative feedback between *WUS* and *CLV*, and the positive feedback of *WUS* and cytokinin signaling determines the fine-scale positioning of the *WUS*-expressing stem cell niche domain (Gordon et al., 2009; Chickarmane et al., 2012).

### CYTOKININS PROMOTE LATERAL INDETERMINACY IN DETERMINATE FMS

In indeterminate inflorescences, the periphery of the meristem produces BMs (and also SPMs and SMs for grasses) or FMs. In many determinate inflorescences, such as in wheat spikes, BM, SM, and FM can also initiate from the IM before its termination in a FM. In contrast to this initiation pattern, FM and BM can also initiate laterally from a terminal flower, either from the axil of a leaf-like organ (such as petals) or can initiate without subtending lateral organs. These types of inflorescence are termed dichasium and pleiochasm (Figure 1C), depending on the number of lateral branches, and can be considered a specialized cyme.



**FIGURE 2 | Conversion of a terminal flower into a pleiochasium-like inflorescence.** Phenotype of *Arabidopsis* (A) *Lansberg erecta* wild-type, (B) *ap1-1* (C) *ap1-1 cal-1* flowers, and (D) a cauliflower with a highly elaborated inflorescence, similar to that seen in the *Arabidopsis ap1-1 cal-1* mutant. Bars = 1 mm.

Common examples include cauliflower and broccoli, which have a phenotype similar to that of the *Arabidopsis apetala1-1 cauliflower-1 (ap1-1 cal-1)* double mutants (Figure 2). In the *ap1-1* single mutant (Figure 2B), secondary flowers laterally initiate from sepal axils and from the pedicel. The *ap1-1 cal-1* double mutants have the same but more complicated inflorescence branching pattern. This lateral inflorescence branching mechanism has many similarities to vegetative stage lateral shoot branching. In contrast to vegetative shoot branching, inflorescences like the raceme and panicle develop iteratively, similar to frond development in ferns (Sanders et al., 2011). Despite these differences, cytokinins also regulate this type of lateral inflorescence branching.

Lateral inflorescence branching is controlled by *AP1* and related MADS-box transcription factor genes in *Arabidopsis* and other Brassicaceae species. In the simple indeterminate inflorescence of *Arabidopsis*, the IM gives rise to FMs and each FM differentiates into four whorls of floral organs that occupy precise positions (Figure 2A). In addition to promoting FM formation and outer

floral whorl specification of sepals and petals, *AP1* inhibits sepal axil meristem activity (Irish and Sussex, 1990; Mandel et al., 1992). In *ap1* mutants, secondary flowers initiate in the axes of sepals, and tertiary flowers can initiate in the sepal axes of secondary flowers, and so on (Figure 2B; Irish and Sussex, 1990; Mandel et al., 1992). This forms a dichasium or pleiochasium-like inflorescence (Figure 1C). The inflorescence phenotype in *ap1* is enhanced by *cauliflower* and *fruitful* mutants to form a cauliflower-like, highly elaborated pleiochasium inflorescence (Figure 2C; Ferrandiz et al., 2000). Indeed, cauliflower has lost a homolog of *AP1* (Figure 2D; Kempin et al., 1995), suggesting that *AP1* function is required to inhibit conversion of a simple raceme to a pleiochasium.

A recent study has shown that *AP1* inhibits lateral inflorescence branching by reducing cytokinin levels. During vegetative stages, leaf axil axillary meristem formation requires cytokinin signaling (Wang et al., 2014) and during reproductive stages, lateral FM formation similarly requires cytokinin signaling (Han et al., 2014). The *ap1* flowers have enhanced cytokinin signaling, as shown by examination of cytokinin-responsive reporter genes, and these flowers also have elevated levels of certain types of cytokinins. In addition, cytokinin treatment or ectopic expression of the cytokinin biosynthesis enzyme *IPT8* in the *AP1*-expressing domain phenocopies the sepal axil secondary flower phenotype (Venglat and Sawhney, 1996; Han et al., 2014). This secondary flower phenotype can be rescued by mutations of cytokinin receptors. Further molecular dissection showed that *AP1* suppresses the cytokinin biosynthetic gene *LOG1* and activates the cytokinin degradation gene *CKX3*, through direct binding to the target gene promoters, thus reducing cytokinins levels in the outer whorls of developing flowers. Restoring the expression levels of either *LOG1* or *CKX3* can partially rescue the *ap1* secondary flower phenotype. In addition to affecting cytokinin homeostasis, *AP1* also directly downregulates a group of flowering time-related MADS-box genes, including *SHORT VEGETATIVE PHASE* (*SVP*), *AGAMOUS-LIKE 24* (*AGL24*), and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), to suppress secondary FM formation. Similar to *IPT8* overexpression, overexpression of *SVP*, *AGL24*, or *SOC1* leads to sepal axil secondary FM formation (Liu et al., 2007). There appears to be crosstalk between cytokinin signaling and these flowering time-related MADS-box genes in the regulation of sepal axil secondary FM formation.

Taken together, the results described above show that cytokinins promote inflorescence complexity in different ways, by promoting meristem activity of IMs and BMs in inflorescences that branch iteratively, and by promoting indeterminate lateral meristem formation in inflorescences that branch laterally. Manipulating cytokinin levels directly or indirectly in crops is expected to change inflorescence complexity to increase yields (Kempin et al., 1995; Ashikari et al., 2005; Kurakawa et al., 2007; Zhang et al., 2012; Li et al., 2013).

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# Regulation of floral stem cell termination in *Arabidopsis*

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In *Arabidopsis*, floral stem cells are maintained only at the initial stages of flower development, and they are terminated at a specific time to ensure proper development of the reproductive organs. Floral stem cell termination is a dynamic and multi-step process involving many transcription factors, chromatin remodeling factors and signaling pathways. In this review, we discuss the mechanisms involved in floral stem cell maintenance and termination, highlighting the interplay between transcriptional regulation and epigenetic machinery in the control of specific floral developmental genes. In addition, we discuss additional factors involved in floral stem cell regulation, with the goal of untangling the complexity of the floral stem cell regulatory network.

**Keywords:** *Arabidopsis*, floral meristem, stem cell, determinacy, flower development

## INTRODUCTION

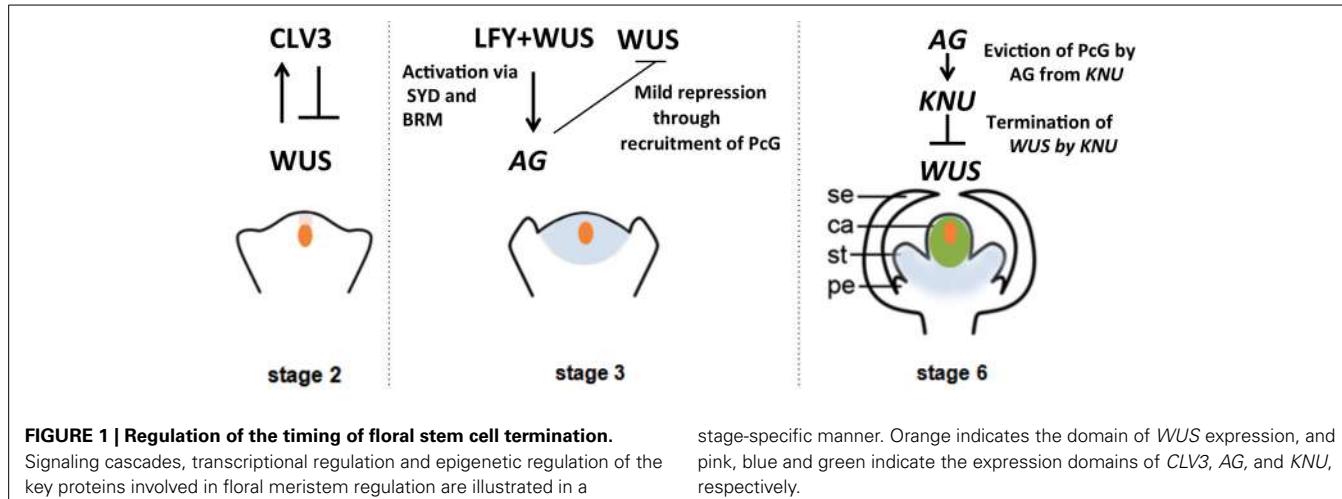
The flower is an elegant structure produced by angiosperms for effective reproduction. In *Arabidopsis*, floral organs are built in four whorls of concentric circles. From outermost to innermost, they consist of four sepals, four petals, six stamens and two fused carpels. The molecular mechanism specifying the identity of each whorl of floral organs is explained by the genetic ABCE model (Krizek and Fletcher, 2005). All four whorls of floral organs are derived from a self-sustaining stem cell pool named the floral meristem (FM), which arises from the peripheral regions of the shoot apical meristem (SAM). Much like the stem cells in the SAM, the stem cells in the FM are maintained by a signaling pathway involving the homeodomain protein WUSCHEL (WUS) and the CLAVATA (CLV) ligand-receptor system (Fletcher et al., 1999; Brand et al., 2000; Schoof et al., 2000). WUS is expressed in the organizing center, and it specifies and maintains the stem cell identity of the overlying cells. Expansion of WUS expression is prevented by the CLV signaling pathway, in which the CLV3 peptide is transcriptionally induced by WUS in the stem cells (Yadav et al., 2011; Daum et al., 2014). Due to the negative feedback regulatory loop of CLV3 and WUS, the stem cell pool remains constant in the initial floral developmental stages (stage 1~2) (Smyth et al., 1990; Schoof et al., 2000).

In the stage 3 floral bud, the C class gene AGAMOUS (AG) is induced by LEAFY (LFY) together with WUS in whorls 3 and 4 (Lenhard et al., 2001; Lohmann et al., 2001). AG has two major roles. It specifies reproductive organs, and it also regulates floral stem cell activity (Lenhard et al., 2001; Lohmann et al., 2001). In stage 6, floral stem cells are terminated in an AG-dependent manner to ensure proper development of the carpels. With respect to floral stem cell regulation, the major two pathways, the AG-WUS pathway and the CLV-WUS pathway, seem

to function independently. The double mutant *ag clv1* shows an additive phenotype of *ag* and *clv1*, and it expresses WUS in a broader domain than the *ag* mutant flower (Lohmann et al., 2001). In fact, the CLV-WUS pathway regulates floral stem cells spatially to restrict and maintain the stem cell pool in the early floral stages (stage 1~6), whereas the AG-WUS pathway provides temporal regulation to shut off stem cell activity at floral stage 6 (**Figure 1**). The precise timing of WUS repression is a key factor that determines the number of cells produced for reproductive organ development.

## DIRECT AND INDIRECT ROLES OF AG IN WUS REPRESSION

AG is reported to directly bind to the WUS locus to repress WUS expression (Liu et al., 2011). Based on an ethyl methanesulfonate mutagenesis screening of enhancer mutants of a weak allele, *ag-10*, which has only a moderate effect on floral meristem determinacy, one CURLY LEAF (CLF) mutant allele, *clf-47*, was identified (Liu et al., 2011). This suggests that CLF is required for floral meristem determinacy. CLF is a core component of polycomb repressive complex 2 (PRC2), which suggests that WUS repression is associated with the deposition of the repressive mark H3 lysine 27 tri-methylation (H3K27me3), a mark that is mediated by the polycomb group proteins (PcG). Consistent with this, one mutant allele of TERMINAL FLOWER 2 (TFL2), a PRC1 factor in *Arabidopsis*, can enhance the *ag-10* indeterminate phenotype (Liu et al., 2011). The *ag-10 tfl2-2* double mutant flowers show enlarged carpels bearing ectopic internal organs, as observed in *ag-10 clf-47*. These results indicate that WUS is a target of PcG during flower development. AG binds to the two CArg boxes in the WUS 3' non-coding region, and TFL2 occupancy at WUS is largely compromised in the *ag-1* null mutant background. These results suggest that AG has a role in the recruitment of PcG



to repress *WUS*. However, whether *AG* recruits Pcg directly is still an open question.

*35S::AG* transgenic plants do not show any obvious floral meristem defects (Mizukami and Ma, 1997), and *WUS* is only mildly repressed after stage 3 directly by *AG*. For the termination of *WUS* at floral stage 6, a C2H2 zinc finger repressor protein, KNUCKLES (*KNU*), plays a pivotal role (Payne et al., 2004; Sun et al., 2009). *KNU* expression starts in stage 5–6, and mutation of *KNU* leads to enlarged carpels and repeated ectopic growth of stamens and carpels. This indeterminate floral phenotype is caused by the prolonged activity of *WUS*, showing that *KNU* is necessary for floral stem cell termination. *KNU* is directly induced by *AG*, and mutations in three CArG box sequences on the *KNU* promoter can abolish *KNU* induction (Sun et al., 2009). Timed induction of *KNU* by *AG* in stage 6 of flower development ensures floral meristem termination and proper development of the female reproductive organs. The timing of *KNU* expression is important for balancing floral stem cell proliferation and differentiation. Delayed *KNU* expression leads to indeterminate flowers with more stamens, and ectopic *KNU* activity can terminate floral meristem precociously and produce flowers without carpels. *KNU* is also regulated by Pcg-mediated H3K27me3, and the removal of the repressive marks of H3K27me3 is *AG*-dependent. It takes approximately 2 days for *AG* to induce *KNU* in stage 6. During these 2 days, the H3K27me3 level on the *KNU* locus is progressively reduced, revealing a potential link between the transcriptional activation of *KNU* by *AG* and *AG*-dependent removal of H3K27me3 from the *KNU* chromatin (Sun et al., 2009).

## EPIGENETIC REGULATION OF TERMINATION TIMING IN FLORAL STEM CELLS

In floral meristems, cell division take 1–2 days on average (Reddy et al., 2004). Therefore, the 2-day of delay in *KNU* induction corresponds to 1–2 rounds of cell division. Through cell division, the pre-existing H3K27me3 on the *KNU* locus may be passively diluted by incorporation of unmodified histone H3, enabling *KNU* expression (Sun et al., 2014). The core components of Pcg, FIE and EMF2 are associated with specific promoter regions of

*KNU*, which include the binding sites of *AG*. Indeed, this region contains a 153 bp fragment that is the minimal sequence of a functional polycomb response element (PRE). This sequence is both necessary and sufficient for Pcg-mediated silencing of a ubiquitous promoter. This raises the possibility that *AG* plays a role in removing Pcg to activate *KNU*. By simulating *AG*'s physical blocking of the site with an artificially-designed TAL protein (a effector-based synthetic DNA binding protein designed to recognize the sequences around the first *AG* binding site), we showed that a YFP reporter could be activated in a cell cycle-dependent manner, even though it had been silenced by the minimal PRE sequence.

PRE was first identified in the fruit fly *Drosophila*, and it is targeted by the Pho-repressive complex (PhoRC) (Muller and Kassis, 2006). In *Arabidopsis*, homologs of PhoRC have not been identified, but in a genome-wide analysis of FIE binding sites, GA-repeat motifs appeared frequently, much like the *Drosophila* PRE (Deng et al., 2013). The *KNU* PRE is located near the 1kb upstream promoter region of the *KNU* transcriptional start site (Sun et al., 2014). Although the entire *KNU* locus is found to be bound by FIE and EMF2, only the transcribed region is covered by the repressive mark H3K27me3, and the PRE is not covered by the repressive mark. The indispensable role of the *KNU* PRE in recruiting PRC2 and establishing the FIE and EMF2 binding pattern on *KNU* indicates that Pcg is first recruited to the *KNU* PRE and may later act on the *KNU* transcribed region to establish the H3K27me3 marks by sliding or by DNA looping. When the *AG* protein binds to the CArG box sequences that overlap the *KNU* PRE, the occupancy of *AG* triggers the displacement of PRC2, which leads to the loss of the H3K27me3 marks on *KNU*. Through cell division, H3K27me3 is diluted due to the lack of Pcg activity, and *KNU* become de-repressed. Delayed reporter induction has been reported following artificial removal of a PRE by a cre-lox system in *Drosophila*, supporting this model of *KNU* de-repression (Beuchle et al., 2001; Muller et al., 2002).

Alternatively, the H3K27me3 mark can be erased by the JmjC-domain-containing histone demethylases REF6, EFL6, JMJ30 and JMJ32 (Lu et al., 2011; Crevillen et al., 2014; Gan et al., 2014). It has been reported that *AG*, REF6 and some other MADS-domain

proteins may form a large protein complex whose function has not been characterized (Smacznia et al., 2012). Therefore, it is also possible that, in parallel with H3K27me3 passive dilution, AG may recruit REF6 to the *KNU* promoter to actively remove H3K27me3. However, this hypothesis does not explain why cell cycle progression is required for AG to induce *KNU*. Also, the known mutants for these demethylases show no meristematic defects. Hence, we propose that REF6 might be involved in the regulation of some other direct downstream targets that are induced by AG.

To remove H3K27me3 marks and activate gene expression, other transcription factors or chromatin remodeling factors may perform functions similar to those that AG does. One such example is LFY in the control of the *AG* locus. For *AG* expression, the repressive mark H3K27me3 is removed by LEAFY (LFY), which recruits the SWI/SNF chromatin remodeling factors SPLAYED (SYD) and BRAHMA (BRM) on the *AG* second intron (Wu et al., 2012). Notably, GA-repeat motifs located near PREs are enriched at LFY targets (Wu et al., 2012; Zhang, 2014).

*WUS*, which is required in the organizing center to stimulate the maintenance of stem cell properties in the overlying cells (Yadav et al., 2011; Daum et al., 2014), is negatively regulated by Pcg-mediated H3K27me3 (Zhang et al., 2007). In the SAM, the *WUS-CLV* signaling pathway works to maintain an appropriately sized stem cell. The signaling pathway remains active in floral stem cells and works to maintain their identity. It is interesting that *WUS* is re-activated and the signaling pathway is re-established in the stage 1 floral primordia (Mayer et al., 1998) and that the SWI/SNF chromatin remodeling factor SYD plays an important role in *WUS* activation (Wagner and Meyerowitz, 2002; Kwon et al., 2005). In floral stage 6, *WUS* is terminated by *KNU* and later silenced by Pcg-mediated H3K27me3 marks (Sun et al., 2009; Liu et al., 2011). Because transcriptional repression of *WUS* and epigenetic silencing of *WUS* both occur at floral stage 6, we suggest that the transcriptional repressor *KNU* may integrate

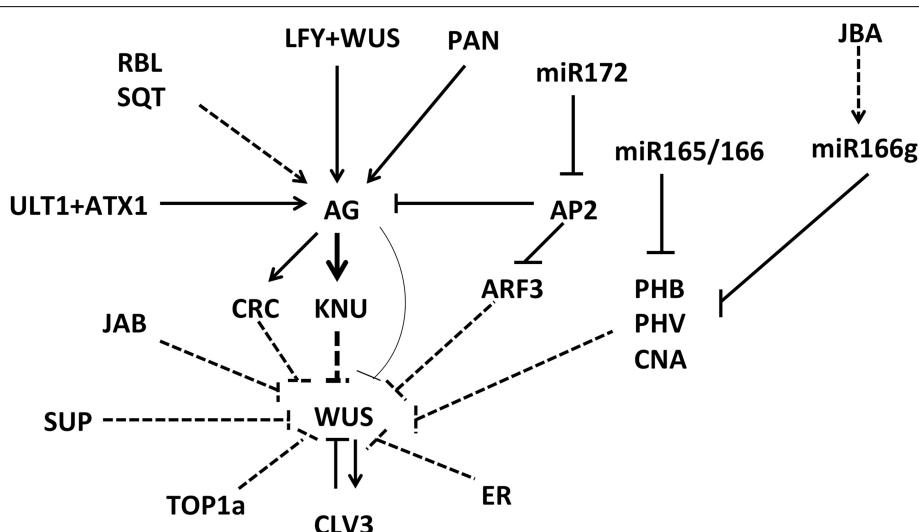
the two processes. During reproductive development, *WUS* is activated in developing stamens at stages 7–8, and later it is activated in developing ovules (Gross-Hardt et al., 2002; Deyhle et al., 2007). How the repressive mark H3K27me3 is removed from the *WUS* locus in those specific tissues and cell types is another open question that will require further investigation.

## OTHER FACTORS INVOLVED IN FLORAL MERISTEM REGULATION

In addition to the known *CLV-WUS* signaling pathway that is responsible for the spatial maintenance of the floral stem cell niche, and in addition to the *AG-KNU-WUS* pathway for the timed termination of floral stem cells, other factors are known to be required for fine-tuning floral stem cell activities (Figure 2).

ULTRAPETALA1 (ULT1), a SAND domain containing protein (Carles and Fletcher, 2009), functions to induce *AG* in floral stem cells in an LFY-independent manner (Engelhorn et al., 2014). *ULT1* may negatively regulate floral stem cell proliferation. The *ult1* mutant flowers have bigger floral meristems and prolonged *WUS* activity, resulting in five petals instead of the usual four (Fletcher, 2001). Thus, both genetic and molecular studies indicate that *ULT1* negatively regulates the *WUS* expressing domain in floral buds, potentially through the *AG-WUS* regulatory pathway (Carles et al., 2004). *ULT1* is reported to be a trithorax group (trxG) protein that can physically interact with another trxG protein, ATX1, a H3K4me3 methyltransferase (Alvarez-Venegas et al., 2003; Carles and Fletcher, 2009). By binding directly to *AG* regulatory sequences, *ULT1* may recruit ATX1 to actively modulate the methylation status of nucleosomes at the *AG* locus.

Two other factors, REBELOTE (RBL) and SQUINT (SQN), can redundantly regulate floral stem cells in addition to *ULT1* (Prunet et al., 2008). Reiterative reproductive floral organs are observed in flowers of the double mutants *rbl sqn*, *rbl ult1*, and *sqn ult1*. In the double mutant flowers, *WUS* activity is prolonged. Presumably, *RBL* and *SQN* both regulate the floral meristem by



**FIGURE 2 | Various factors involved in floral meristem control.** Many regulators control the expression of the stem cell identity gene *WUS* in direct and indirect ways. Solid lines indicate direct activation or repression, and dashed lines indicate a proposed type of regulation that has yet to be confirmed.

reinforcing *AG* expression. As a cyclophilin protein, *SQN* was recently found to bind the protein chaperone Hsp90 and promote microRNA activity via AGO1 (Earley and Poethig, 2011). The *sqn* single mutant displays increased carpel number relative to wild-type, and it has abnormal phyllotaxy of the flowers. This phenotype increased expression of *SPL* family transcription factors, which are targeted by the microRNA miR156 (Smith et al., 2009). *PERIANTHIA* (*PAN*), a bZIP transcription factor, also affects floral stem cell activity through direct activation of *AG* (Running and Meyerowitz, 1996; Chuang et al., 1999; Das et al., 2009; Maier et al., 2009). In *pan* mutant flowers, *AG* mRNA levels are reduced in short-day conditions, resulting in flowers with an increased number of floral organs. In addition, increased floral meristem indeterminacy is observed in *lfy pan* and *seuss (seu) pan* double mutant flowers. Ectopic floral organs continue to grow inside the fourth whorl floral organs of *lfy pan* and *seu pan* plants, suggesting a potential effect of the floral identity gene *LFY* and the adaptor-like transcriptional repressor *SEU* in floral meristem regulation (Das et al., 2009; Wynn et al., 2014).

*SUPERMAN* (*SUP*), which encodes a C2H2 zinc finger protein with a C-terminal EAR-like repression motif, is thought to function as a transcriptional repressor during flower development (Hiratsu et al., 2002). Loss-of-function mutants of *SUP* produce supernumerary stamens at the expense of carpels, indicating that *SUP* has a role in maintaining the boundary between the 3rd and 4th whorl floral organs (Sakai et al., 1995). Compared to the *ag-1* mutant flowers, flowers of the double mutant *ag-1 sup* produce greatly enlarged floral meristems, generating reiterating whorls of petals, indicating the role of *SUP* in floral stem cell regulation in parallel with *AG* (Bowman et al., 1992).

*CRABS CLAW* (*CRC*), which is a direct downstream target of *AG*, is reported to be involved in floral meristem control. Null mutants for *crc-1* do not show floral meristem defects; instead, the apical part of the mutant carpel is unfused. However, in combination with certain other mutants, supernumerary whorls of floral organs are observed; this occurs in *crc-1 spatula-2*, *crc-1 ag-1/+*, *crc-1 rbl-1*, *crc-1 sqn-4*, *crc-1 ult1-4*, *crc-1 pan-3* and *crc-1 jaiba* double mutant flowers (Prunet et al., 2008; Zuniga-Mayo et al., 2012). *CRC* encodes a YABBY family transcription factor, and its expression begins in floral stage 5–6 on the abaxial side of the carpel primordia. *CRC* may regulate *WUS* activity in a non-cell autonomous manner (Bowman and Smyth, 1999; Lee et al., 2005).

Various microRNAs are reported to be involved in floral meristem determinacy control. For instance, *miR172* promotes termination of floral stem cells by reducing the expression of its target, *AP2* (Chen, 2004). Over-expression of a *miR172*-resistant version of *AP2* (35S::*AP2m1/3*) leads to indeterminate stamens and petals (Chen, 2004; Zhao et al., 2007). The class III HD-ZIP genes, including *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*), are targeted by *miR165/166*. Over-expression of *miR165/166* in an *ag-10* background, a weak allele of *ag*, or alleles of *PHB* and *PHV* that are resistant to *miR165/166* can lead to indeterminate growth of floral organs (Ji et al., 2011). A proper balance of *PHB/PHV* and *mir165/166* is important for floral meristem determinacy control. Consistent with this, in the triple mutant of *phb phv cna*, floral carpel number is increased (Prigge et al., 2005). Similarly,

enlarged shoot meristems caused by increased *WUS* expression are observed in the *jabba1-D* mutant, a dominant allele of *JABBA* (*JBA*) that produces an increased amount of *miR166g* to regulate *PHB*, *PHV* and *CORONA* (*CNA*) expression (Williams et al., 2005).

The *ERECTA* (*ER*) receptor kinase-mediated regulation of *WUS* expression was recently reported to be mediated by a pathway parallel to the *WUS-CLV* pathway in both SAM and FM (Mandel et al., 2014). As a secondary signaling factor, *ER* works together with the nuclear protein *JBA* to repress *WUS*. In a *jba-1D/+ er-20* double mutant background, the SAM and floral meristem are greatly enlarged, and the spiral vegetative phyllotaxy switches to whorled patterns. In the *jba-1D/+ er-20* background, *AG* is ectopically expressed at a level that produces ectopic fused carpels from the inflorescence meristem, indicating an indirect role of *ER* in floral meristem identity control.

Recently, a mutation in the DNA topoisomerase gene *TOPOISOMERASE1a* (*TOP1a*) was shown to increase floral meristem indeterminacy in an *ag-10* background, as the *ag-10 top1a-2* double mutant exhibits an indeterminate floral meristem (Liu et al., 2014a). In floral stem cell regulation, *TOP1a* may function to reduce nucleosome density, thus facilitating Pcg-mediated H3K27me3 deposition on *WUS*. Mutations in another gene *AUXIN RESPONSE FACTOR 3* (*ARF3*), have also been reported to enhance the *ag-10* indeterminate phenotype (Liu et al., 2014b). Double mutant *ag-10 arf3-29* flowers produce additional floral organs that grow inside of the unfused sepaloid carpels, suggesting that *ARF3* may reinforce floral meristem determinacy through *WUS* repression. The *ARF3* locus is directly bound by *AP2*, indicating that *AP2*'s role in floral stem cell regulation is also partially mediated by *ARF3*.

## CONCLUSION

The complex regulatory network controlling floral meristem development produces elegant flowers with defined numbers and whorls of floral organs, thus ensuring that plant reproduction can occur (Figure 2). With knowledge of the spatial and temporal control of floral stem cells, as well as knowledge of the many factors responsible for fine-tuning floral stem cell activity, steady progress will be made in unraveling the mysteries of floral meristem regulation. Recently developed techniques, including ChIP-seq, RNA-seq, TALENs, CRISPR/Cas9, confocal live imaging and mathematical modeling, will help to provide further insights into the intriguing nature of flower development.

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# Epigenetic regulation of rice flowering and reproduction

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Current understanding of the epigenetic regulator roles in plant growth and development has largely derived from studies in the dicotyledonous model plant *Arabidopsis thaliana*. Rice (*Oryza sativa*) is one of the most important food crops in the world and has more recently become a monocotyledonous model plant in functional genomics research. During the past few years, an increasing number of studies have reported the impact of DNA methylation, non-coding RNAs and histone modifications on transcription regulation, flowering time control, and reproduction in rice. Here, we review these studies to provide an updated complete view about chromatin modifiers characterized in rice and in particular on their roles in epigenetic regulation of flowering time, reproduction, and seed development.

**Keywords:** chromatin, epigenetics, flowering time, histone modification, DNA methylation, non-coding RNA, reproduction, *Oryza sativa*

## INTRODUCTION

Epigenetics is defined as nucleotide sequence-independent changes in the gene expression that are mitotically and/or meiotically heritable. The fundamental repeating unit of chromatin is nucleosome. The nucleosome contains 145–147 base pairs (bp) of DNA wrapped around an octamer of histone proteins, comprising two copies of each of the four core histones, H2A, H2B, H3, and H4 (McGinty and Tan, 2014). The linker histone H1 associates with DNA in between the two nucleosomes and participates in higher order chromatin structure formation and remodeling. The structure of chromatin can be subjected to panoply of epigenetic regulations including DNA methylation, histone covalent modifications, histone variants, and ATP-dependent chromatin remodeling. DNA methylation has been widely considered as a heritable epigenetic mark that regulates expression of genes in both plants and mammals (Law and Jacobsen, 2010; Furner and Matzke, 2011; Wu and Zhang, 2014). Histone modifications including methylation, acetylation, phosphorylation, ubiquitination, and sumoylation, play critical roles in regulating chromatin structure and gene expression, mainly by altering nucleosome stability and positioning that affect DNA accessibility for regulatory proteins or protein complexes involved in transcription, DNA replication and repair (Pikaard and Scheid, 2013; To and Kim, 2014; Van Lijsebettens and Grasser, 2014). ATP-dependent chromatin remodeling factors control relocation or dissociation of nucleosomes, and histone chaperones bind histones and play crucial roles in nucleosome assembly/disassembly in diverse chromatin metabolism and epigenetic regulation (Zhu et al., 2012; Gentry and Hennig, 2014).

Rice (*Oryza sativa*) is a worldwide crop and represents a valuable model plant for monocots, to which many of our food crops belong. Compared to the extensively studied dicot model plant *Arabidopsis thaliana*, rice has only been more recently studied in epigenetic modifications (reviewed in Chen and Zhou,

2013). Genome-wide analyses of DNA methylations have revealed conservation as well as distinct differences between rice and *Arabidopsis*, and that a much higher level of DNA methylation is observed in association with more numerous transposable elements present in the rice genome (Yan et al., 2010; Zemach et al., 2010; Chodavarapu et al., 2012; Li et al., 2012). Genome-wide analyses by chromatin immunoprecipitation combined with high-throughput sequencing (ChIP-Seq) have shown that several types of histone modifications, e.g., histone H3 lysine 9 acetylation (H3K9ac) and H4K12ac, H3K4 di-/tri-methylation (H3K4me2/3), H3K27me3, and H3K36me3, are broadly distributed with distinct patterns within the rice genome (He et al., 2010; Malone et al., 2011; Du et al., 2013). In this review, we summarize and discuss regulators involved in different types of chromatin modifications and their roles in rice plant flowering time control and reproduction.

## REGULATION OF DIFFERENT TYPES OF CHROMATIN MODIFICATIONS IN RICE

Different types of chromatin modifications are regulated by specific factors that are generally conserved in rice and other plant species ([www.chromdb.org](http://www.chromdb.org)). So far, only some of the rice chromatin modifiers are functionally characterized by analysis of loss-of-function mutants and RNAi or overexpression transgenic plants (Table 1).

### DNA METHYLATION

In plants, DNA methylation occurs at cytosine residues in symmetric, CG and CHG, as well as asymmetric, CHH, contexts (where H = A, T or C; Law and Jacobsen, 2010). In *Arabidopsis*, CG methylation is maintained by METHYLTRANSFERASE 1 (MET1; Saze et al., 2003), whereas CHG methylation is mediated by CHROMOMETHYLASE 3 (CMT3; Lindroth et al., 2001). The maintenance of CHH methylation is carried

**Table 1 | Chromatin modifiers functionally characterized in rice.**

|                       | <b>Name</b>      | <b>Gene locus</b> | <b>Molecularfunction</b>             | <b>Biological role</b>                        | <b>Reference</b>   |
|-----------------------|------------------|-------------------|--------------------------------------|---|--|
| DNA methylation       | OsMET1b/OsMET1-2 | LOC_Os07g08500    | DNA methyltransferase                | Seed development                              | Hu et al. (2014), Yamauchi et al. (2014)                             |
|                       | OsDRM2           | LOC_Os03g02010    | <i>De novo</i> DNA methyltransferase | Pleiotropic effects on development            | Moritoh et al. (2012), Pang et al. (2013)                            |
|                       | OsDDM1           | LOC_Os09g27060    | DNA methylation maintenance          | Transposon repression, growth inhibition      | Higo et al. (2012)   |
| DNA demethylation     | OsROS1a          | LOC_Os01g11900    | DNA demethylase                      | Plant reproduction                            | Zemach et al. (2010), Ono et al. (2012)                              |
|                       | OsROS1c          | LOC_Os05g37350    | DNA demethylase                      | Transposon activation                         | La et al. (2011)   |
| Histone methylation   | SDG714           | LOC_Os01g70220    | H3K9 methyltransferase               | Transposon repression, trichome development   | Ding et al. (2007b)  |
|                       | SDG728           | LOC_Os05g41170    | H3K9 methyltransferase               | Transposon repression, seed development       | Qin et al. (2010)  |
|                       | SDG725           | LOC_Os02g34850    | H3K36 methyltransferase              | Hormone regulatory gene activation, flowering | Sui et al. (2012, 2013)  |
| Histone demethylation | SDG724           | LOC_Os09g13740    | H3K36 methyltransferase              | Flowering                                     | Sun et al. (2012)  |
|                       | SDG723/OsTrx1    | LOC_Os09g04890    | H3K4 methyltransferase               | Flowering                                     | Choi et al. (2014)   |
|                       | JMJ706           | LOC_Os10g42690    | H3K9 demethylase                     | Floral organ development                      | Sun and Zhou (2008)  |
| Polycomb silencing    | JMJ705           | LOC_Os01g67970    | H3K27 demethylase                    | Biotic stress response, plant reproduction    | Li et al. (2013)   |
|                       | JMJ703           | LOC_Os05g10770    | H3K4 demethylase                     | Stem elongation, transposon repression        | Chen et al. (2013), Cui et al. (2013)                                |
|                       | JMJ701           | LOC_Os03g05680    | H3K4 demethylase                     | Flowering                                     | Yokoo et al. (2014)  |
| Others                | OsiEZ1/SDG718    | LOC_Os03g19480    | H3K27 methyltransferase              | Flowering                                     | Liu et al. (2014)  |
|                       | OsCLF/SDG711     | LOC_Os06g16390    | H3K27 methyltransferase              | Flowering                                     | Liu et al. (2014)  |
|                       | OsFIE1           | LOC_Os08g04290    | <i>Drosophila</i> ESC homolog        | Pleiotropic effects on development            | Zhang et al. (2012b), Nallamilli et al. (2013), Folsom et al. (2014) |
| Histone deacetylation | OsFIE2           | LOC_Os08g04270    | <i>Drosophila</i> ESC homolog        | Organ generation, reproduction                | Luo et al. (2009), Li et al. (2014)                                  |
|                       | OsEMF2b          | LOC_Os09g13630    | <i>Drosophila</i> Su(z)12 homolog    | Floral organ development                      | Yang et al. (2013), Conrad et al. (2014)                             |
|                       | OsHDT1/HDT701    | LOC_Os05g51840    | H4 deacetylase                       | Biotic stress response, heterosis             | Li et al. (2011a), Ding et al. (2012a)                               |
| Others                | OsSRT1           | LOC_Os04g20270    | H3K9 deacetylase                     | Cell death, transposon repression             | Huang et al. (2007), Zhong et al. (2013)                             |
|                       | CHD3/CHR729      | LOC_Os07g31450    | Chromodomain and PHD-domain protein  | Pleiotropic effects on development            | Hu et al. (2012)   |
|                       | MEL1             | LOC_Os03g58600    | AGO-family protein                   | Meiosis progression                           | Nonomura et al. (2007), Komiya et al. (2014)                         |
| Others                | SHO1             | LOC_Os04g43050    | Homolog of DICER-LIKE 4              | Pleiotropic effects on development            | Abe et al. (2010)  |
|                       | SHL2             | LOC_Os01g34350    | RDR6 homolog                         | Floral organ development                      | Toriba et al. (2010)   |
|                       | WAF1             | LOC_Os07g06970    | HEN1 homolog                         | Pleiotropic effects on development            | Abe et al. (2010)  |
|                       | BRK1             | LOC_Os07g32480    | H2A phosphorylation                  | Meiosis progression                           | Wang et al. (2012)   |

out by CMT2 and DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), an ortholog of mammalian Dnmt3 (Law and Jacobsen, 2010; Stroud et al., 2014). DRM2 is required for *de novo* cytosine methylation in both symmetric and asymmetric sequence contexts, which is guided to the target region by RNA-directed DNA methylation (RdDM) pathway (Cao and Jacobsen, 2002; Law and Jacobsen, 2010; Stroud et al., 2014). While *Arabidopsis* contains only one *MET1* gene, rice has two *MET1* genes, *MET1a* (also named *OsMET1-1*) and *MET1b/OsMET1-2* (Teerawanchpan et al., 2004; Yamauchi et al., 2008). The transcripts of *MET1b* accumulate more abundantly than those of *MET1a* in all of the examined rice tissues, indicating that *MET1b* may play a more important role in maintaining DNA methylation (Yamauchi et al., 2008). Consistently, more recent studies demonstrate that *MET1b* is an essential gene and its loss causes genome-wide reduction of CG methylation in rice seedlings (Hu et al., 2014; Yamauchi et al., 2014). Rice contains also one *DRM2* gene, *OsDRM2*, and the recombinant OsDRM2 protein expressed in *Escherichia coli* or *Saccharomyces cerevisiae* exhibits stochastic *de novo* DNA methyltransferase activity *in vitro* at CG, CHG, and CHH (Sharma et al., 2009; Pang et al., 2013). Interestingly, OsDRM2 was found to interact with the ATP-dependent RNA helicase, OseIF4A, in both *in vitro* and *in vivo* assays (Dangwal et al., 2013). The interaction specifically depends on the ubiquitin-associated domain of OsDRM2, pointing to a mechanism in which OsDRM2 is recruited to specific chromatin sites by eIF4A together with other cellular proteins for catalyzing DNA methylation (Dangwal et al., 2013). Similar to the *Arabidopsis DECREASE IN DNA METHYLATION1 (DDM1)*, which encodes a nucleosome remodeling ATPase, *OsDDM1* is also necessary for maintenance of DNA methylation in transposons and repetitive sequences (Higo et al., 2012). The rice genome contains three putative CMT3 homologs (Sharma et al., 2009), yet their functions remain to be characterized.

DNA methylation can be removed passively through dilution during replication as well as actively through catalysis by demethylation enzymes (La et al., 2011; Ono et al., 2012). In *Arabidopsis*, active demethylation is catalyzed by REPRESSOR OF SILENCING 1 (ROS1; Gong et al., 2002; Agius et al., 2006), DEMETER (DME; Choi et al., 2002; Gehring et al., 2006), and DEMETER-LIKE 2 (DML2) and DML3 (Choi et al., 2002; Ortega-Galisteo et al., 2008). Phylogenetic analysis showed that the rice genome encodes six putative bi-functional DNA glycosylases that catalyze cytosine DNA demethylation: four ROS1 orthologs (ROS1a to ROS1d) and two DML3 orthologs (DML3a and DML3b), but no DME orthologs (Zemach et al., 2010). *ROS1c* has been shown to be involved in DNA demethylation and control of the retrotransposon *Tos17* activity (La et al., 2011). Quantitative RT-PCR analysis revealed that *ROS1a*, *ROS1d*, and *DML3a* are expressed in different examined plant tissues, including anthers and pistils, whereas *ROS1b* and *DML3b* are scarcely expressed in these tissues (Ono et al., 2012). Future studies are necessary to investigate the role of these different genes in rice genome DNA methylation.

## HISTONE METHYLATION

Histone methylation marks are established on lysine (K) and arginine (R) residues by distinct enzymes, namely histone lysine

methyltransferases (HKMTs) and protein arginine methyltransferases (PRMTs), respectively (Liu et al., 2010; Yao and Shen, 2011). In general, H3K9, H3K27, and H4K20 methylations are associated with transcriptional repression, whereas methylation on H3K4 and H3K36 correlates with gene activation. Furthermore, each K residue can be mono-, di-, or tri-methylated, and different methylation status may have different functional implications (Yu et al., 2009).

All known plant HKMTs contain an evolutionarily conserved SET domain (reviewed in Berr et al., 2011). The rice genome encodes at least 37 SET domain proteins, grouped into distinct families (Ng et al., 2007; Huang et al., 2011; Thorstensen et al., 2011). To date, several members belonging to different families are characterized (Table 1). Analyses of SET DOMAIN GROUP 714 (SDG714) and its close homologs (e.g., SDG728) showed that these rice SDG proteins have either specific or redundant functions in regulating histone H3K9 methylation and retrotransposon repression (Ding et al., 2007a,b, 2010; Qin et al., 2010). Knockdown of *SDG714* leads to decreased H3K9 methylation levels accompanied by a reduction of CG and CHG methylation, suggesting that H3K9 methylation and DNA methylation act closely together to stably repress the transposition of transposons to maintain genome stability (Ding et al., 2007b). Ectopic expression of *SDG714* in *Arabidopsis* can cause a global elevation of H3K9me2 (Ding et al., 2010). Knockdown of *SDG725* impairs deposition of H3K36me2/3 at several examined gene loci (Sui et al., 2012, 2013). SDG724 is also involved in H3K36me2/3 deposition (Sun et al., 2012). SDG723/OsTrx1 is a close homolog of the *Arabidopsis* H3K4-methyltransferase ATX1 and can methylate *in vitro* H3 within oligonucleosomes (Choi et al., 2014). The rice genome contains two genes encoding putative H3K27 methyltransferases, *OsiEZ1/SDG718* (also named *OsSET1*) and *OsCLF/SDG711*, which likely work in protein complexes in Polycomb silencing pathway (see Section below).

Histone lysine methylation can be removed by histone demethylases, which consist of two classes: Lysine Specific Demethylase 1 (LSD1) and Jumonji C (jmjC) domain-containing proteins (Tsukada et al., 2006; Mosammaparast and Shi, 2010). LSD1, a flavin-dependent amine oxidase, has been the first histone demethylase reported (Shi et al., 2004) and *Arabidopsis* contains three LSD1 homologs, which are involved in flowering time regulation (Jiang et al., 2007; Liu et al., 2007; Shafiq et al., 2014). Three rice genes (*Os02g0755200*, *Os04g0560300*, and *Os08g0143400*) encode LSD1 homologs, but their functions remain uncharacterized. There are at least 20 jmjC domain-containing proteins in rice, and the first characterized JMJ706 specifically demethylates H3K9me2/me3 (Sun and Zhou, 2008). More recently, several other rice jmjC-encoding genes have been characterized. *JMJ705* encodes a histone lysine demethylase that specifically removes H3K27me2/3, and the expression of *JMJ705* is induced by stress signals and during pathogen infection (Li et al., 2013). For active histone marks, *JMJ703* is involved in the removal of H3K4me1/me2/me3 (Chen et al., 2013; Cui et al., 2013), and *JMJ701* in removal of H3K4me3 (Yokoo et al., 2014). So far, however, histone demethylase(s) involved in removal of H3K36 methylation is(are) unknown.

## POLYCOMB SILENCING

Polycomb Group (PcG) proteins were first identified as master regulators and suppressors of homeotic genes in *Drosophila melanogaster*. Polycomb Repressive Complex 2 (PRC2) has four core components: ENHANCEROF ZESTE (E[z]), SUPPRESSOR OF ZESTE 12 (Su[z]12), EXTRA SEX COMBS (ESC), and the 55 kDa WD40-repeat protein N55 (Schuettengruber and Cavalli, 2009). PRC2 mediates H3K27me3 deposition via the catalytic subunit E[z], a SET-domain containing protein (Czernin et al., 2002). The four core subunits of the PRC2 complex are well conserved in animals as well as in plants (Chen and Rasmuson-Lestander, 2009; He et al., 2013). While in *Drosophila* all but one subunit is encoded by a single gene, most of the plant PRC2 core subunits are encoded by small gene families. In *Arabidopsis*, MEDEA (MEA)/FERTILIZATION INDEPENDENT SEED 1 (FIS1), CURLY LEAF (CLF), and SWINGER (SWN) are the three homologs of E[z]; FIS2, VERNALIZATION 2 (VRN2), and EMBRYONIC FLOWER 2 (EMF2) are the three homologs of Su[z]12; MULTICOPY SUPPRESSOR OF IRA1 (MSI1) to MSI5 are the five homologs of N55; and FERTILIZATION INDEPENDENT ENDOSPERM (FIE) is the only homolog of ESC. Remarkably, MEA/FIS1 and FIS2, which are important for endosperm and seed development in *Arabidopsis*, are absent from rice, and rice has two E[z] homologs: OsIEZ1/SDG718 and OsCLF/SDG711, two Su[z]12 homologs: OsEMF2a and OsEMF2b, but also two FIE homologs: OsFIE1 and OsFIE2 (Luo et al., 2009). Functional roles of some of these rice PcG proteins have been characterized (Table 1). The expression of OsIEZ1/SDG718 and OsCLF/SDG711 is induced by and represses flowering genes in long day and short day, respectively (Liu et al., 2014). While OsFIE2 is expressed broadly in all examined rice tissues, OsFIE1 is expressed specifically in the rice endosperm and its expression in vegetative tissues is likely to be silenced by promoter DNA methylation (Zhang et al., 2012b; Nallamilli et al., 2013). Furthermore, OsFIE1 is imprinted and only the maternal allele is expressed in endosperm (Luo et al., 2009). More recently, it was reported that OsFIE1 is responsive to temperature changes and its expression negatively correlates with the duration of the syncytial seed developmental stage during heat stress (Folsom et al., 2014). DNA methylation, H3K9me2 and/or H3K27me3 are likely involved in regulation of varied repressive status of OsFIE1 (Zhang et al., 2012b; Nallamilli et al., 2013; Folsom et al., 2014). Functional characterization of OsEMF2b revealed that PRC2 plays a major role in modulation of the expression of E-function MADS-box transcription factor genes required for floral organ specification and floral meristem determinacy (Luo et al., 2009; Yang et al., 2013; Conrad et al., 2014). Very importantly, OsFIE2 interacts with OsIEZ1/SDG718 and the OsFIE2-associated complex purified from transgenic rice suspension cells (containing OsEMF2b, OsCLF, OsIEZ1/SDG718) can methylate H3K27 in *in vitro* histone methyltransferase assay (Nallamilli et al., 2013).

## HISTONE ACETYLATION

Histone lysine acetylation is generally associated with transcription activation and is dynamically regulated by the antagonistic activities between histone acetyltransferases (HATs) and histone deacetylases (HDACs; Chen and Tian, 2007). All four core histones

can be acetylated and a nucleosome contains 26 putative acetylation sites (Lusser et al., 2001). Global analysis of lysine acetylation demonstrates the involvement of protein acetylation in diverse biological processes in rice (Nallamilli et al., 2014). The rice genome contains eight HATs and 19 HDACs (Hu et al., 2009; Liu et al., 2012). The eight HATs can be divided into four groups, namely the CREB-Binding Protein (CBP) group, the TAFII-associated factor (TAFII250) group, the GCN5-related N-terminal acetyltransferase (GNAT) group, and the MYST (named for the founding members MOZ, Ybf2/Sas3, Sas2, and Tip60) group (Liu et al., 2012). The 19 HDACs are grouped into three distinct families, namely the Reduced Potassium Deficiency 3 (RPD3) family, the Silent Information Regulator 2 (SIR2) family, and the type-II HDAC (HD2) family which is plant specific (Ma et al., 2013). Reversible and dynamic changes of H3 acetylation occurs at submergence-inducible genes, *alcohol dehydrogenase 1 (ADH1)* and *pyruvate decarboxylase 1 (PDC1)* in rice (Tsuiji et al., 2006). Forward genetic analysis has identified a rice mutant, *rice plasticity 1 (rpl1)*, which displays increased environment-dependent phenotypic variations and an elevation of overall H3K9 acetylation (Zhang et al., 2012a). Down-regulation of *OsHDT1/HDT701*, which encodes a histone H4 deacetylase, causes elevated levels of H4 acetylation and increased transcription of pattern recognition receptor (PRR) and defense-related genes (Ding et al., 2012a). Knockdown of *OsSRT1*, a member of SIR2-like HDAC family, results in an increase of H3K9 acetylation (H3K9ac), leading to DNA fragmentation and cell death, and the *OsSRT1* protein binds to loci with relative low level of H3K9ac and regulates expression of many genes related to stress and metabolism as well as several families of transposable elements (Huang et al., 2007; Zhong et al., 2013).

## READERS OF HISTONE MODIFICATIONS

Specific recognition of histone modifications by readers can recruit various components of the nuclear signaling network to chromatin, mediating fundamental processes such as gene transcription, DNA replication and recombination, DNA repair and chromatin remodeling (Musselman et al., 2012). Some readers are reported in *Arabidopsis* (reviewed in Berr et al., 2011), and more recent works have identified several novel chromodomain (CHD)- and/or plant homeodomain (PHD)-containing proteins as readers of H3K4me2/me3 and H3K36me3 (Bu et al., 2014; Lopez-Gonzalez et al., 2014; Molitor et al., 2014; Xu et al., 2014). Interestingly, the rice CHD3 protein can bind both the active mark H3K4me2 and the repressive mark H3K27me3 via its CHD and PHD domain, respectively (Hu et al., 2012). Knockdown of CHD3 caused reduction of H3K4me3 and H3K27me3 at many genes. It was thus suggested that the rice CHD3 may act as a bifunctional reader capable to recognize and modulate both H3K4 and H3K27 methylations (Hu et al., 2012).

## SMALL AND LONG NON-CODING RNAs

Non-coding small RNAs (sRNA) of 21–24 nucleotides (nt) in length as well as long non-coding RNAs (lncRNAs, >200 nt in length) are known to be involved in chromatin modifications and thus epigenetic inheritance (reviewed in Castel and Martienssen, 2013; Bond and Baulcombe, 2014). Genome-wide

profiling have identified several hundreds of different sRNAs, and differences exist at their expression levels between different rice subspecies, reciprocal hybrids, different plant tissues, and under different growth conditions (Chen et al., 2010; He et al., 2010; Jeong et al., 2010; Zhang et al., 2014). Remarkably, the most abundant sRNAs identified in rice panicles are 24 nt in length and mainly correspond to transposon-associated or repeat-associated small interfering RNAs (siRNAs; Jeong et al., 2011). The most intriguing role of siRNAs is in repression of transposons and repeat elements in reproductive tissues and epigenomic reprogramming during gametogenesis (Gutierrez-Marcos and Dickinson, 2012; Castel and Martienssen, 2013; Bond and Baulcombe, 2014). ARGONAUTE (AGO) proteins play important roles in microRNA-mediated post-transcriptional gene silencing (PTGS) and siRNA-mediated RdDM (Vaucheret, 2008). A germ line specific AGO-encoding gene, *MEIOSIS ARRESTED AT LEPTOTENE1* (*MEL1*), has been reported in rice, and the *mel1* mutant shows chromosome abortion during early meiotic stages, leading to impaired male and female fertilities (Nonomura et al., 2007). More recently, forward genetic analysis has identified a lncRNA, which could be subsequently processed to small RNAs, as a key regulator of male fertility in rice (Ding et al., 2012b,c). Meanwhile, Zhou et al. (2012) reported that a spontaneous mutation of a small RNA could cause male sterility in rice. Nevertheless, the precise role of lncRNA and sRNA, particularly at rice chromatin structure levels, requires future investigations.

## EPIGENETIC REGULATION OF RICE FLOWERING

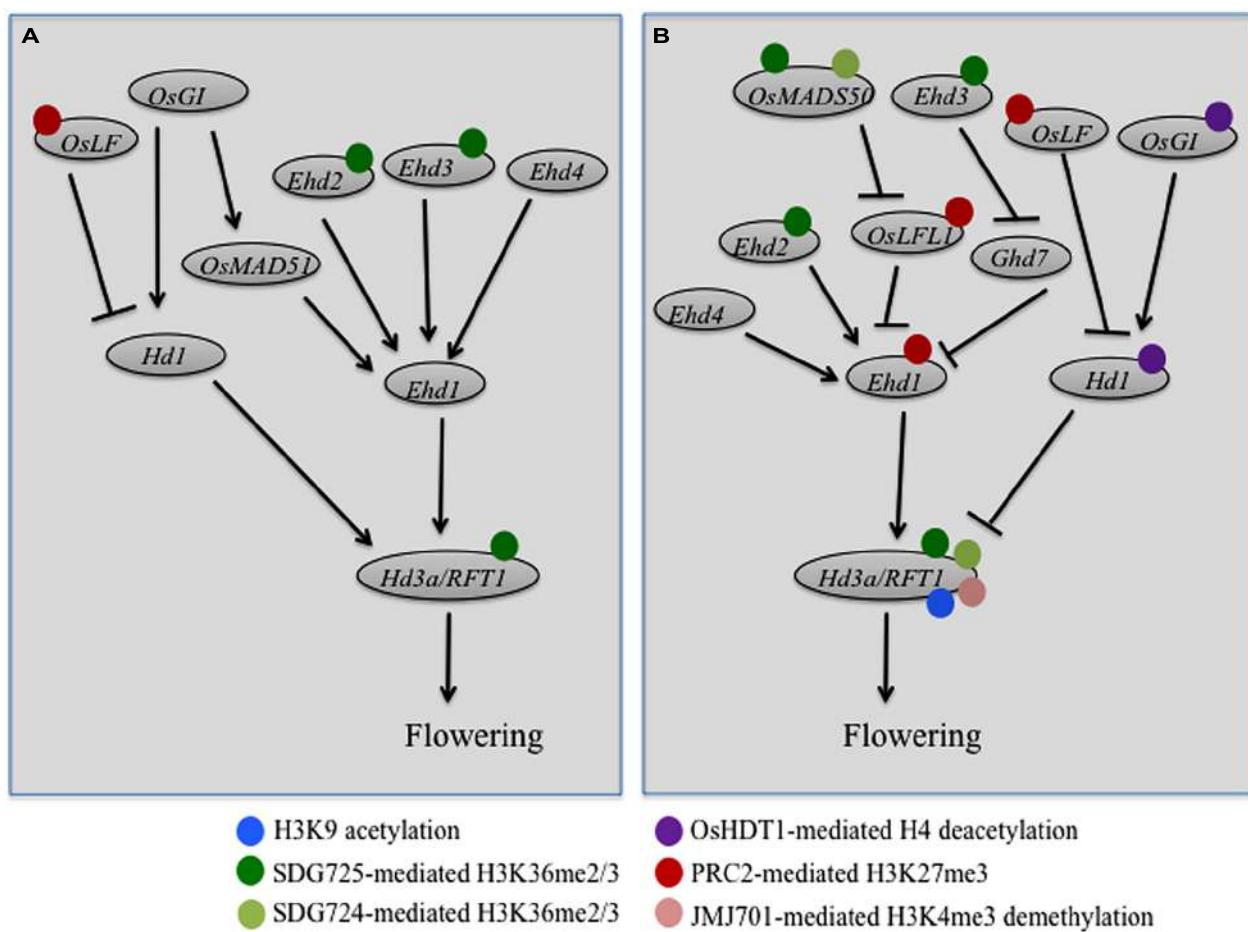
Flowering represents the transition from vegetative to reproductive growth, a key developmental switch during the plant life cycle. Flowering time is precisely controlled by complex gene network that integrates environmental signals, such as day length (photoperiod), light intensity and quality, and ambient temperature, as well as endogenous cues involving plant hormones (Albani and Coupland, 2010; Shrestha et al., 2014). Photoperiod is one of the most predictable cues in nature, and according to photoperiod responsiveness plants can be categorized into three groups: long-day (LD) plants, short-day (SD) plants, and day-neutral plants. *Arabidopsis* is a facultative LD plant whose flowering is accelerated when grown under LD photoperiods. Furthermore, flowering of most *Arabidopsis* ecotypes is promoted by a prolonged exposure to the cold of winter (a process known as vernalization), which has an epigenetic basis of competence memory (Ream et al., 2012; Song et al., 2012). During recent years, many chromatin modifiers have been shown as involved in *Arabidopsis* flowering time regulation, with majority of them acting via the transcriptional regulation of *FLOWERING LOCUS C* (*FLC*), a key flowering repressor at which vernalization and autonomous pathways converge (Berr et al., 2011; He, 2012; Letswaart et al., 2012). In contrast to *Arabidopsis*, rice is a facultative SD plant and does not require vernalization to induce flowering and does not contain a *FLC* homolog. The complex gene network of rice flowering pathways primarily consists of flowering activators, and remarkably several chromatin modifiers have been shown recently as involved in rice flowering time control (Figure 1).

## KEY TRANSCRIPTION FACTORS OF RICE FLOWERING PATHWAYS

Within the rice flowering pathways, the close paralogs *Heading date 3a* (*Hd3a*) and *RICE FLOWERING LOCUS T1* (*RFT1*) are specifically upregulated upon the inductive SD photoperiods in leaf phloem tissue and encode small globular proteins named florigens, which move to the shoot apex to promote flowering (Tsuji et al., 2013; Sun et al., 2014). There are at least two pathways that control the *Hd3a/RFT1* expression under either SD (Figure 1A) or LD (Figure 1B) photoperiods: the *Early heading date 1* (*Ehd1*) and the *Hd1* pathways (Tsuji et al., 2013; Sun et al., 2014). *Ehd1* encodes a B-type transcription factor that plays a key role in activation of both *Hd3a* and *RFT1* expression. The expression of *Ehd1* is modulated by at least three different types of function factors (Sun et al., 2014). The first type comprises day length-independent activators, including *Ehd2*, also known as *Rice Indeterminate1* (*RID1*) or *Os Indeterminate1* (*OsId1*), and *Ehd4*, which encode two different zinc-finger transcription factors and act in both SD and LD conditions in *Ehd1* induction (Figure 1). The second type comprises SD-preferential activators, including the PHD-finger factor *Ehd3* and the MADS-box family transcription factor *OsMADS51*, which induce *Ehd1* expression specifically in SD conditions (Figure 1A). And the third type comprises LD-preferential repressors, including *Grain number, plant height, and heading date7* (*Ghd7*) that encodes a CCT-domain protein and *LEC2-FUSCA3-Like 1* (*OsLFL1*) that encodes a B3-type transcription factor, both repress *Ehd1* expression specifically in LD conditions (Figure 1B). Further upstream, the LD-preferential regulator *OsMADS50* promotes flowering via repression of *OsLFL1*. Interestingly, *Ehd3*, which acts as an activator of *Ehd1* to promote flowering in SD conditions (Figure 1A), displays a repressor function on *Ghd7* and thus also promotes flowering in LD conditions (Figure 1B). The rice circadian clock related protein *GIGANTEA* (*OsGI*) activates the *Ehd1* pathway partly via induction of *OsMAD51* expression (Figure 1B). While the *Ehd1* pathway is more unique to rice, the *OsGI-Hd1-Hd3a* pathway is very similar to the *Arabidopsis* GI-CO-FT pathway, composing of the respective orthologous proteins in the two plant species (Tsuji et al., 2013; Sun et al., 2014). An atypical helix-loop-helix (HLH) protein (*OsLF*) also is involved in the *OsGI-Hd1-Hd3a* pathway via *Hd1* repression. *Hd1* acts as an activator to promote rice flowering in SD conditions (Figure 1A) but as a suppressor of rice flowering in LD conditions (Figure 1B). Phytochrome signaling is crucial in conversion of *Hd1* activity because mutation of *Phytochrome B* (*PHYB*) or phytochrome deficiency (e.g., in *photoperiod sensitivity5* mutant) maintains *Hd1* as an activator independent of day length. Under LD conditions, the red-light photoreceptor *PHYB* pathway may convert and maintain *Hd1* as a repressor possible via post-translational modification and/or protein complex formation. Because of space limitation, the one who is interested in more details about rice flowering pathways can read the two excellent review articles here cited (Tsuji et al., 2013; Sun et al., 2014) and the original research papers referred therein.

## ACTIVE CHROMATIN MARKS ARE INVOLVED IN RICE FLOWERING TIME REGULATION

Understanding how the rice flowering pathway genes are regulated in the chromatin context has great importance. Recent studies have



**FIGURE 1 |** Regulatory networks of genetic and epigenetic control of rice flowering under short-day (A) and long-day (B) photoperiod conditions.

Rice flowering network is integrated by two florigen genes *Hd3a* and *RFT1*, which are regulated by at least two pathways: the *Hd1*-dependent and the *Ehd1*-dependent pathways. Expressions of *Hd1* and *Ehd1* are further

regulated by more upstream genes as indicated by different names in the circles. Arrows indicate for transcriptional activation, whereas bars indicate for transcriptional repression. Different color spheres surrounding the flowering gene circles indicate for different regulations by the indicated histone modifications at the gene locus, currently described in literatures.

found that histone acetylations, H3K4 and H3K36 methylations are involved in active transcription of several genes within the rice flowering pathways (Figure 1). It was reported that overexpression of the HD2-family HDAC gene *OsHDT1* in hybrid rice leads to early flowering under LD conditions, probably through transcriptional repression of *OsGI* and *Hd1* (Li et al., 2011a). Interestingly, the expression of *OsHDT1* displays a circadian rhythm under SD conditions, peaked at the end of day, which coincides with rhythmic expression of *OsGI* and advances that of *Hd1*. Ectopic *OsHDT1* expression in transgenic rice attenuates the overdominance rhythmic expression of *OsGI* and *Hd1* in hybrid rice, which may explain the early flowering phenotype specifically observed in hybrid but not parental rice lines (Li et al., 2011a). Histone H4 acetylation levels were observed to positively correlate with the rhythmic expression of *OsGI* and *Hd1*, and *OsHDT1* overexpression was shown to impair the acetylation increase at the peak time (Li et al., 2011a).

A positive DNA/histone methylation role in rice flowering promotion was first indicated by the study of the

S-adenosyl-L-methionine synthetase gene mutants (Li et al., 2011b). S-Adenosyl-L-methionine is a universal methyl group donor for both DNA and protein methylations. Its deficiency caused late-flowering of rice plants and reduction of *Ehd1*, *Hd3a*, and *RFT1* expression, which is associated with reduced levels of H3K4me3 and DNA CG/CHG-methylations at these flowering gene loci (Li et al., 2011b). More recently, it was reported that suppression of *OsTrx1*, an ortholog of the *Arabidopsis* H3K4-methyltransferase gene *ATX1*, delays rice flowering time under LD conditions (Choi et al., 2014). The *OsTrx1* suppression did not affect the *OsMADS50* and *Hd1* pathways, but elevated *Ghd7* expression and drastically reduced *Ehd1*, *Hd3a* and *RFT1* expression, which is consistent with the plant late-flowering phenotype (Figure 1B). The PHD domain of *OsTrx1* can bind to native histone H3 and the SET domain of *OsTrx1* can methylate histone H3 from oligonucleosomes *in vitro* (Choi et al., 2014). Yet the role of *OsTrx1* in histone methylation *in vivo* remains undemonstrated. Because the *OsTrx1* and *Ehd3* proteins bind each other, the authors propose that *OsTrx1* may promote rice flowering via interaction

with Ehd3 (Choi et al., 2014). Mutant characterization of *Photoperiod sensitivity-14* (*Se14*), which encodes the JmjC-domain protein JMJ701, revealed that H3K4me3 elevation at the *RFT1* promoter region increases *RFT1* expression, leading to rice plant early flowering under LD conditions (Yokoo et al., 2014). It is currently unknown whether or not OsTrx1 and JMJ701 could work as a couple in an antagonistic manner to control H3K4me3 levels at the *RFT1* locus.

H3K36me3 is generally considered as acting more downstream of H3K4me3 during transcription processes (Berr et al., 2011). The first H3K36-methyltransferase characterized in rice is SDG725, which has been shown to specifically methylate H3K36 from mononucleosomes *in vitro* and is required for H3K36me2/me3 deposition at chromatin regions of genes related to brassinosteroid biosynthesis or signaling pathways (Sui et al., 2012). Knockdown of *SDG725* caused a rice plant late-flowering phenotype (Sui et al., 2012), and subsequent investigation revealed that SDG725 is necessary for H3K36me2/3 deposition at several flowering genes including *Ehd3*, *Ehd2*, *OsMADS50*, *Hd3a*, and *RFT1* (Sui et al., 2013). Characterization of the late-flowering mutant named *long vegetative phase 1* (*lvp1*) together with map-based cloning has uncovered *SDG724* as an essential regulator of the *OsMADS50-Ehd1-RFT1* pathway (Sun et al., 2012). The recombinant SDG724 protein can methylate H3 (with K site undetermined) from oligonucleosomes and the *lvp1* mutant plants show global reduction of H3K36me2/me3 levels. Remarkably, ChIP analysis revealed specific reduction of H3K36me2/me3 at *OsMADS50* and *RFT1* but not at *Ehd1* and *Hd3a* in the *lvp1* mutant plants (Sun et al., 2012). Both the *lvp1* (*sdg724*) mutant and the SDG725-knockdown mutant exhibit late-flowering phenotypes under either SD or LD conditions (Sun et al., 2012; Sui et al., 2013), pointing to a crucial role of H3K36me2/me3 in promoting rice plant flowering irrespective of photoperiods. It is noteworthy that in *Arabidopsis* the SDG8-mediated H3K36me2/me3 also plays a major role in flowering time control, but in that case in prevention of early flowering (Shafiq et al., 2014). Future studies are necessary to investigate mechanisms underlying the overlap and specific targets of SDG724 and SDG725 in the rice flowering time control.

#### REPRESSIVE CHROMATIN MARKS ARE INVOLVED IN RICE FLOWERING TIME REGULATION

The repressive mark H3K27me3 is known to play a key role in *FLC* repression in vernalization-induced *Arabidopsis* plant flowering (He, 2012; Letswaart et al., 2012). Interestingly, recent studies have shown that H3K27me3 deposited by PRC2-like complexes also plays an important role in vernalization-independent rice flowering time control (Figure 1). Loss-of-function of the PRC2 gene *OsEMF2b* causes late-flowering, which is associated with an increase of *OsLFL1* expression and a decrease of *Ehd1* expression (Yang et al., 2013). The *OsEMF2b* protein physically interacts with *OsVIL3* (named as *OsVIL2* in Yang et al., 2013, but here corrected to the first nomenclature used in Zhao et al., 2010; also called LC2), a PHD-domain protein showing homologies to the *Arabidopsis* VIN3-group proteins including VERNALIZATION INSENSITIVE 3 (VIN3), VIN3-LIKE 1 (VIL1)/VRN5, and VIL2/VEL1. The *Arabidopsis* VIN3-group proteins are known to be associated and to work together with the PRC2 core complex (constituting

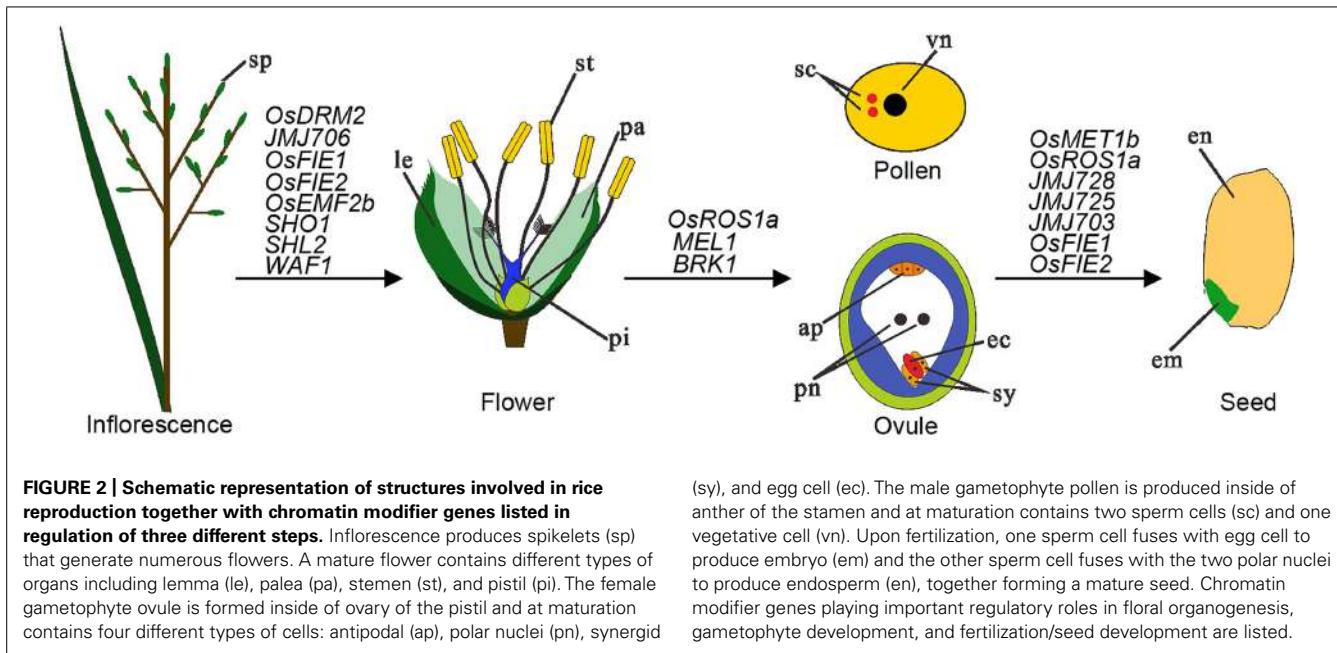
the so-called PHD-PRC2 complexes) and the *VIN3* expression is induced early during vernalization (reviewed in He, 2012; Letswaart et al., 2012). Consistent with the idea that *OsVIL3/LC2* works together with PRC2, knockdown of *OsVIL3/LC2* results in rice late-flowering, increase of *OsLFL1* and *OsLF* expression, and decrease of *Ehd1* as well as *Hd3a* and *RFT1* expression (Wang et al., 2013; Yang et al., 2013). The *OsVIL3/LC2* protein binds at the *OsLFL1* and *OsLF* chromatin regions and the H3K27me3 enrichments at *OsLFL1* and *OsLF* are impaired in the *osvil3/lc2* mutant (Wang et al., 2013; Yang et al., 2013). In addition to *OsVIL3/LC2*, *OsVIL2* plays a similar but non-redundant role in rice flowering time control. Expression of both *OsVIL3/LC2* and *OsVIL2* is induced by SD conditions and the *OsVIL3/LC2* and *OsVIL2* proteins physically interact, thus leading to the proposition that the *OsVIN3/LC2-OsVIL2* dimer may recruit PRC2 in H3K27me3 deposition and *OsLF* suppression in rice photoperiod flowering regulation (Wang et al., 2013). Very recently, *OsiEZ1/SDG718* and *OsCLF/SDG711* have been reported to display distinct roles in photoperiod regulation of flowering (Liu et al., 2014). While *OsiEZ1/SDG718* is induced in SD conditions and represses *OsLF* to promote flowering (Figure 1A), *OsCLF/SDG711* is induced in LD conditions and represses *OsLF* and *Ehd1* to inhibit flowering (Figure 1B). The *OsCLF/SDG711* protein has been shown to target *OsLF* and *Ehd1* loci to mediate H3K27me3 deposition and gene repression (Liu et al., 2014).

#### EPIGENETIC REGULATION OF RICE REPRODUCTION AND SEED FORMATION

After flowering, plant sexual reproduction occurs in dedicated floral organs through sporogenesis, gametogenesis, embryo- and endosperm-genesis, resulting in seed formation. Studies in *Arabidopsis* have unraveled diverse epigenetic regulatory mechanisms as involved in different processes during floral organogenesis and plant sexual reproduction (Shen and Xu, 2009; Engelhorn et al., 2014; She and Baroux, 2014). Although more recent, studies in rice also have started to uncover multiple types of epigenetic modifiers involved in the regulation of plant reproduction (Figure 2).

#### EPIGENETIC REGULATION IN RICE REPRODUCTION

Compared to those of *Arabidopsis*, the rice inflorescence and flower have greatly diverged structures that are regulated by a conserved genetic framework together with rice specific genetic mechanisms (Yoshida and Nagato, 2011). Several epialleles are found to affect rice plant reproduction. The metastable epigenetic silencing of *DWARF1*, which is associated with DNA methylation and H3K9me2 at the gene promoter region, causes dwarf tillers, compact panicles (inflorescences) and small round rice grains (Miura et al., 2009). The *abnormal floral organ* (*afo*) epimutation causes increased DNA methylation and suppression of the transcription factor gene *OsMADS1*, leading to pseudovivipary, a specific asexual reproductive strategy (Wang et al., 2010). The transcription factor gene *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 14* (*SPL14*), also known as *IDEAL PLANT ARCHITECTURE 1* (*IPA1*) or *WEALTHY FARMER'S PANICLE* (*WFP*), promotes panicle branching and regulates a large number of genes, and differences in DNA methylation at the locus as well as the micro RNA 156 (*OsmiR156*) contribute to expression differences of



*SPL14/IPA1/WFP* in different rice varieties (Jiao et al., 2010; Miura et al., 2010; Lu et al., 2013). Important roles of sRNA (both miRNAs and siRNAs) in rice floral organ development are also evidenced by mutants of several sRNA-pathway genes, including *SHOOT ORGANIZATION 1 (SHO1)* encoding a DICER-LIKE 4 homolog, *SHOOTLESS 2 (SHL2)* encoding a RDR6 homolog, and *WAVY LEAF 1 (WAF1)* encoding a HEN1 homolog (Abe et al., 2010; Toriba et al., 2010). lncRNAs are also reported as involved in plant reproductive process (Swiezewski et al., 2009; Heo and Sung, 2011). In rice, a point mutation that alter the secondary structure of the lncRNA called Long-Day-specific Male-fertility-Associated RNA (LDMAR) has been found to cause the photoperiod sensitive male sterility (Ding et al., 2012b).

Importance of DNA methylation in regulation of rice reproduction has been further supported by mutant studies. Targeted disruption of the DNA demethylase gene *ROS1a* leads to paternal allele transmission defect, presumably because of a male gametophytic defect prior to fertilization (Ono et al., 2012). Disruption of *OsDRM2* led to pleiotropic developmental defects in both vegetative and reproductive stages including semi-dwarfed stature, reductions in tiller number, and complete sterility (Moritoh et al., 2012). Consistently, transcriptome analysis of isolated rice gametes by deep sequencing indicates that *OsDRM2* is expressed in male cells but low in vegetative cells (Anderson et al., 2013).

Several modifiers of histone modifications are also critical for rice reproduction (Figure 2). Loss-of-function of the rice PRC2 gene *OsEMF2b* results in complete sterility, and severe floral organ defects and indeterminacy that resemble loss-of-function mutants in E-class floral organ specification genes (Conrad et al., 2014). The epimutation of *OsFIE1 (Epi-df)* that is caused by DNA hypomethylation, reduced H3K9me2 and increased H3K4me3 at the gene locus, leads to ectopic expression of *OsFIE1*, resulting in a dwarf stature, diverse floral defects, and alteration of H3K27me3 levels

(sy), and egg cell (ec). The male gametophyte pollen is produced inside of anther of the stamen and at maturation contains two sperm cells (sc) and one vegetative cell (vn). Upon fertilization, one sperm cell fuses with egg cell to produce embryo (em) and the other sperm cell fuses with the two polar nuclei to produce endosperm (en), together forming a mature seed. Chromatin modifier genes playing important regulatory roles in floral organogenesis, gametophyte development, and fertilization/seed development are listed.

at hundreds of target genes (Zhang et al., 2012b). Mutation of the H3K27-demethylase gene *JMJ705* also causes partial sterility (Li et al., 2013). The *OsFIE2* RNAi lines display pleiotropic phenotypes including vegetative and reproductive organ formation, a decreased amount of pollen grains and a high proportion of male sterility (Li et al., 2014). These studies indicate that a balanced level of H3K27me3 is critical and that either its increase or decrease can cause rice reproduction defects. The other chromatin repressive mark H3K9me2/me3 is also important because mutation of the H3K9-demethylase gene *JMJ706* impairs spikelet development, including defective floral morphology and altered organ number (Sun and Zhou, 2008). Pleiotropic defective phenotypes including panicle morphology, rachis branch and spikelet numbers have also been described for mutants of the H3K36-methyltransferase gene *SDG725* and the H3K4-demethylase gene *JMJ703* (Sui et al., 2012; Cui et al., 2013), indicating that chromatin active marks also play important function during rice reproduction.

While precise reproduction processes affected by many of the above mentioned modifiers remain to be elucidated, meiosis is found to be regulated by several epigenetic factors. The rice germline-specific AGO-family protein *MEL1* binds preferentially 21 nt siRNAs derived mostly from intergenic regions (Komiyama et al., 2014), and its loss-of-function impairs both sporophytic germ-cell development and meiosis (Nonomura et al., 2007). The *mell1* mutant displays aberrant vacuolation of spore mother cells, and arrested chromosome condensation at early meiosis stages. H3K9me2 distribution as well as the localization of ZEP1, a component of transverse filaments of the rice synaptonemal complex, are affected in *mell1*, indicating for a role of *MEL1* in chromatin structure organization and homologous chromosome synapsis in early meiosis (Nonomura et al., 2007; Komiyama et al., 2014). Histone phosphorylation is also involved in rice meiosis process. The rice Bub1-Related Kinase 1 (BRK1) is required for H2A phosphorylation and the centromeric recruitment of SHUGOSHIN 1 (SGO1),

which is likely essential for generating proper tension between the homologous kinetochores at metaphase I to facilitate the accurate segregation of homologous chromosomes at anaphase I (Wang et al., 2012).

### EPIGENETIC REGULATIONS IN SEED DEVELOPMENT

Like other angiosperms, sexual double fertilization initiates rice seed development, giving rise to two fertilization products, the embryo and the endosperm. Epigenetic mechanisms are thought to have important contribution to plant hybrid vigor (heterosis), a phenomenon referring to the increased yield and biomass of hybrid offspring relative to the parents (Chen and Zhou, 2013; Groszmann et al., 2013). In line with this idea, divers epigenetic pathways are found as involved in seed development and seed quality control (**Figure 2**).

Genome-wide analyses in rice have revealed that sRNA expression, DNA methylation, and histone modifications (e.g., H3K9ac, H3K4me3, and H3K27me3) significantly differ between hybrids and their parents (He et al., 2010; Chodavarapu et al., 2012; Zhang et al., 2014). Remarkably, the amount of 24 nt siRNAs, with most of them likely involved in regulation of the starch and sucrose biosynthesis pathway, declines with the process of rice grain-filling and this decline is to a lower degree in inferior grains than superior grains (Peng et al., 2013). The siRNAs may act *via* or together with DNA methylation in heterochromatin silencing. In line with this idea, the maternal loss of *ROS1a* causes failure of early stage endosperm development, leading to incomplete embryogenesis producing irregular but viable embryos that failed to complete seed dormancy (Ono et al., 2012). While the *met1a* null mutant displays a normal phenotype, the *met1b* mutant exhibits abnormal seed phenotypes, which is associated with either viviparous germination or early embryonic lethality (Hu et al., 2014; Yamauchi et al., 2014). Levels of DNA methylation in *met1b* are broadly reduced at genome-wide scale and in particular at repetitive centromeric and transposon sequences as well as at the *OsFIE1* gene locus in the embryos (Hu et al., 2014; Yamauchi et al., 2014).

*OsFIE1* is an imprinted gene in rice endosperm but the *osfie1* mutant does not display any autonomous endosperm proliferation without fertilization, differing from the *Arabidopsis fie*, *mea* and *fis* mutants that are generally recognized with an autonomous endosperm proliferation phenotype (Luo et al., 2009). Nevertheless, over-expression of *OsFIE1* causes precocious cellularization and reduced seed size, and it has been proposed that that *OsFIE1* has a role in regulating seed enlargement under heat stress (Folsom et al., 2014). In addition, *OsFIE2* has a critical role in normal endosperm development and grain-filling. Down-regulation of *OsFIE2* results in small seeds and partial loss of seed dormancy, likely because of down-regulation of genes encoding the starch synthesis rate limiting step enzymes and multiple storage proteins (Nallamilli et al., 2013). Future studies are necessary to precise similarities and differences of PRC2-mediated H3K27me3 repression mechanisms involved in seed development between *Arabidopsis* and rice.

Involvement of other histone methylation marks in seed development are also evidenced from mutant studies (**Figure 2**). Down-regulation of the H3K9-methyltransferase gene *SDG728* reduces seed size and alters seed morphology (Qin et al., 2010).

Loss-of-function of the H3K4-demethylase gene *JMJ703* causes abnormal grain phenotypes, including reduced length, width, and thickness (Cui et al., 2013). Also, knockdown of the H3K36-methyltransferase gene *SDG725* results in small seed size and reduced seed weight (Sui et al., 2012).

### CONCLUSION REMARKS

The availability of full genome sequences and diverse improved powerful genomic and analytic tools have greatly advanced our knowledge about rice epigenetic modifiers and their biological roles. There are still a large number of modifiers uncharacterized, and molecular mechanisms of function of many chromatin modifiers remain to be investigated into details. It remains to be uncovered how the general histone modification and DNA methylation enzymes exert specific functions in plant growth and developmental processes and what effectors are involved. In particular, H3K27me3 is recognized as a crucial epigenetic mark associated with gene transcriptional repression, and the classical model proposes a sequential mode of action of the two Polycomb complexes: PRC2 is responsible H3K27me3 establishment, and PRC1 recognizes the H3K27me3 mark and further catalyzed downstream H2A monoubiquitination. While PRC1-like components and histone monoubiquitination have been recently studied in *Arabidopsis* (reviewed in Molitor and Shen, 2013; Feng and Shen, 2014), effectors acting together with H3K27me3 in rice remain unknown so far. Utilization of advanced technologies in proteomics, deep sequencing, and gene knockdown will facilitate future studies in functional characterization of interesting genes, investigation of protein complex composition and function, and gene networks controlling rice flowering and reproduction. The extensive agriculture breeding has greatly enriched the rice germplasm resources with large collections of cultivated rice and their wild relatives. Comparative studies of different rice varieties and hybrids will likely impact on knowledge of genetics, epigenetics, and inheritance of agriculture traits as well as fundamental understanding of conservation and diversification of molecular mechanisms.

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# Interpreting lemma and palea homologies: a point of view from rice floral mutants

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In contrast to eudicot flowers which typically exhibit sepals and petals at their periphery, the flowers of grasses are distinguished by the presence of characteristic outer organs. In place of sepals, grasses have evolved the lemma and the palea, two bract-like structures that partially or fully enclose the inner reproductive organs. With little morphological similarities to sepals, whether the lemma and palea are part of the perianth or non-floral organs has been a longstanding debate. In recent years, comparative studies of floral mutants as well as the availability of whole genome sequences in many plant species have provided strong arguments in favor of the hypothesis of lemma and palea being modified sepals. In rice, a feature of the palea is the bending of its lateral region into a hook-shaped marginal structure. This allows the palea to lock into the facing lemma region, forming a close-fitting lemma–palea enclosure. In this article, we focus on the rice lemma and palea and review some of the key transcription factors involved in their development and functional specialization. Alternative interpretations of these organs are also addressed.

**Keywords:** sepal, grass, MADS-box gene, perianth, bract, prophyll

## EQUATING FLOWER ARCHITECTURES

Flowers are biological wonders. Flowering plants, or angiosperms, have evolved into an impressive number of species (the lowest estimations are well above 200,000; Scotland and Wortley, 2003) and are found in almost all ecological niches around the world. Flowers exist in a staggering variety of forms, colors and architectures and yet an exhaustive catalog is still a long way ahead (Endress, 2011). The ecological dominance and evolutionary success of the angiosperms is partly explained by the flexibility of their flower-based mode of reproduction which has allowed sustained species diversification over time (Crepet and Niklas, 2009).

At the molecular level, flowers are formed upon the action of numerous transcription factors, the majority belonging to the MIKC<sup>c</sup>-type MADS-box family (Gramzow and Theissen, 2010; Wellmer and Riechmann, 2010). The current and widely-accepted model that describes how these transcription factors interact to direct the development of floral organs, the ABCDE model, is based on early mutant studies in two eudicot species, *Antirrhinum majus* (Plantaginaceae; Schwarz-Sommer et al., 1990) and *Arabidopsis thaliana* (Brassicaceae; Bowman et al., 1991). Consequently, conceptual thinking of flower development is rooted in the typical dicotyledonous, four-concentric whorl flower architecture in which each whorl is occupied by one type of organ with the following sequence: sepal, petal, stamen, and carpel (from the outermost to the innermost whorl). The model is flexible enough however to be extended to various floral architectures (Bowman, 1997; Erbar, 2007; Theissen and Melzer, 2007); and derived models, such as the “fading borders” model, have been

generated to describe the flowers of species as phylogenetically distant from *A. thaliana* as the basal angiosperms (Buzgo et al., 2004). In several monocot species for example, sepals and petals are not distinguishable and are collectively referred to as tepals. Nevertheless, the relation between tepals and sepals/petals can be accounted for in the ABCDE model by shifts in the domain of expression of B-function homeotic genes (Bowman, 1997).

There are several species however where the interpretation of the floral architecture itself, and most particularly the outer whorls and peripheral organs, is problematic to begin with. Within the monocots, this is the case for members of the grass family which bear characteristic flowers, termed florets, that differ substantially from the one described in the ABCDE model. The periphery of the grass flower is occupied by elongated and leafy organs, evocative of small bracts, in a striking contrast to a typical monocot perianth. The nature of these organs and the identity of their counterparts in non-grass related species, if any, have been subject to much debate for more than a century (Clifford, 1986). The identity of the bract-like organs closest to the inner flower, called lemma and palea, has been the most controversial. While various interpretations have been formulated (Clifford, 1986), the lemma and palea have been commonly interpreted as a bract and a prophyll, respectively (Linder, 1987; Rudall and Bateman, 2004). Alternatively, the palea has been interpreted as two fused sepals (adaxial tepals; Schuster, 1910; Stebbins, 1951) and the lemma has been rarely interpreted as a sepal (calyx; Francis, 1920).

*Oryza sativa* (common rice) is one of the best documented grass species and many rice mutants have been described in the

literature. Focusing on *O. sativa*, in the following are reviewed some of the key pieces of data that have surfaced in the last 30 years or so which have shed light on the controversial nature of the lemma and palea.

## LEMMA AS BRACT AND PALEA AS PROPHYL EQUIVALENTS?

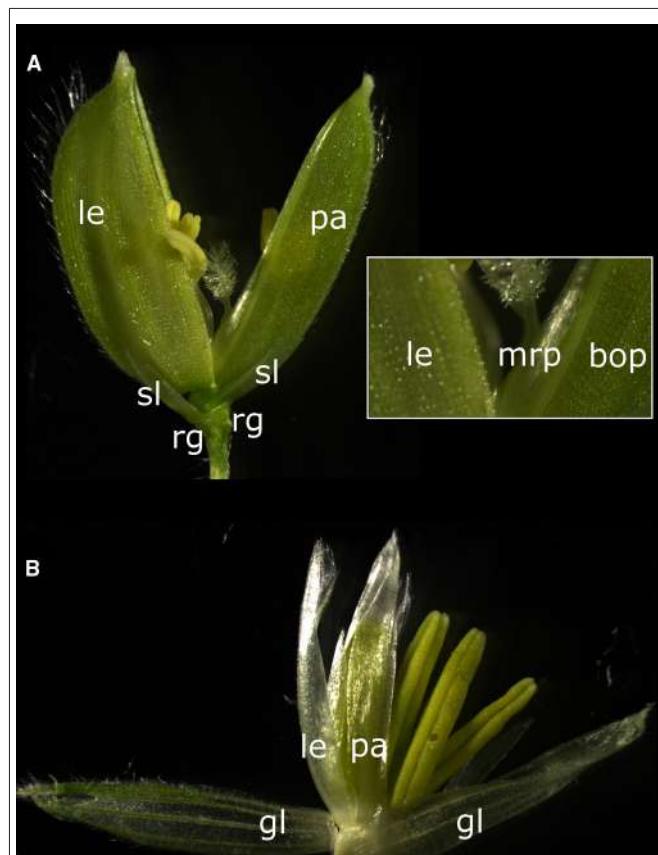
The structure of the rice flower is commonly described and organs designated as following (**Figure 1**): On a short axis, the rachilla, are proximally attached two cupule-shaped small outgrowths called rudimentary glumes. Above the rudimentary glumes are found a pair of scales called sterile lemmas or empty glumes depending on how they are interpreted. The floret is the unit above the sterile lemmas that comprises the lemma, the palea and the enclosed inner floral organs. The floret is commonly considered as the grass equivalent of the eudicot flower and the addition of the floret, the sterile lemmas and the rudimentary glumes forms the spikelet.

The above nomenclature stems from an early and common interpretation which is based on the observation that the lemma arises on the main axis, which is distinct from the floret axis. The lemma is thus regarded as a modified leaf which subtends the

floral meristem in its axil (Arber, 1934; Bell, 1991; Kellogg, 2001). The distinct origin of the lemma is illustrated in the *leafy lemma* barley mutant in which the lemma is specifically transformed into a leaf-like organ (Pozzi et al., 2000). While modified leaves growing near inflorescences, or bracts, can take petal-like vivid colors in some species (Buzgo and Endress, 2000), they do not belong to the perianth by definition and are therefore extra-floral organs. Facing the lemma, the palea originates on the floret axis and, since it is the first “leaf” arising from the meristem subtended by the lemma, it is commonly considered a prophyll. The basal bracts that subtend the spikelet are called glumes and in rice the term has been applied indiscriminately to both the rudimentary glumes and the empty glumes, bringing some confusion to which are the spikelet-subtending bracts. Based on serial sections, Arber (1934) concluded that the rudimentary glumes are the true basal bracts of the rice spikelet, only in an extremely reduced, vestigial form. Consequently the “empty glumes” are interpreted as sterile lemmas, since they do not bear any flowers in their axils.

## LEMMA AND PALEA AS SEPAL EQUIVALENTS?

The interpretation of the lemma as bract and palea as prophyll equivalents relies for the most part on early morphological comparative studies (Arber, 1934; Stebbins, 1951; Clifford, 1986; Bell, 1991). However, more recent progress in the genetics of flower development highlighting the universal role of MADS-box genes as floral homeotic genes suggest that both lemma and palea are sepal equivalents, in the sense that they are outer perianth organs corresponding to the tepals/sepal of most other flowers. Such equivalency does not imply however that sepals and both lemma and palea are derived from the same ancestral organ. The sequencing of the genome of *Amborella trichopoda*, a species belonging to the sister lineage to all other extant flowering plants, has revealed that each of the eight major lineages of MADS-box genes were represented in the most recent common ancestor of the angiosperms (Amborella Genome Project, 2013). MADS-box genes are thus invaluable molecular markers toward determining floral organ identity. In the interpretation where the lemma and palea are floral organs, homeotic genes associated with floral identity are expected to be expressed in these structures. Conversely, such gene expression is expected to be lacking in bracts and other non-floral structures. In rice, inflorescence meristem identity is specified by *AP1/FUL*-like genes and a *SEP* gene (Kobayashi et al., 2012). Furthermore no significant expressions of floral MADS-box genes can be detected in the bracts of grasses, strongly suggesting that the lemma and palea are distinct from these structures (Kyozuka et al., 2000; Malcomber and Kellogg, 2004; Prasad et al., 2005; Preston and Kellogg, 2007). Expression analysis of key MADS-box genes in *Streptochaeta angustifolia*, a non-spikelet-bearing grass species, and in the grass outgroup monocot *Joinvillea ascendens* allowed Preston et al. (2009) to infer the putative floral architecture of the grass common ancestor: three categories of structure (glume-, sepal-, and petal-equivalents) would each express a different combination of *AP1/FUL*-like, *LHS1*-like and B-class genes. In any case, expression of any of these genes is neither expected in the bracts of the grass common ancestor nor detected in the bracts of any of the investigated monocot species. The authors suggest that the ancestral sepal-equivalent structures



**FIGURE 1 | Structure of the rice and maize spikelets. (A)** Rice spikelet. Inset is a close-up view of the basal region of the lemma and palea. le, lemma; pa, palea; mrp, marginal region of palea; bop, body of palea; sl, sterile lemma; rg, rudimentary glume. **(B)** Maize tassel (male) spikelet. le, lemma; pa, palea; gl, glume.

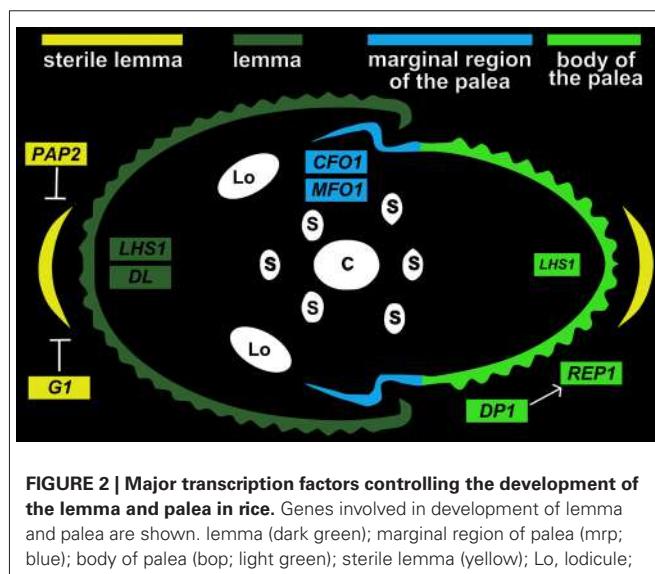
which express *AP1/FUL*-like and *LHS1*-like genes are the organs from which the lemma and palea are derived (Preston et al., 2009).

According to the ABCDE model, perianth whorls develop under the action of A-class genes (sepals) or cumulative action of A-and B-class genes (petals). Petals are therefore expected to homeotically transform into sepals or at least acquire some degree of sepal identity when B-class genes are disrupted, as documented in the *apetala3* (*ap3*) mutant of *A. thaliana* (Goto and Meyerowitz, 1994). The role of genes for B function has been shown to be conserved across the angiosperms (Whipple et al., 2007) and in maize, disruption of the B-class *SILKY1* gene leads to a homeotic conversion of the lodicules (organs commonly considered as petal equivalents) into lemma/palea-like structures (Ambrose et al., 2000). A similar homeotic conversion is observed in the loss-of-function alleles of the *SUPERWOMAN1* (*SPW1*) gene, the rice ortholog of *AP3* (Nagasaki, 2003). Following the ABCDE model, these results strongly suggest that the lemma and palea are equivalent to the sepals of most other flowers.

The phenotype of maize *branched silkless* (*bd1*), in which transition from the spikelet meristem to the floret meristem is blocked, supports that lemma and palea are floral organs. The mutant is able to produce glumes but neither lemma nor palea is formed (Colombo et al., 1998), indicating that the whorls holding the lemma and the palea originate from a floral meristem.

## PALEA AS A DIFFERENTIATED LEMMA

Irrespective of the homology of the lemma and palea, the genetic mechanisms that control their development are distinct (summarized in Figure 2). There are mutants in which either the palea or the lemma is specifically affected, such as the *leafy lemma* mutant of barley or the *depressed palea1* (*dp1*) mutant of rice, which palea is dramatically reduced but its lemma remains unchanged (Pozzi et al., 2000; Luo et al., 2005). Ambrose et al. (2000) hypothesized that the lemma and the palea reside in two distinct whorls, which would account for some level of genetic independence and explains the asymmetrical phenotypes.



Depending on the grass species, the palea can be distinguished from the lemma by various morphological features, such as the number of vascular bundles, size, or surface structure. In *O. sativa*, the differentiation of the palea is particularly pronounced. Edges of the palea curl outwardly at its base in a hook-shaped marginal structure which fits together with the inwardly curled facing lemma. The marginal region of the palea is smooth and light colored, in contrast to the body of the palea which is populated with silicified cells bearing trichomes. Phenotypes of several mutants suggest that the rice palea can be considered as a composite of two types of domain: the body and the marginal region. In this hypothesis, the body is further interpreted as a structure with a lemma identity and the marginal regions as distinct structures with palea identity (Yoshida and Nagato, 2011). Phenotypes of the *mfo1* and *cfo1* mutants further support this idea (Ohmori et al., 2009; Sang et al., 2012; see below).

## TRANSCRIPTION FACTORS INVOLVED IN PALEA DIFFERENTIATION

The *AGL6*-like MADS-box gene *MOSAIC FLORAL ORGANS1* (*MFO1*; *MADS6*) is a major determinant of the rice palea architecture. In *mfo1*, the palea acquires features of the lemma, namely inward curling, loss of the marginal region and ectopic expression of *DROOPING LEAF* (*DL*), a gene normally expressed in the lemma (Ohmori et al., 2009). In addition to its role in palea differentiation, *MFO1* has a central role in spikelet development and is involved in floral meristem determinacy. A phylogenetic analysis has revealed that expression of *MFO1* in the palea has appeared later in the evolution and correlated with the origin of the grass spikelet (Reinheimer and Kellogg, 2009). In maize, the *bearded-ear* (*bde*) gene is orthologous to *MFO1* and is also expressed in the palea but not in the lemma, suggesting a conserved role for *AGL6*-like genes in the palea across the grasses (Thompson et al., 2009). This hypothesis could be tested by investigating the role of *MFO1/bde* orthologs in other grass species.

Similarly to *mfo1*, *chimeric floral organs1* (*cfo1*; the mutant of rice *MADS32*) shows variable defects in the inner whorls but a rather consistent, somewhat similar phenotype to *mfo1* in the palea. The marginal region in *cfo1* mutants is enlarged and silicified and ectopic expression of *DL* is also observed. However, unlike in *mfo1* paleas, there is no lemma-like inward curling (Sang et al., 2012). *CFO1* was thought to be a grass-specific gene until the recent sequencing of *Amborella trichopoda* revealed the presence of an ortholog, implying that the gene has been lost outside of the grass group. The evolution of *CFO1* and its ancestral function remain to be elucidated and it would be particularly interesting to know if, similarly to *MFO1*, the gene was recruited in the palea to support its differentiation in grasses.

*RETARDED PALEA1* (*REP1*) encodes a *CYCLOIDEA* (*CYC*)-like TCP transcription factor which promotes the growth of the body of the palea, presumably by defining the boundaries between the marginal region and the body (Yuan et al., 2009). In *rep1* the body is strongly reduced, resulting in a much smaller palea, whereas the marginal region is widened. Over-expression lines show the opposite phenotype, that is an overgrown body and narrower marginal region.

*RETARDED PALEA1* is hypothesized to be downstream of the *DP1* gene which encodes an AT-hook transcription factor (Jin et al., 2011). The *dp1* mutant shows a more severe phenotype than *rep1*: The body is lost entirely, leaving two marginal leafy organs which are likely to be transformed marginal regions. The only putative ortholog to *DP1* described so far is the maize *BARREN STALK FASTIGIATE1* (*BAF1*) gene. The *BAF1/DP1* function is hypothesized to be conserved in all of the grasses (Gallavotti et al., 2011), and would contribute to the differentiation of the grass flower. The phenotypes of *rep1* and *dp1* mutants are consistent with the interpretation of the rice palea being composed of two types of domain: a lemma-identity structure (the body) and two differentiated lateral structures (the marginal regions; Jin et al., 2011).

### TRANSCRIPTION FACTORS INVOLVED IN LEMMA DIFFERENTIATION

A common feature of both *mfo1* and *cfo1* mutants is the palea ectopic expression of *DL* in the abnormal paleas. Mutant alleles of *dl* have been well documented, mostly for the striking loss of carpel identity, a function which is conserved in *A. thaliana* via the *CRABS CLAW* (*CRC*) ortholog, and for the inability to maintain erect leaves (Bowman and Smyth, 1999; Yamaguchi et al., 2004). *DL* promotes cell proliferation in the leaf midrib structure and in the lemma, along its longitudinal axis. This is illustrated in the *dl-sup1* mutant which grows a shorter lemma; and for the requirement of the gene in awn development (Toriba and Hirano, 2014). In a *dl cfo1* double mutant, the altered marginal region phenotype of *cfo1* is rescued, suggesting that the defects observed in *cfo1* marginal regions are due to the ectopic activity of *DL*. The marginal region is not altered however in a *dl mfo1* double mutant, so the precise mechanisms by which ectopic *DL* expression disturbs palea development remain to be elucidated (Li et al., 2011).

Another gene involved in lemma differentiation is the rice *LHS1* (*MADS1*) gene. Ectopic expression of *LHS1* in the sterile lemma confers the organ lemma-like morphological and anatomical traits. Conversely, silenced lines of *LHS1* show transformation of their lemmas into sterile lemma-like organs with poor cellular differentiation (Prasad et al., 2005). The palea is only slightly affected in these mutants, suggesting that *LHS1* functions essentially as a lemma differentiation gene.

### LEMMAS AND STERILE LEMMAS

Eighty years ago, Arber hypothesized that the sterile lemmas are the remaining organs of two additional spikelets, lost from an ancestral rice with a three-floret spikelet (Arber, 1934). The LONG STERILE LEMMA1 (G1) protein contains an ALOG domain and belongs to a recently described class of transcription factor. The *g1* mutant shows the striking phenotype of sterile lemmas transformed into lemmas, bringing genetic evidence to the long-standing hypothesis by Arber (1934; Yoshida et al., 2009). This idea is supported by similar phenotypes of *panicle phytomer2* (*pap2*; *mads34*; Lin et al., 2014).

The spikelet of the wild rice *O. grandiglumis* bears elongated sterile lemmas which are in a striking resemblance to the ones of *g1* or *pap2*. Nucleotide sequences of *O. grandiglumis* G1 and *PAP2*

show some polymorphism in key functional domains, suggesting that the long sterile lemma phenotype of *O. grandiglumis* is the result of natural variations in the *G1* and/or *PAP2* sequences.

### LEMMA AND PALEA ILLUSTRATE THE ANGIOSPERM FLOWER PLASTICITY

The large diversity in flower shape and architecture across the angiosperms makes unraveling the evolution of morphological features a laborious and challenging task. Identification and analysis of floral transcription factors have uncovered how subtle genetic alterations can result in dramatic morphological changes. Duplication, recruitment and/or sub-functionalization of the MADS-box transcription factors have been shown to correlate with floral diversification (Shan et al., 2009; Yockteng et al., 2013), and undoubtedly, the complexity and flexibility of floral feature evolution had been underestimated during the pre-molecular era (Endress and Matthews, 2012).

Before the advent of molecular biology, the lemma and palea of grasses have been arguably most commonly interpreted as a bract and prophyll, respectively, although a handful of authors over the last century have suggested that they might be modified perianth parts. While the lemma and palea of grasses show significant morphological variations depending on the observed species, expressions of *AP1/FUL*-like genes as well as *LHS1*-like genes are detected in these structures. This implies that the lemma and palea are emerging on a floral meristem and that they are very likely to be distinct from glumes since the expression of *LHS1*-like genes has not been observed in the glumes of any grasses yet (Preston et al., 2009). Some mutants affected in B function, which is likely to be conserved across angiosperms (Whipple et al., 2007), show a homeotic transformations of their second whorl organs into lemma/palea-like organs. Taken together, these data suggest that the lemma and palea of grasses are likely to be sepal equivalents.

Biotic-pollinated plants must accommodate for bud protection and attract pollinators at the same time, and their perianth has evolved under these constraints. In wind-pollinated grasses however, elongated and covering outer organs provide advantageous protection against pests and physical damage. Under the assumption that the grass lemma and palea are sepal equivalents, these organs, and most particularly in the case of rice, can be regarded as a remarkable illustration of the evolutionary potency of the angiosperms.

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# Sterility Caused by Floral Organ Degeneration and Abiotic Stresses in *Arabidopsis* and Cereal Grains

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Natural floral organ degeneration or abortion results in unisexual or fully sterile flowers, while abiotic stresses lead to sterility after initiation of floral reproductive organs. Since normal flower development is essential for plant sexual reproduction and crop yield, it is imperative to have a better understanding of plant sterility under regular and stress conditions. Here, we review the functions of ABC genes together with their downstream genes in floral organ degeneration and the formation of unisexual flowers in *Arabidopsis* and several agriculturally significant cereal grains. We further explore the roles of hormones, including auxin, brassinosteroids, jasmonic acid, gibberellic acid, and ethylene, in floral organ formation and fertility. We show that alterations in genes affecting hormone biosynthesis, hormone transport and perception cause loss of stamens/carpels, abnormal floral organ development, poor pollen production, which consequently result in unisexual flowers and male/female sterility. Moreover, abiotic stresses, such as heat, cold, and drought, commonly affect floral organ development and fertility. Sterility is induced by abiotic stresses mostly in male floral organ development, particularly during meiosis, tapetum development, anthesis, dehiscence, and fertilization. A variety of genes including those involved in heat shock, hormone signaling, cold tolerance, metabolisms of starch and sucrose, meiosis, and tapetum development are essential for plants to maintain normal fertility under abiotic stress conditions. Further elucidation of cellular, biochemical, and molecular mechanisms about regulation of fertility will improve yield and quality for many agriculturally valuable crops.

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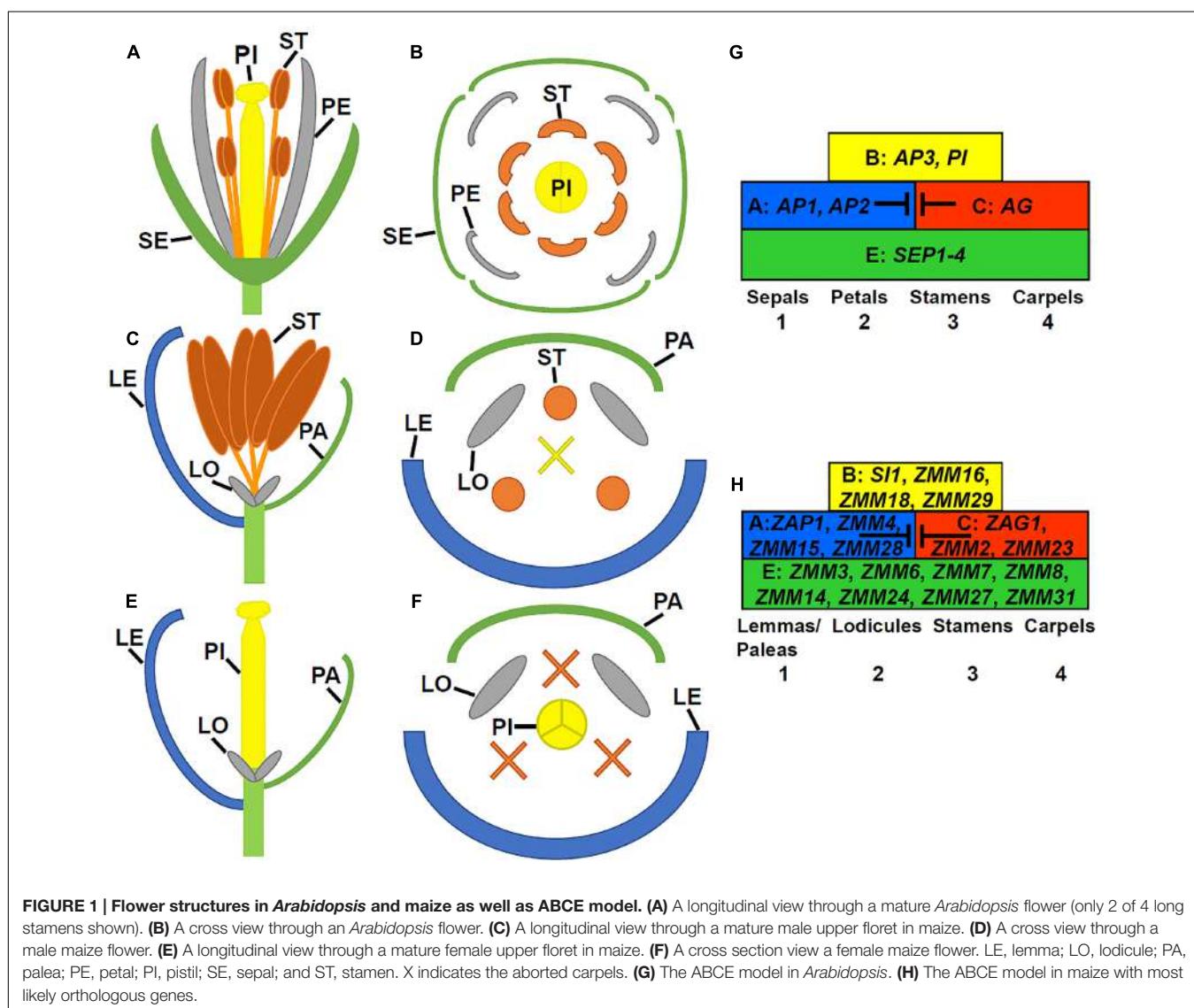
## INTRODUCTION

Flower development is a long and complex process, which is mainly classified into four stages: flowering transition, floral meristem identity, floral organ identity, and floral organ morphogenesis. Mainly using model species *Arabidopsis thaliana* and snapdragon (*Antirrhinum majus*), extensive molecular genetic studies have identified numerous genes required for flower development, particularly during early stages. *Arabidopsis* plants produce raceme-type indeterminate inflorescences where flowers are indefinitely generated. A typical *Arabidopsis* flower contains four protective sepals in the first whorl, four petals in the second whorl, six stamens (male reproductive organs) in the third whorl, and two fused carpels (female reproductive structure)

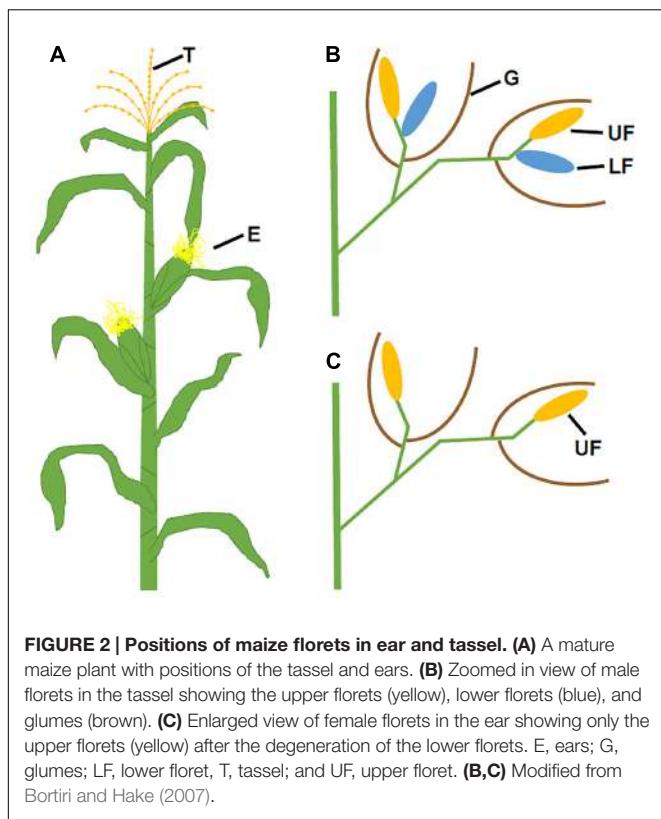
that form the gynoecium in the fourth whorl (**Figures 1A,B**). Different from *Arabidopsis*, Poaceae plants, commonly known as grasses, produce determinate panicles where flowers (or florets) are organized into spikelets. In maize, these spikelets are grouped into separate male and female inflorescences (**Figure 2A**). The highly branched male inflorescence, the tassel, is composed of spikelet pairs, each of which comprises an upper and a lower floret surrounded by the leaf like structures known as glumes (**Figure 2B**). Similarly, spikelet pairs are formed in the female inflorescence, but the lower floret in each spikelet pair is aborted (**Figure 2C**) (Zhang and Yuan, 2014). Poaceae flowers have stamens and carpels similar to eudicot flowers, such as *Arabidopsis*. In maize, the male floret contains three stamens (**Figures 1C,D**), while the female floret produces three central carpels which are fused to form the pistil (**Figures 1E,F**) (Zhang and Yuan, 2014). Maize florets do not contain sepals and petals. Instead, the sepal-analogous organs lemma and palea are produced (**Figures 1C–F**) (Schmidt and Ambrose, 1998;

Lombardo and Yoshida, 2015). Additionally, the petal analogous structures known as lodicules are essential for pollination via opening the bract organs (Yoshida, 2012).

Within cereal grains, floral organ degeneration is not unique to maize. During development in grain crops such as wheat (*Triticum aestivum*), rice (*Oryza sativa*), and sorghum (*Sorghum bicolor*), the arrest of stamen or carpel primordia, or both potentially results in reduced fertility or completely sterile flowers (Bommert et al., 2005; Yoshida and Nagato, 2011; Aryal and Ming, 2014). In the floret pair of sorghum, one floret is bisexual fertile, whereas the other one is bisexual sterile. Similarly, wild barley (*Hordeum vulgare*) produces a central fertile floret surrounded by a pair of sterile florets, and even oats (*Avena sativa*) are known to form sterile flowers at the apex of the rachilla (Schmidt and Ambrose, 1998). Additionally, abiotic stresses cause flower sterility, which consequently results in yield loss. In this review, we will focus on discussing molecular genetic and physiological mechanisms underlying sterility caused by floral



**FIGURE 1 |** Flower structures in *Arabidopsis* and maize as well as ABCE model. **(A)** A longitudinal view through a mature *Arabidopsis* flower (only 2 of 4 long stamens shown). **(B)** A cross view through an *Arabidopsis* flower. **(C)** A longitudinal view through a mature male upper floret in maize. **(D)** A cross view through a male maize flower. **(E)** A longitudinal view through a mature female upper floret in maize. **(F)** A cross section view a female maize flower. LE, lemma; LO, lodicule; PA, palea; PE, petal; PI, pistil; SE, sepal; and ST, stamen. X indicates the aborted carpels. **(G)** The ABCE model in *Arabidopsis*. **(H)** The ABCE model in maize with most likely orthologous genes.



organ degeneration and abiotic stresses mainly in *Arabidopsis* and key cereal grain plants.

## MOLECULAR GENETIC REGULATION OF FLORAL ORGAN DEGENERATION

Degeneration or abortion of developing stamens and/or pistil is a main mechanism used by plants to produce unisexual flowers or sterile flowers. In *Arabidopsis*, there are four major classes of genes that specify floral organ identity. Class A genes [*APETALA1* (*API*) and *AP2*], class B genes [*AP3* and *PISTILLATA* (*PI*)], the class C gene [*AGAMOUS* (*AG*)], and the semi-redundant class E genes [*SEPALLATA1-4* (*SEP1-4*)]. Class A and E genes are required for specifying sepals in the first whorl. Class A, B, and E genes in combination control petal identity in the second whorl. Class B, C, and E genes direct the stamen identity in the third whorl, and class C and E genes specify carpels in the fourth whorl (Figure 1G) (Bowman et al., 1991; Rounseley et al., 1995; Pelaz et al., 2000; Ditta et al., 2004). The ABC model can also be applied to flower development in other plants including the Poaceae, although many variations exist (Figure 1H; Table 1). Altered expression patterns of B and C class genes can result in floral organ degeneration and sterility.

The extensive roles and interactions of ABC genes in flower development are summarized in Prunet and Jack (2014). What is less clear and receives less attention is that after the establishment

of floral organ identity, how floral organ identity genes play a role in development of functional floral organs. *AG* is required throughout reproductive development for establishing fertility. Specifically, *AG* is expressed in stamen and carpel primordia initially, and later in specific cell types of stamens and carpels (Bowman et al., 1991). *AG* (along with *PI* and *AP3*) controls stamen development via directly activating the expression of *SPOROCYTELESS/NOZZLE* (*SPL/NZZ*), which in turn regulates microsporogenesis (Ito et al., 2004; Liu et al., 2009). *AG* also upregulates the expression of the *DEFECTIVE IN ANTER DEHISCENCE 1* (*DAD1*) gene that encodes a jasmonic acid (JA) biosynthesis enzyme (Ito et al., 2007). The *dad1* mutant produces immature pollen, resulting in male sterility. If *AG* is not expressed in flowers prior to stage 7 in *Arabidopsis*, plants fail to undergo microsporogenesis, while increased duration of *AG* expression enhances normal stamen and pollen production (Ito et al., 2007). Similarly, in maize *branched silkless1-2* (*bd1-2*) mutants, loss of expression of class C and D (ovule specification) genes like *ZAG1* (*Zea mays AG1*), *ZAG2*, and *ZMM2* (*Zea mays MADS2*) may cause female sterility (Colombo et al., 1998).

Flowers destined to be male or female often begin as hermaphroditic flowers, but later undergo a programmed degeneration of the gynoecium or androecium, respectively, in early reproductive development. This degeneration is often accompanied by down regulation of B and C class genes (Ainsworth et al., 2005). Unisexual flowers in plants like asparagus (*Asparagus officinalis*) undergo abortion late in development at the onset of meiosis, although remnants of male or female organs remain (Dellaporta and Calderon-Urrea, 1993; Aryal and Ming, 2014). In female asparagus flowers, the expression of B class gene *AODEF* (*Asparagus officinalis DEFICIENS*) is decreased in the stamen, which may cause stamen degeneration (Park et al., 2003). Loss of class B gene function also leads to stamen degeneration in the tulip (*Tulipa gesneriana*) mutant *viridiflora* (Kanno et al., 2007). In male sorrel (*Rumex acetosa*) flowers, both class B and C genes are present in early male flower formation. In later stages, the expression of a class C gene is not detectable in the region that would specify carpels in a female or hermaphroditic flower (Ainsworth et al., 2005). In white champion (*Silene latifolia*), the class C gene *SLM1* (*Silene latifolia MADS1*) is expressed until meiosis in male and female floral organs. Later in female flower development, stamens undergo degeneration. The expression of *SLM1* is not detected in aborted stamens, while its expression persists in the undeveloped gynoecium of male flowers (Hardenack et al., 1994). Moreover, in *S. latifolia*, *Arabidopsis* orthologs of *SHOOT MERISTEMLESS* (*SLSTM1* and *SLSTM2*) and *CUP SHAPED COTYLEDON* (*SLCUC1* and *SLCUC2*) likely control sex determination via regulating cellular proliferation in the third whorl (Zluvova et al., 2006).

Growing evidence supports that the early loss of class B and C genes leads to the arrest of development in reproductive organ primordia and ultimately the inability of these flowers to form functional carpels or stamens. It is clear that during development class B and C genes must be expressed in the correct location for a sufficient duration. Without the normal expression, flowers exhibit a wide array of phenotypes, ranging from floral organs

**TABLE 1 | Key genes for maize unisexual flower development.**

| Gene                           | Function  | Reference                                    |
|--------------------------------|---|--|
| ZAP1                           | Class A function  | Mena et al., 1995                            |
| ZMM4                           | Class A function  | Fischer et al., 1995                         |
| ZMM15, ZMM28                   | Class A function  | Münster et al., 2002                         |
| IDS1*, SID1*                   | Class A function  | Chuck et al., 1998, 2008                     |
| SI1                            | Class B function  | Ambrose et al., 2000; Whipple et al., 2004   |
| ZMM16, ZMM18, ZMM29            | Class B function  | Münster et al., 2001; Whipple et al., 2004   |
| ZMM2                           | Class C function  | Theissen et al., 1995                        |
| ZMM23                          | Class C function  | Münster et al., 2002                         |
| ZAG1                           | Class C function  | Schmidt et al., 1993                         |
| ZAG2                           | Class D function  | Schmidt et al., 1993                         |
| ZMM1                           | Class D function  | Theissen et al., 1995                        |
| ZMM25                          | Class D function  | Münster et al., 2002                         |
| ZAG3                           | AGL6 function   | Mena et al., 1995; Thompson et al., 2009     |
| ZMM6                           | Class E function  | Fischer et al., 1995                         |
| ZMM24, ZMM27, ZMM31            | Class E function  | Münster et al., 2002                         |
| ZmLHS1a, ZM1HS1b (ZMM8, ZMM14) | Class E function  | Cacharrón et al., 1999                       |
| ZMM3                           | Class E function  | Fischer et al., 1995; Kobayashi et al., 2010 |
| ZMM7                           | Class E function  | Fischer et al., 1995                         |
| TS1                            | TS2 expression and JA production                              | Calderon-Urrea and Dellaporta, 1999          |
| TS2, TS4, TS6                  | Pistil abortion   | DeLong et al., 1993; Irish, 1997             |
| SK1                            | Protects pistils  | Calderon-Urrea and Dellaporta, 1999          |
| RMR6                           | Protects tassel by repressing SK1                             | Parkinson et al., 2007                       |
| NA1                            | Promotes stamen development, BR production                    | Hartwig et al., 2011                         |
| OPR7, OPR8                     | Promote carpel abortion and stamen development; JA production | Yan et al., 2012                             |
| AN1                            | promotes stamen arrest; GA production                         | Bensen et al., 1995                          |
| D1, D2, D3, D5                 | Promote stamen arrest; GA production                          | Fujioka et al., 1988; Spray et al., 1996     |

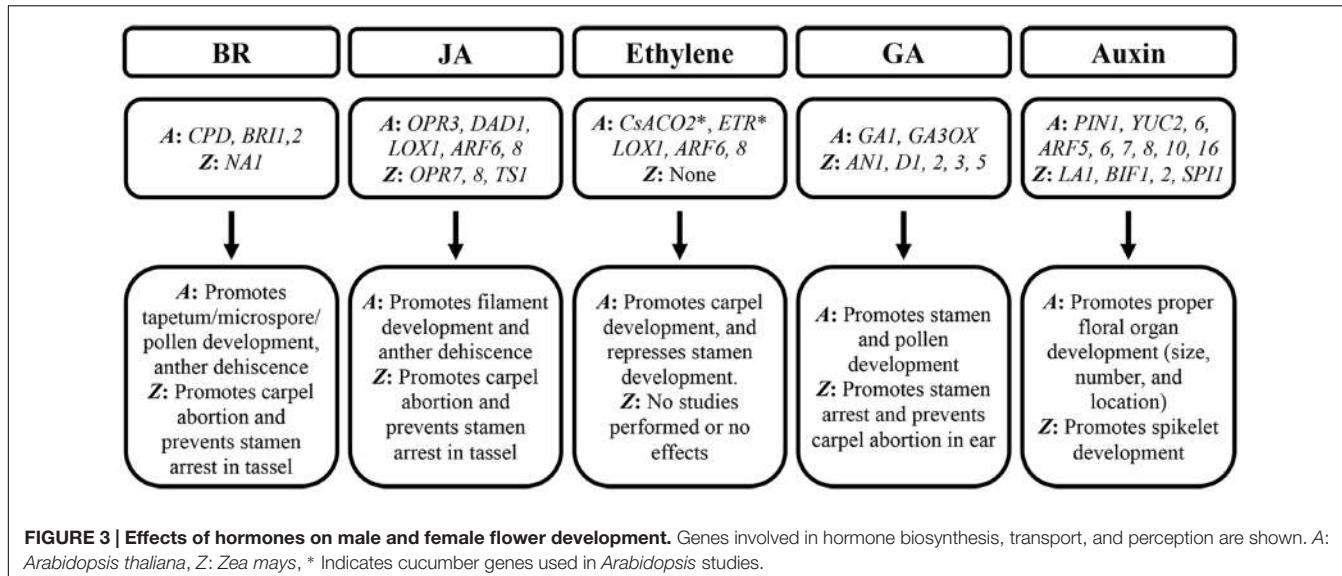
\*Cannot compensate for the AP2 mutation in *Arabidopsis*.

present in incorrect whorls, to unisexual flower development, and even complete sterility.

Besides class B and C genes, many additional genes are essential for the establishment of the unisexual state in monoecious plants. Male and female flower development in plants like maize begins as identical, but degeneration of gynoecium primordia in the male flowers and degeneration of stamen primordia in the female flowers result in the production of two distinct flower types (Dellaporta and Calderon-Urrea, 1993; Irish, 1996). TASSEL SEED (TS) genes are responsible for normal pistil abortion in the tassel. In recessive *ts1* and *ts2* mutants, feminization of tassels occurs and pistillate flowers are formed. The *ts1* mutant phenotype is attributable to a mutation in a lipoxygenase that produces JA (DeLong et al., 1993; Malcomber and Kellogg, 2006; Acosta et al., 2009). *TS2* (a short-chain alcohol dehydrogenase) triggers the programmed cell death (PCD) of pistils (DeLong et al., 1993; Parkinson et al., 2007). In *silkless1* (*sk1*) mutants, pistils are not developed in female florets, while male florets are unaffected (Malcomber and Kellogg, 2006). In the ear, *SK1* protects pistils from undergoing PCD caused by *TS2* (Calderon-Urrea and Dellaporta, 1999). Similarly, in the maize relative *Tripsacum*, the *TS2* homolog *GYNOMONOECIOUS SEX FORM1* is expressed in pistils prior to abortion (Li et al., 1997).

Moreover, in *required to maintain repression6* (*rnr6*) mutants, pistils fail to abort, which causes the feminization in tassels (Parkinson et al., 2007). *RMR6* (encoding the largest subunit of RNA polymerase IV, an orthologue of *Arabidopsis* NRPD1a) acts by limiting the activity of *SK1* to the primary ear floret, resulting in PCD of the gynoecium in the tassel and the secondary ear floret (Parkinson et al., 2007; Erhard et al., 2009). In each of the dominant single mutants of *Ts3*, *Ts5*, and *Ts6*, as well as the recessive mutant *ts4*, a variety of phenotypes are observed in the tassel, such as reduced tassel size, bisexual flowers, and feminization of the tassel (Veit et al., 1993; Irish et al., 1994). Key genes involved in unisexual flower development in maize are summarized in **Table 1**.

In addition to the formation of unisexual flowers, completely sterile flowers are also commonly produced in grasses due to floral organ degeneration. In some cereal grains, a fertility conversion of sterile flowers is possible. In the sorghum *multiseeded1* (*msd1*) mutant, the development of bisexually sterile flowers become normal, leading to the formation of all fertile flowers (Burow et al., 2014). In barley, the *vrs1* (*six-rowed spike1*) mutation results in fully fertile barley known as six-rowed barley. In the wild-type barley, the *VRS1* gene suppresses lateral spikelet development, causing a central fertile floret surrounded by two sterile florets (Komatsuda et al., 2007).



## ROLES OF HORMONES IN FLORAL ORGAN DEVELOPMENT

Hormones have strong influences on flower sexuality and fertility. Some hormones are essential for both male and female organ development, while others are male or female specific (Figure 3). Brassinosteroids (BRs) and JA promote male but suppress female organ development in both *Arabidopsis* and maize (Clouse et al., 1996; Szekeres et al., 1996; Clouse and Sasse, 1998; Stintzi and Browse, 2000; Zhao and Ma, 2000; Li et al., 2001; Nagpal et al., 2005; Mandaokar et al., 2006; Acosta et al., 2009; Yan et al., 2012). Ethylene has been shown to act as a feminizing agent in plants like cucumber, but its role in *Arabidopsis* and maize is less understood (Yin and Quinn, 1995; Duan et al., 2008; Wang et al., 2010). The function of gibberellic acid (GA) is conflicting, as it is critical for proper male organ development in *Arabidopsis*, but it antagonizes stamen development in the maize ear (Fujioka et al., 1988; Dellaporta and Calderon-Urrea, 1994; Bensen et al., 1995; Spray et al., 1996; Goto and Pharis, 1999; Michaels and Amasino, 1999; Cheng et al., 2004; Yu et al., 2004; Hu et al., 2008). Differently, auxin is necessary for both male and female floral organ development (Okada et al., 1991; Sessions et al., 1997; Nagpal et al., 2005; Cheng et al., 2006; Wu et al., 2006; Dong et al., 2013).

### Brassinosteroid

Brassinosteroids are widely involved in cell expansion, cell division, senescence, vascular differentiation, and stress responses. Overall, BRs promote the formation of stamens and pollen in both *Arabidopsis* and maize, and the abortion of pistils in staminate maize flowers. The *constitutive photomorphogenesis and dwarfism* (*cpd*) mutant which fails to form the ecdysone-like brassinosteroids, produces pollen defective in pollen tube elongation (Szekeres et al., 1996). Both *cpd* and *brassinosteroid-insensitive1* (*bri1*) mutants make far fewer pollen grains per locule with limited viability. Similarly, the *brassinosteroid-insensitive2* (*bin2*) mutant is male sterile (MS; Li et al., 2001). Further studies

show that BRs control male fertility via regulating expression of genes critical for anther and pollen development, such as *SPL/NZZ*, *DEFECTIVE IN TAPETAL DEVELOPMENT AND FUNCTION 1* (*TDF1*), *ABORTED MICROSPORES* (*AMS*), and *MS1* and *MS2* genes (Ye et al., 2010). Additionally, BRs are required for sex determination in maize. In the maize *nana plant 1* (*nal*) mutant tassel, some carpels fail to abort, resulting in both staminate and pistillate flowers (Hartwig et al., 2011). The *nal* mutation occurs in *ZmDET2*, a homolog of the *Arabidopsis DE-ETIOLATED2* (*DET2*) which encodes the important BR biosynthesis enzyme 5 $\alpha$ -steroid reductase, suggesting an important role of BRs in the formation of tassel flowers in maize.

### Jasmonic Acid

In *Arabidopsis* and the maize tassel, JA is crucial for stamen and pollen maturation. In *Arabidopsis*, the *12-oxophytoenoic acid reductase 3* (*opr3*) mutant is deficient in JA synthesis at the conversion of linolenic acid to JA. The *opr3* mutant produces stamens that are abnormal in filament elongation and dehiscence (Stintzi and Browse, 2000; Zhao and Ma, 2000; Mandaokar et al., 2006). Maize has a series of *OPR* genes, among which *OPR7* and *OPR8* represent the *Arabidopsis OPR3* orthologs (Zhang et al., 2005; Yan et al., 2012). The *opr7-5 opr8-2* double mutant plants form feminized tassels devoid of stamen formation and are capable of seed production if pollinated with wild-type pollen. This phenotype can be reversed by exogenous application of JA (Acosta et al., 2009; Yan et al., 2012). A similar phenotype is observed when the *AG* expression is lost, as *AG* promotes the *DAD1* (JA biosynthesis enzyme) expression (Ito et al., 2007). AUXIN RESPONSE FACTOR (ARF) transcription factors ARF6 and ARF8 are required for JA production. Disruption of ARF6 and ARF8 genes results in delayed stamen development, and consequently the complete male sterility (Nagpal et al., 2005). As discussed above, feminization in the *ts1* tassel is attributed to loss of JA synthesis (Acosta et al., 2009). In the tomato

*JAs-insensitive1 (jai-1)* mutant, the male fertility is also greatly affected with about 28% of pollen being viable and only 10% actually germinating (Li et al., 2004). However, it is believed that the additional female sterility may be caused by arrest in embryo/seed development.

The effects of JA on stamen development and male fertility in maize and *Arabidopsis* are consistent. In both organisms JA promotes male organ development, but suppresses female organ development. Unlike in *Arabidopsis*, JA is also important for female fertility in tomato, indicating potentially divergent and complex roles of JA in plant sexual reproduction that warrant further exploration. Of particular interest, AG is required for the *DAD1* expression, suggesting interaction between the JA signaling and the class C gene AG.

## Ethylene

Ethylene promotes the formation of female flowers in cucumber (*Cucumis sativus*). *CsACO2 (OXIDASE GENE2)* encodes an ACC OXIDASE which oxidizes ethylene intermediates to form ethylene. Transgenic *Arabidopsis* plants expressing *CsACO2* under control of the AP3 promoter display repressed stamen development and male sterility (Yin and Quinn, 1995; Duan et al., 2008). Down-regulation of the ethylene receptor gene *ETR1* results in the decrease of the ETR1-interacting kinase CTR1, which a repressor of the ethylene signaling. Loss of ETR1 fails the formation of ETR1-CTR1 complex. Consequently, de-suppression of the ethylene response pathway causes the production of female flowers in *Arabidopsis* (Wang et al., 2010). So far, little is known about the effects of ethylene on flower development in monocots, including maize.

## Gibberellin

In *Arabidopsis*, gibberellin (GA) deficiencies greatly impact male fertility, resulting in partial or complete male sterility. Conversely, GA deficiencies promote stamen maturation in the maize ear, leading to the formation of perfectly bisexual flowers.

In *Arabidopsis*, the GA deficient mutant *ga1-3*, which fails to catalyze the first step in GA biosynthesis due to a deletion in ent-KAURENE SYNTHASE, exhibits abnormal microsporogenesis and retarded growth of all floral organs, e.g., stamens with greatly shortened filaments that cannot pollinate pistils (Michaels and Amasino, 1999; Cheng et al., 2004; Yu et al., 2004). Similarly, the *ga1-1* mutant has severe defects in stamen and pollen maturation as well as petal and sepal growth (Goto and Pharis, 1999). DELLA proteins (transcriptional repressors), such as RGA, RGA-LIKE1 (RGL1), and RGL2, repress stamen development (Cheng et al., 2004). The DELLA degradation triggered by GA activates JA biosynthesis genes *DAD1* and LIPOXYGENASE 1 (*LOX1*; Cheng et al., 2009).

In *Arabidopsis*, during GA biosynthesis, four *GIBBERELLIN 3-OXIDASE (GA3OX)* genes are responsible for the final GA activation. The *ga3ox1 ga3ox3* double mutant shows high frequency of sterility on the lowest siliques with fertility restoration after the 20<sup>th</sup> to 25<sup>th</sup> silique, whereas triple mutants *ga3ox1 ga3ox3 ga3ox4* and *ga3ox1 ga3ox2 ga3ox3*, on average, underwent a later conversion. This sterility is caused by abnormal anther dehiscence and shortened anther filaments, highlighting

that GA is required for stamen development in *Arabidopsis* (Hu et al., 2008).

In the maize ear, GA promotes the arrest of stamens, but prevents carpel abortion. The maize *ANTHER EAR1 (ANI)* gene is necessary for the production of ent-kaurene during GA biosynthesis. Besides short stature and delayed maturity, the *an1* mutant develops perfectly bisexual flowers in ears, indicating the inability of the *an1* plant to successfully abort stamens in the ear (Bensen et al., 1995). In addition, maize dwarf mutants *d1*, *d2*, *d3*, and *d5*, which are deficient in GA production, also form stamens in flowers of the ear (Fujioka et al., 1988; Dellaporta and Calderon-Urrea, 1994; Spray et al., 1996).

Taken together, in both dicots and monocots the male organ development is sensitive to GA, however, its effects are opposite.

## Auxin

In *Arabidopsis* and maize, auxin is required for the formation of all floral organs, as disruption of genes associated with auxin signaling, biosynthesis, and transport leads to flowers with various abnormalities (Okada et al., 1991; Nagpal et al., 2005; Cheng et al., 2006; Wu et al., 2006; Cecchetti et al., 2008). ARFs activate or repress expression of auxin response genes. In *Arabidopsis*, the *arf6 arf8* double mutant and plants expressing miR167 resistant versions of *ARF6* and *ARF8* exhibit many flower defects, such as shortened petals, gynoecium, and stamen filaments, failure to release pollen, as well as abnormal ovules (Nagpal et al., 2005; Wu et al., 2006). In the *arf3/ett* (*ettin*) mutant, a decreased number of stamens are observed (Sessions et al., 1997). In the *Arabidopsis floral organs in carpels (foc)* mutant, increased expression of *ARF10*, *16*, and *17* due to the lack of its negative regulator miR160 results in floral organ loss and abnormal female fertility (Liu et al., 2010). In rice, expressing the miR160 resistant version of *OsARF18* causes the formation of abnormal flowers and reduced seed set (Huang et al., 2016a). Mutations in *arf5/mp* (*monopteros*) lead to either small or absent lateral flowers (Przemeck et al., 1996). *YUCCA* (*YUC*) genes in *Arabidopsis* encode auxin biosynthesis enzymes. Stamens in the *yuc2yuc6* double mutant fail to elongate but produce pollen grains, while flowers in the *yuc1yuc4* double mutant cannot form functional reproductive organs (Cheng et al., 2006). Ectopic expression of the small protein ligand TAPETUM DETERMINANT1 (*TPD1*) causes abnormal ovule and seed development via altering auxin signaling (Huang et al., 2016b,c). In maize, mutation in the *SPARSE INFLORESCENCE1 (SPI1)* gene, which functions as a *YUC*-like gene, results in tassels with small ears and few kernels (Gallavotti et al., 2008a).

Many genes in the auxin transport pathway play key roles in maintaining fertility and normal floral organ development. In *Arabidopsis*, PIN-FORMED (PIN) transporters function in polar auxin transport. In the *pin1-1* mutant, various phenotypes, such as missing stamens, the formation of sterile pistil-like structures, and abnormal petal shape, are observed (Okada et al., 1991). In maize, *BARREN INFLORESCENCE1 (BIF1)* and *BARREN INFLORESCENCE2 (BIF2)* are involved in regulating polar auxin transport. BIF1 likely acts upstream of polar auxin transport via up-regulating the expression of *ZmPIN1a* (Gallavotti et al., 2008b). The tassels and ears in *bif1* and *bif2* mutants have reduced

number of spikelets/florets and floral organs, and consequently fewer kernels (McSteen and Hake, 2001; Barazesh and McSteen, 2008; Skirpan et al., 2009). The rice gene *LAZY1* (*LA1*), which encodes a novel grass specific protein, is a negative regulator of polar auxin transport (Li et al., 2007). In maize, the *la1-ref* mutant carries a mutation in the maize ortholog of *LA1* (Dong et al., 2013). Spikelets in *la1-ref* either are not fully developed or undergo abortion especially in the tassel tip. Similarly, in the ear, silk production is decreased and spikelets are aborted in the ear tip (Dong et al., 2013).

The role of auxin in flower development is challenging to interpret. Due to the effects of auxin on the entirety of plant, it is possible that some phenotypes are the consequence of larger changes occurring in the plant. For example, as mentioned above, the *LA1* gene regulates polar auxin transport in *Arabidopsis*. *LA1* also affects plant architecture, since the tiller morphology is altered in mutants like *la1-ZF802* in a pattern known as tiller-spreading (Li et al., 2007). These architectural changes may alter photosynthesis which consequently affects fertility and yield (Wu et al., 2013). Thus, more work should be done to look into specific roles of all involved hormones in flower development and fertility. Effects of hormones on female and male flower development are summarized in **Figure 3**.

## STERILITY AND ABIOTIC STRESSES

The loss of yield caused by abiotic stresses is partially attributed to defects in flower development. Even a mild or a short-term abiotic stress can cause a significant decrease in fertility. The majority of studies focus on the effects of abiotic stresses, including heat, cold, and drought, on fertility at the morphological level in *Arabidopsis* and cereal grains; however, the molecular mechanisms behind are not clear.

### Heat Stress

Many stages of flower development, particularly the late stages of stamen development, are sensitive to heat stress. In *Arabidopsis* and cereal grains, sensitive stages include meiosis of pollen mother cells (PMC), tapetum development, anther dehiscence/pollen release, anthesis, and fertilization (Dupuis and Dumas, 1990; Kim et al., 2001; Abiko et al., 2005; Oshino et al., 2007; Thakur et al., 2010; Zinn et al., 2010; De Strome and Geelen, 2014). The overall effects of heat stress on male sterility depend on duration, timing, and temperature (Schoper et al., 1986, 1987a,b). The female organ is not as susceptible as the male organ to the heat stress.

The tapetum in the anther is particularly vulnerable to heat stress (Parish et al., 2012). In *Arabidopsis*, the tapetum consists of a monolayer of cells, which surrounds successive stages of microsporocytes, tetrads, microspores, and developing pollen as anther development progresses. Tapetal cells undergo three stages: differentiation, maturation, and PCD. First, the early differentiated tapetal cells secrete the callase enzyme that is required for the release of haploid microspores from meiotic tetrads. Second, mature tapetal cells produce a large amount of specialized non-photosynthetic plastids (elaioplasts) and

tapetosomes, which provide lipids, proteins, and sporopollenin essential for pollen wall formation. Finally, tapetal cells are degenerated via PCD, and the remnants are important for the completion of pollen wall formation (McCormick, 1993; Wu and Cheung, 2000; Parish and Li, 2010).

The abnormal tapetum or altered timing of its degeneration causes pollen defects and consequently male sterility. Barley and wheat grown at elevated temperatures (barley: 30–35°C day/20–25°C night, wheat: 30°C for 1–3 days, or varied 30/20°C day/night at meiosis) display precocious tapetum degradation (Saini and Aspinall, 1982; Saini et al., 1984; Abiko et al., 2005; Oshino et al., 2007; Omidi et al., 2014). In rice, tapetal genes like *YY1* and *YY2* are down regulated following heat stress [39/30°C (day/night) for 5 days], affecting tapetum function and consequently pollen viability (Endo et al., 2009). Additionally in rice, male sterility in the thermos-sensitive genic male-sterile (TGMS) line 95850ms is caused by premature tapetum PCD and consequent pollen grain collapse (Ku et al., 2001, 2003). A recent study shows that the TGMS trait in the *thermosensitive genic male sterile 5* (*tms5*) mutant is caused by the loss of function of RNase *Z<sup>1</sup>*, which processes mRNAs of three ubiquitin fusion ribosomal protein genes (*UbL40*) (Zhou et al., 2014). At restrictive temperatures, high level of *UbL40* results in abortive pollen and therefore male sterility. *Arabidopsis* plants under heat stress (31 and 33°C) show reduced expression of *YUCCA* genes especially in tapetum and PMC. Inactivation of *YUC2* and *YUC6* leads to decreased male fertility, while which can be reversed by exogenous application of auxin (Sakata et al., 2010, 2014). More work needs to be done to understand the genetic pathways leading to decreased fertility during heat stress, especially the role of auxin in male fertility and tapetum development.

Another sensitive stage is the PMC meiosis. Wheat and rice exposed to high or varied temperatures [wheat: high 30°C (1–3 days), varied 30°/20°C (day/night), rice: 39/30°C (day/night; 5 days)] at and prior to the onset of PMC meiosis exhibit greatly reduced grain set (Saini and Aspinall, 1982; Saini et al., 1984; Endo et al., 2009; Omidi et al., 2014). Impairments in rice PMC division occur even 5°C over the ambient temperature [28.3/21.3°C (day/night)], resulting in decreased pollen production especially in susceptible cultivars (Prasad et al., 2006).

Anther dehiscence, anthesis, and fertilization are sensitive to elevated temperatures too. Heat stress applied to wheat [two-day intervals of 36/31°C (day/night)] from floral emergence to 3 days post anthesis results in male sterility due to abnormal pollen grains (Tashiro and Wardlaw, 1990; Ferris et al., 1998). Similarly, rice that receives a short-term (33.7°C, 1 h) or a long-term heat stress (35°C, 38°C, and 41°C, 5 days) at anthesis display reduced fertility, but with a better fertility when stress was applied before or after anthesis (Satake and Yoshida, 1978; Jagadish et al., 2007). Heat stressed rice [35/25°C (day/night)] has decreased anther dehiscence and pollen count (Das et al., 2014). Pollen germination is also very vulnerable to high temperature stress. When maize tassels and rice spikelets are subjected to high heat stress [maize: 6 h of 40°C, rice: 35/25°C (day/night) or greater for 3 days], the ability of pollen to fertilize the ear is lost, which is attributed to the failure of pollen tube growth (Dupuis and Dumas, 1990; Das et al., 2014). In *Arabidopsis*, disruption of

*THERMOSENSITIVE MALE STERILE 1 (TMS1)*, which encodes the heat shock protein HSP40, causes pollen tubes to burst and decreased pollen tube length (Yang et al., 2009).

Although the cause of the heat-induced sterility is not clear, it might be related to heat shock proteins like HSP40 mentioned above (Yang et al., 2009). Mutations in the small heat shock protein gene *BOBBERT1 (BOB1)* result in a range of phenotypes, such as irregular flowers and sterile siliques (Perez et al., 2009). In maize, pollen infertility may be due to the lack of production of major protective HSPs (Dupuis and Dumas, 1990; Hopf et al., 1992), supported by the fact that pollen grains do not express HSP RNAs at dehiscence (Dietrich et al., 1991; Young et al., 2001). In wheat, heat-stress induces many HSPs, including HSP17, HSP26, and HSP70, as well as microRNAs targeted *HSP* genes (Kumar et al., 2015).

## Cold Stress

Extensive research has been done about the effects of below optimal temperature conditions on growth and development of *Arabidopsis* and cereal crops. In *Arabidopsis*, a large number of genes are identified with differential expression after chilling stress, and many of which play roles in pollen development (Lee et al., 2005; Zou and Yu, 2010). The *COLD REGULATED (COR)* genes are induced at low temperatures. The WRKY transcription factors repress *COR* expression via binding to their c-repeat binding factors (CBF; Zou et al., 2010). Plants harboring mutated *WRKY* genes show increased pollen viability under the chilling stress. In *Arabidopsis*, freezing treatment ( $0^{\circ}\text{C}$  for 72 h) induces the acclimation of *COR*, lipid transfer proteins, and  $\beta$ -amylase in vegetative tissues, but not in pollen, which may explain the inability of pollen to withstand the chilling stress (Lee and Lee, 2003). In rice, DEHYDRATION RESPONSIVE ELEMENT BINDING PROTEIN1F (*OsDREB1F*) activates the expression of *COR15a*. Overexpression of *OsDREB1F* causes increased cold and drought tolerance, which aids spike development, further highlighting the role of *COR* genes in cold tolerance (Wang et al., 2008). For a review on freezing tolerance genes Thomashow (1999).

In cereal grains, the establishment of reproductive development, branching, and spikelet pair formation are sensitive to low temperature stress. Maize plants grown at cold conditions ( $10^{\circ}\text{C}$  for 3 days or longer) during the reproductive transition produce less tassel branches and spikelet pairs (Bechoux et al., 2000). In the maize inbred line Dent11, chilling stress [ $17/6^{\circ}\text{C}$  (day/night)] leads to the reduction of 43 and 29% of pollen when stress is applied at branch and spikelet initiation, respectively (Tranel et al., 2009).

In anthers, meiosis and tapetum development are particularly cold sensitive. Sorghum and rice display male sterility under cold conditions during meiosis and microspore development (Downes and Marshall, 1971; Mamun et al., 2006; Wood et al., 2006; Gothandam et al., 2007; Sakata et al., 2014). Abnormal tapetum development and degradation under chilling stress results in aberrant pollen (Sakata et al., 2014). Plants insensitive to GA or deficient in GA production exhibit more severe problems in tapetal cell hypertrophy and pollen production under chilling. In the tapetum, chilling stress represses both the cell wall bound

acid invertase gene *OSINV4* and the monosaccharide transporter gene *OSMST8*, which causes failed transport of sugar to the tapetum and developing pollen (Oliver et al., 2005; Mamun et al., 2006). ABA application also leads to abnormal pollen, possibly by repressing *OSINV4* and *OSMST8* (Oliver et al., 2005). More work needs to be done in the future to determine genes responsible for the abnormal development of tapetum under chilling conditions.

Later in development, anthesis and pollen germination are also cold sensitive. In wheat, chilling conditions [ $8/2^{\circ}\text{C}$  (day/night)] applied to anthesis result in high levels of male sterility (Subedi et al., 1998). In *Arabidopsis*, freezing stress causes reduced pollen tube growth and decreased seed production. Similarly, mutations in G protein-coupled receptor-type G proteins (GTGs) lead to decreased pollen germination, abnormal pollen tube elongation, and consequent seed loss (Jaffé et al., 2012). In rice, the QTL *COLD1*, which encodes a regulator of G-protein signaling, acts to sense chilling (Ma et al., 2015). *COLD1* is important for maintaining grain yield, further suggesting that G-protein signaling plays a key role in chilling tolerance during sexual reproduction. In young rice panicles, the *Ctb1* QTL harbors an F-box protein gene that is responsible for chilling tolerance (Saito et al., 2010). Additionally, upregulation of the *CORN CYSTATIN* genes *CC8* and *CC9* is observed under cold stress ( $14$  and  $14/7^{\circ}\text{C}$ ). *CC8* is found in kernel and the immature tassel, while *CC9* is detected in immature and mature tassels, silk, and kernels (Massonneau et al., 2005). Future study looking into the roles of these cystatins in fertility could be valuable.

Collectively, similar to what is observed under heat stress, the stamen development is sensitive to cold stress, particularly during meiosis, tapetum development, pollen germination, and anthesis. The female organ development remains relatively unaffected to cold stress. However, not all studies agree with this finding. In maize, prolonged exposure to cold stress ( $10^{\circ}\text{C}$  for 7 days) results in the abortion of the ear (Lejeune and Bernier, 1996). The effects of ear abortion may be prevented by applying benzyladenine (a synthetic cytokinin) exogenously (Lejeune et al., 1998). Genes like *COR* and those involved in GA and G-protein signaling may play important roles in chilling tolerance during sexual reproduction.

## Drought Stress

Similar to heat, drought stress affects flower development and consequently impairs fertility. In stamen development, drought stress causes shortened anther filaments, delayed anther development and dehiscence, as well as reduced pollen viability (Su et al., 2013; Tunc-Ozdemir et al., 2013; Ma et al., 2014). Female fertility is less sensitive to drought stress (Su et al., 2013). Younger buds are sacrificed during early drought stress and water is likely allocated to older flowers (Su et al., 2013).

Under moderate and severe drought stresses, thousands of genes are differentially expressed (Ma et al., 2014). Genes like *DREB1*, *ABA-RESPONSIVE ELEMENTS BINDING FACTORS (ABF)*, *NAC DOMAIN CONTAINING PROTEIN019 (NAC019)*, *RESPONSIVE TO DESSICATION20 (RED20)*, and *RD29A* were upregulated (Su et al., 2013). A great number of genes involved in ABA and JA signaling are also upregulated, which may affect stamen filament elongation as well as overall stamen and pistil

development (Su et al., 2013). *CYCLIC NUCLEOTIDE-GATED CHANNEL16* (*CNGC16*) is important for stress response, as disruption of *CHGC16* leads to reduced pollen viability (Tunc-Ozdemir et al., 2013).

Some cereals like rice and wheat are sensitive to drought stress, whereas others such as sorghum are quite drought tolerant. In maize, female organ development, particularly prior to pollination, is sensitive to drought stress, which is often attributed to problems with carbohydrate transport and metabolism. When comparing well watered with drought treated plants, carbohydrate transport to ovary is decreased in drought conditions and expression of carbohydrate (e.g., starch and sucrose) metabolism genes is altered (Mäkelä et al., 2005; Kakumanu et al., 2012). Many genes in maize kernels show differential expression under drought stress, such as those important for carbohydrate metabolism (*SU1P*, *ISA1*, *DULL1*, *FRK2*, *GLU1*, and *AAG1*), stress response and regulation (*ZmDJ1*, *SOD1*, and *STII*), and transcriptional regulation of drought inducible genes (*EREBP1*, *MYB-IF35*, *MYB-IF25I*, and *RISBZ4*; Marino et al., 2009). Under drought conditions, genes involved in cell cycle, cell division, and antioxidant formation are down regulated in ovaries, while genes essential for stress responses like ABA are expressed at higher levels. Increased ABA may lead to a reduction of invertase in ovaries, limiting sucrose use and subsequent ovary abortion (Zinselmeier et al., 1999; Andersen et al., 2002; Boyer and Westgate, 2004; McLaughlin and Boyer, 2004; Kakumanu et al., 2012).

During meiosis, drought stress results in differential expression of many genes, for example, genes encoding  $\beta$ -carotene hydroxylase and cytochrome P450 monooxygenase which might protect against oxidative damage. Altered expression was also observed in genes that encode histone H2A and dehydrin DHN1, suggesting the importance of chromatin stabilization and dehydration prevention under drought stress (Zhuang et al., 2008). After pollination, elevated expression of senescence genes may be the cause of embryo abortion (McLaughlin and Boyer, 2004). Interestingly, pollen development was relatively unaffected by drought stress in maize (Schoper et al., 1986; Westgate and Boyer, 1986).

Drought stress is detrimental to pollen production in wheat, resulting in a 40–50% of reduction in yield (Dorion et al., 1996). Drought-induced degeneration of tapetal cells may contribute to the failure of microspore and pollen development. The timing of tapetum degeneration is crucial, as its early degeneration results in loss of orientation, and late degeneration leads to microspores that do not receive essential nutrients (Saini et al., 1984; Lalonde et al., 1997; Ji et al., 2010). In addition, pollen developed under drought condition is devoid of starch, limiting fertilization, and pollen tube growth (Ji et al., 2010). In wheat, drought stress decreases the level of invertases in developing pollen and microspores, (Dorion et al., 1996; Lalonde et al., 1997; McLaughlin and Boyer, 2004; Koonjul et al., 2005). Drought tolerant lines have a normal invertase expression (Ji et al., 2010). In wheat, no effects are observed on female fertility under moderate drought stress (Saini and Aspinall, 1981; Ji et al., 2010).

In rice, male sterility is common under drought stress conditions. If drought conditions are applied during PMC

meiosis, the pollen production is severely affected (Sheoran and Saini, 1996), which is potentially caused by tapetal cell vacuolization/degeneration and abnormal starch deposition (Nguyen and Sutton, 2009; Jin et al., 2013). Under drought conditions, the presence of reactive oxygen species (ROS) results in a depletion of ATP and therefore leads to PCD and pollen abortion in rice (Nguyen et al., 2009). Furthermore, expression of genes critical for tapetal cell PCD and pollen wall formation is altered along with increased ABA signaling and decreased GA signaling (Jin et al., 2013). Both invertase and starch synthase gene expression are reduced under drought stress (Sheoran and Saini, 1996; Nguyen et al., 2010). Conversely, genes involved in sugar transport are upregulated (Sheoran and Saini, 1996; Nguyen et al., 2010; Fu et al., 2011). The accumulation of sugar may help maintain water levels in the anther due to low water potential (Fu et al., 2011).

It is not clear why the stamen development and male fertility appear more susceptible to abiotic stresses in plants. It will be necessary to identify genes that first respond to abiotic stresses and genes that later build strength for plants to cope with long-term abiotic stresses. Hormones, such as ABA and auxin, are heavily involved in abiotic stresses. It is well known that cross-talks are important for plant hormonal signaling; however, little is known about cross-talks among hormones in response to different abiotic stresses. The effects on fertility and some potential genes involved under abiotic stresses are summarized in Table 2 and Figure 4.

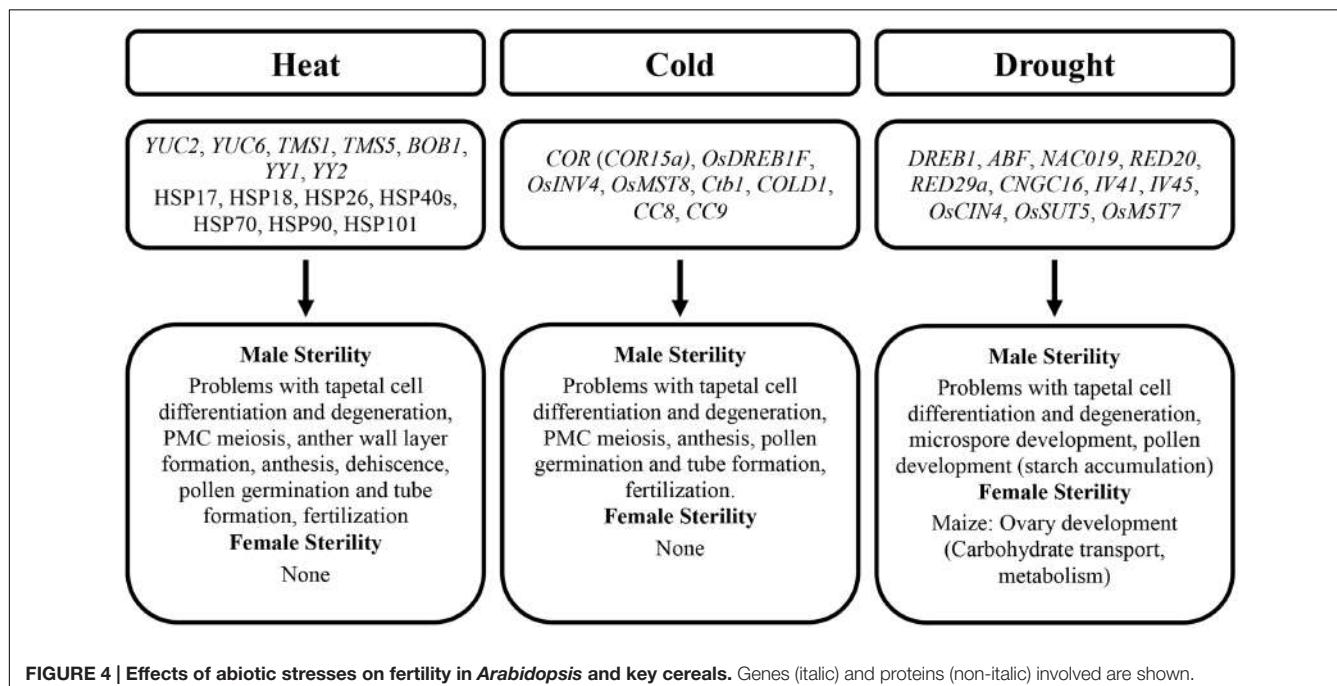
## CONCLUSION AND FUTURE DIRECTIONS

Floral organ degeneration or abortion under the normal condition results in the formation of unisexual flowers, such as in maize; or completely sterile flowers, such as in sorghum. In addition to other genes, class B and C genes are involved in floral reproductive organ degeneration via losing their functions in floral organ identity or in regulating expression of downstream target genes. Moreover, hormones play important roles in establishing the male and female state. Genes underlying JA and BR signaling and their biosynthesis promote stamen development and carpel abortion, whereas genes involved in GA signaling and their biosynthesis induce carpel development and stamen abortion (with exception of the maize ear). Auxin is essential for the formation of all floral organs, including stamen and carpels. Interactions between flower and hormone regulation genes are essential for flower organ establishment and fertility.

It is evident that maintaining ideal temperature and soil moisture is crucial for fertility in *Arabidopsis*, maize, wheat, and rice. Abiotic stresses commonly lead to male sterility, while female viability is well maintained under most mild abiotic stresses. In nearly all plants under all observed abiotic stresses, the most sensitive stages causing sterility are during tapetum development, male meiosis, microsporogenesis, anthesis, and fertilization. Hormones play important roles in male and female organ development during abiotic stresses. Auxin application can reverse some effects of heat stress, whereas decreased GA in

**TABLE 2 | Effects of abiotic stresses on sterility and genes involved.**

| Stress   | Organism           | Key effects of stress  | Genes involved  |
|----------|--------------------|--|---|
| Heat     | <i>Arabidopsis</i> | Abnormal microsporogenesis, irregular pollen and male sterility  | <i>TMS1, BOB1, YUC2, YUC6</i>   |
| Heat     | Maize              | Decreased pollen germination and pollen tube growth, decreased number of silks and florets, kernel abortion          | <i>HSP70, HSP18, HSP101</i>   |
| Heat     | Wheat              | Early tapetum degradation, decreased pollen viability, reduced anther size, irregular embryo sac development         | <i>ARF, TPR</i>   |
| Heat     | Rice               | Abnormal microsporogenesis, decreased pollen production and viability, altered flower timing                         | <i>YY1, YY2</i>   |
| Chilling | <i>Arabidopsis</i> | Decreased pollen viability and pollen tube growth  | <i>COR, WRKY, CBF1</i>  |
| Chilling | Maize              | Decreased number of tassel branches and spikelet pairs, decreased pollen production, ear abortion                    | <i>CC8, CC9</i>   |
| Chilling | Wheat              | Pollen death and male sterility, no affects on female development  | None  |
| Chilling | Rice               | Abnormal microspore, pollen development, and tapetum degradation   | <i>COLD1, OsINV4, OsMST8, OsDREB1F</i>  |
| Drought  | <i>Arabidopsis</i> | Decreased anther filament length, delays in anther development and dehiscence, decreased pollen viability            | <i>DREB1, ABF, NAC019, RED20, RD29A, CNGC16</i>   |
| Drought  | Maize              | Decreased number of kernels and increased embryo abortion  | <i>TM00030371, TM00036151, H2A, DHN1, SIP1, SU1P, ISA1, DULL1, FRK2, GLU1, AAG1, ZmDJ1, SOD1, ST11, EREBP1, MYB-IF35, MYB-IF251, RISBZ4</i> |
| Drought  | Wheat              | Abnormal microspore and tapetum development, pollen devoid of starch, decreased fertilization and pollen tube growth | <i>IVR1, IVR5</i>   |
| Drought  | Rice               | Decreased pollen viability, abnormal tapetal degeneration and starch deposits in pollen                              | <i>OsDREB1F, OsmiR408, OsCIN4, OsSUT5, OsM577</i>   |

**FIGURE 4 | Effects of abiotic stresses on fertility in *Arabidopsis* and key cereals.** Genes (italic) and proteins (non-italic) involved are shown.

stressed plants worsens tapetum defects and consequently further reduces pollen production.

Overall, abiotic stress induced sterility causes the major loss of crop yield. By 2050 the global population is expected to reach 9.1 billion. Additionally, if the use of grains for biofuel production is intensified, the demand for crop products will be further increased. High-yield wheat ideotypes are currently being studied and improved based on long-term climate projections (Semenov and Stratonovitch, 2013). To develop high-yield crops

that have ideal agronomic traits and can cope with anticipated environmental changes using traditional and molecular breeding approaches, it is necessary to decipher molecular genetic mechanisms that cause flower sterility under normal and stress conditions.

## AUTHOR CONTRIBUTIONS

AS and DZ conceived the idea and wrote the manuscript.

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# Reproduction and the pheromonal regulation of sex type in fern gametophytes

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The fern life cycle includes a haploid gametophyte that is independent of the sporophyte and functions to produce the gametes. In homosporous ferns, the sex of the gametophyte is not fixed but can vary depending on its social environment. In many species, the sexual phenotype of the gametophyte is determined by the pheromone antheridiogen. Antheridiogen induces male development and is secreted by hermaphrodites once they become insensitive to its male-inducing effect. Recent genetic and biochemical studies of the antheridiogen response and sex-determination pathway in ferns, which are highlighted here, reveal many similarities and interesting differences to GA signaling and biosynthetic pathways in angiosperms.

**Keywords:** antheridiogen, sex determination, ferns, GA signaling, GA biosynthesis

## INTRODUCTION

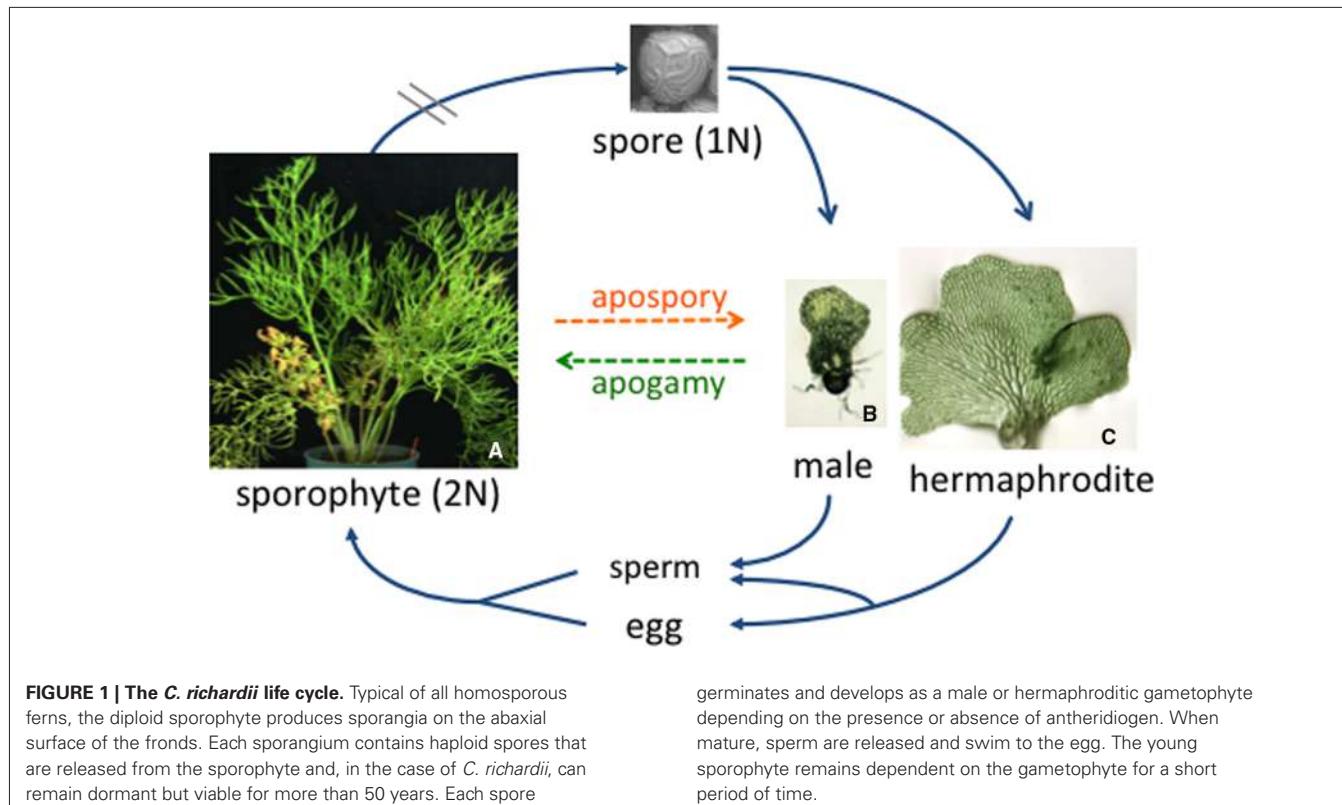
The fern life cycle, illustrated in **Figure 1**, features two distinct body types: the large diploid sporophyte and the tiny haploid gametophyte. From a reproduction point of view, the sole function of the sporophyte is to produce then release haploid spores, while the gametophyte, which grows from a spore, functions to produce the gametes. Some ferns, like all angiosperms, are heterosporous and produce both mega- and microspores that are destined to develop as female and male gametophytes, respectively. Most ferns species are homosporous and produce only one type of spore. While textbook drawings of homosporous fern gametophytes typically show a heart-shaped hermaphrodite, fern gametophytes can be male, female, male then female, female then male, hermaphroditic or asexual, depending on the species. In this review we highlight old and recent studies that have revealed the fascinating cross-talk that occurs between neighboring gametophytes in determining what their sexual phenotype will be.

## ASEXUAL REPRODUCTION IN FERN GAMETOPHYTES

In addition to reproducing sexually, there are many examples of fern gametophytes that circumvent sex and reproduce asexually. The most common type of asexual reproduction is apogamy, whereby a sporophyte plant develops from a gametophyte without fertilization, similar to apomixis in angiosperms. In naturally occurring apogamous species, the viable spores produced by the sporophyte have the same chromosome number as the sporophyte (Walker, 1962, 1979). Obligate apogamy often occurs naturally in species of ferns that produce no or only one type of gametangia. Because water is required for the flagellated sperm to swim to the egg in ferns, apogamous species are typically found in dry habitats where water is limiting (White, 1979). Apogamy also can be artificially induced in many ferns by adding sucrose to the culture media in which gametophytes are grown (Whittier and Steeves, 1962; White,

1979). By optimizing the conditions for inducing apospory in *Ceratopteris richardii* gametophytes, a recent study has established *C. richardii* as a useful experimental system for studying this phenomenon (Cordle et al., 2007). Induced apogamous sporophytes of *C. richardii* have features typical of the sporophyte, including stomata, vascular tissue and scale-like ramenta; however, they are abnormal compared to sexually-derived diploid sporophytes, which could be a consequence of being haploid. To better understand how sucrose promotes the development of a sporophyte from cells of the gametophyte, the same researchers identified 170 genes whose expression is up-regulated during the period of apogamy commitment. Many of them are associated with stress and metabolism or are homologs of genes preferentially expressed in seed and flower tissues (Cordle et al., 2012). Understanding apogamy, coupled with studies of apospory in *C. richardii*, where diploid gametophytes develop from cells of sporophyte leaves without meiosis (DeYoung et al., 1997), should provide useful insights into genes and molecular mechanisms that regulate the alternation of gametophyte and sporophyte generations in ferns in the absence of meiosis and fertilization.

A second form of asexual reproduction in homosporous ferns involves vegetative propagation of the gametophyte. While relatively rare, such gametophytes typically do not produce sex organs. The fern *Vittaria appalachiana*, for example, is only known from its gametophytes (Farrar and Mickel, 1991). Each gametophyte forms vegetative buds, or gammae, that allow gametophytes to multiply and form mats in dark, moist cavities and rock shelters in the Appalachian Mountains. While the origin of *V. appalachiana* (is it a recent hybrid or ancient relict?) and why it is unable to form sporophytes are unknown at this time, its persistent gametophyte suggest that fern gametophytes, like bryophyte gametophytes, can persist and thrive for very long periods of time.



## SEXUAL REPRODUCTION

Most homosporous ferns that reproduce sexually ultimately form hermaphroditic gametophytes that have antheridia and archegonia. While hermaphroditism increases the probability that a single gametophyte will reproduce, self-fertilization of a hermaphrodite (which is genetically similar to a doubled haploid in angiosperms) results in a completely homozygous sporophyte. Given that this absolute inbreeding could have negative consequences to the individual and reduce genetic variation in populations, it is not surprising that homosporous ferns have evolved mechanisms to promote outcrossing. One such mechanism that is common to many species of ferns involves the pheromonal regulation of sexual identity, where the sexual phenotype of an individual gametophyte depends on its social environment.

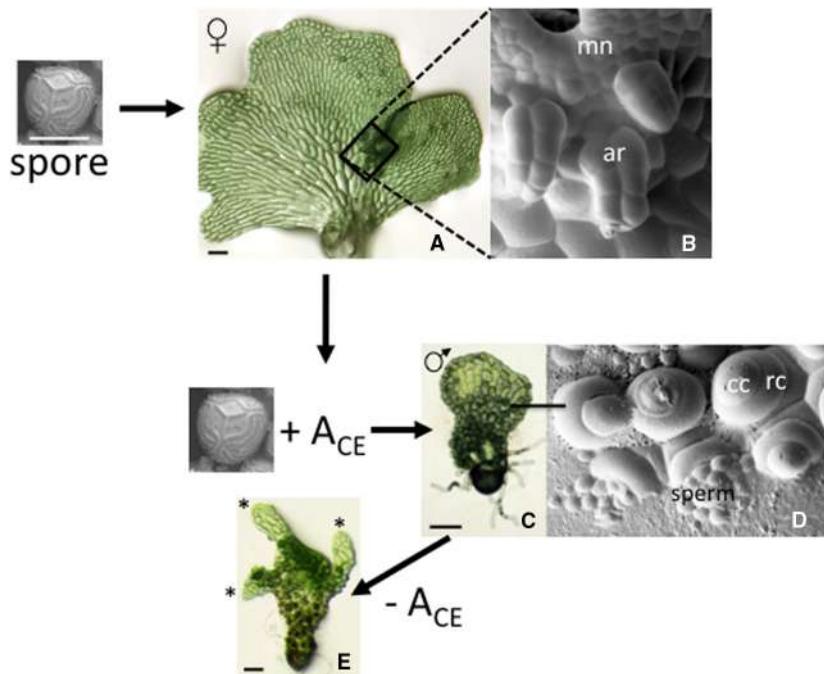
## ONE GENOTYPE—TWO OR MORE PHENOTYPES

In the late 1800's, botanists began noting that fern gametophytes are often sexually dimorphic, with larger gametophytes bearing archegonia and smaller gametophytes bearing antheridia (Prantl, 1881; Yin and Quinn, 1995). The size difference between them was attributed to the presence or absence of a meristem, with females or hermaphrodites being "meristic" (with a meristem) and males "ameristic" (without a meristem). In a major discovery, Döpp noted that the medium harvested from cultures of *Pteridium aquilinum* gametophytes contained a pheromone that promoted the development of males in juvenile gametophytes (Döpp, 1950); this pheromone is referred to as antheridiogen. Antheridiogens or antheridiogen responses have since been identified in over 20 species of

ferns (Yamane, 1998; Kurumatani et al., 2001; Jimenez et al., 2008).

Much of what is known about the biology of antheridiogen responses can be attributed to studies by Näf and Schraudolf during the 1950s and 1960s (reviewed in Näf, 1959, 1979). This response is illustrated here for the fern *C. richardii*, originally characterized by Hickok et al. (1995). In this species, an individual spore always develops as a relatively large hermaphrodite (**Figure 2A**) that produces egg-forming archegonia (**Figure 2B**), sperm-forming antheridia and multicellular lateral meristem. The hermaphrodite also secretes antheridiogen, or  $A_{CE}$  (for antheridiogen *Ceratopteris*) into its surroundings. If the hermaphrodite is removed then replaced with a genetically identical spore, the new spore will develop as an ameristic male gametophyte (**Figure 2C**) with many antheridia (**Figure 2D**) in response to  $A_{CE}$  secreted by the hermaphrodite. In a population of spores, spores that germinate first become hermaphrodites that secrete  $A_{CE}$ , while slower-growing members of the population become male in response to the secreted  $A_{CE}$ . In comparison to chromosomal based sex determination, this mechanism of sex-determination is unusual because it allows the ratio of males to hermaphrodites to vary depending on population size and density and it is inherently flexible rather than fixed.

Typical of other ferns, a *C. richardii* gametophyte is able to respond to  $A_{CE}$  for a limited period of time, prior to the establishment of a lateral meristem. The lateral meristem not only confers indeterminate growth to the gametophyte, but its formation coincides with a loss in ability to respond to  $A_{CE}$  as well as the secretion of  $A_{CE}$ . Archegonia invariably initiate



**FIGURE 2 | The antheridiogen response in *C. richardii*.** A single spore always develops as a hermaphrodite when grown in the absence of  $A_{CE}$ . The hermaphrodite consists of a single sheet of cells with a distinct multicellular meristem that forms a meristem notch and multiple archegonia that develop adjacent to the meristem notch, which are highlighted in the SEM (boxed area of the hermaphrodite). Hermaphrodites secrete  $A_{CE}$ ; in the presence of  $A_{CE}$ , spores develop as

males. The male lacks a meristem and almost all cells differentiate as antheridia. The SEM shows six antheridia, each having a ring cell and a cap cell that pops open to release sperm. When a male gametophyte is transferred to media lacking  $A_{CE}$ , some cells divide and begin to form a hermaphroditic prothallus. The “switched” male shown is forming three such prothalli. mn: meristem notch; ar: archegonia; cc: cap cell; rc: ring cell.

close to the meristem notch of the hermaphrodite, well after the lateral meristem is well developed. While the hermaphroditic program of expression cannot be reversed, the male program of expression is reversible. Cells of the male gametophyte prothallus, when transferred to media lacking  $A_{CE}$ , will divide to ultimately form one or more new hermaphroditic prothalli (**Figure 2E**). Antheridiogen thus serves multiple functions in male gametophyte development: it represses divisions of the prothallus that establish the lateral meristem; it promotes the rapid differentiation of antheridia; it represses its own biosynthesis; and it serves to maintain in the gametophyte an ability to respond to itself.

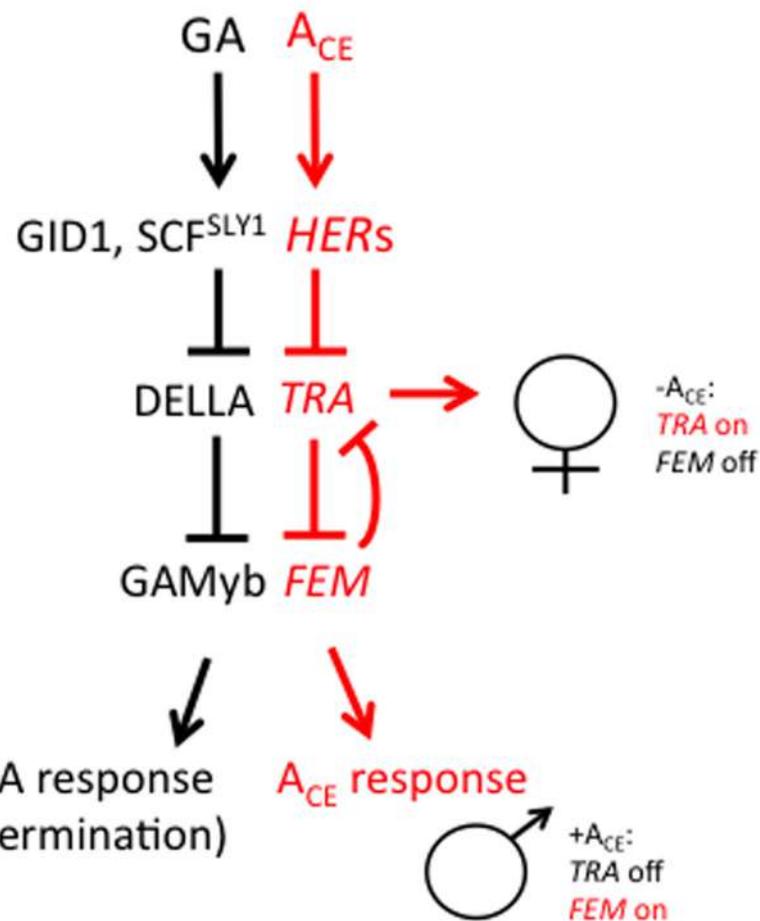
All of the antheridiogens that have been structurally characterized from ferns are gibberellins (GAs) (Yamane et al., 1979; Furber et al., 1989; Takeno et al., 1989; Yamane, 1998). Although the structure of  $A_{CE}$  is unknown, GA biosynthetic inhibitors reduce the proportion of males in a population of *C. richardii* gametophytes suggesting that  $A_{CE}$  and GA share a common biosynthetic pathway (Warne and Hickok, 1989). ABA, a known antagonist of GA responses in angiosperms, completely blocks the  $A_{CE}$  response in *C. richardii*, also indicating that  $A_{CE}$  is likely a GA (Hickok, 1983).

### THE SEX-DETERMINING PATHWAY IN *Ceratopteris*

Most recent studies aimed at understanding how antheridiogen determines the sex of the gametophyte have focused on

two species of homosporous ferns: *C. richardii* and *Lygodium japonicum*. *Ceratopteris richardii* is a semi-tropical, annual species and is useful as a genetic system for many reasons. Large numbers of single-celled, haploid spores (typically  $10^6$ ) can be mutagenized and mutants identified within 2 weeks after mutagenesis. Gametophytes can be dissected and regrown, making it possible to simultaneously self-fertilize and out-cross a single mutant gametophyte. Because self-fertilization of a gametophyte results in a completely homozygous sporophyte that produces  $>10^7$  spores within a 6-month period, suppressor mutants are also easy to generate. Because *C. richardii* gametophytes are sexually dimorphic, mutations affecting the sex of the gametophyte are especially easy to identify (Hickok, 1977, 1985; Hickok et al., 1985, 1991; Warne and Hickok, 1986; Warne et al., 1988; Hickok and Schwarz, 1989; Vaughn et al., 1990; Scott and Hickok, 1991; Chun and Hickok, 1992; Banks, 1994, 1997a,b; Eberle and Banks, 1996; Strain et al., 2001; Renzaglia et al., 2004). Over 70 mutants affecting sex determination have been characterized, most falling into three major phenotypic groups: the *hermaphroditic* (*her*) mutants, which are hermaphroditic in the presence or absence of  $A_{CE}$ , the *transformer* (*tra*) mutants, which are male in the presence or absence of  $A_{CE}$ , and the *feminization* (*fem*) mutants, which are female in the presence or absence of  $A_{CE}$  and produce no antheridia. Through test of epistasis (i.e., comparing mutant phenotypes of single and various combinations of double and triple mutants), a genetic model of the sex determination pathway has been developed and

## Arabidopsis GA pathway *Ceratopteris* SD pathway



**FIGURE 3 | A comparison of the GA signaling pathway in angiosperms and the sex-determining (SD) pathway in *C. richardii*.** The SD pathway in *C. richardii* is based solely on the epistatic interactions among

sex-determining mutants but it is consistent with recent molecular and biochemical studies in the fern *L. japonicum*. T bars represent repressive events whereas arrows indicate activating events.

is illustrated in **Figure 3** (Eberle and Banks, 1996; Banks, 1997a,b; Strain et al., 2001). This pathway reveals that there are two major regulators of sex: *TRA*, which is necessary for lateral meristem and archegonia development (female traits), and *FEM*, which is necessary for antheridia development (the male trait). *FEM* and *TRA* negatively regulate each other such that only one can be expressed in the gametophyte. What determines whether *FEM* or *TRA* is expressed in the gametophyte is *A<sub>CE</sub>*. *A<sub>CE</sub>* activates the *HERs*, which, in turn, repress *TRA*. Because *TRA* cannot repress *FEM*, *FEM* is expressed and the gametophyte develops as a male. In the absence of *A<sub>CE</sub>*, *HER* is not active and is thus unable to repress *TRA*. *TRA* promotes the development of a gametophyte with female traits and represses the development of antheridia by repressing the *FEM* gene that promotes male development. Additional genetic experiments have revealed that the repression of *FEM* by *TRA* and of *TRA* by *FEM* is indirect and involves other genes (Strain et al., 2001). What is remarkable about this pathway is that it is inherently flexible, which is consistent with what is understood about sex determination in this species by

*A<sub>CE</sub>*. This “battle of the sexes”—deciding whether to be male or female—depends on which of the two major regulatory sex genes prevails in the young gametophyte, a decision that is ultimately determined by the presence or absence *A<sub>CE</sub>*.

While this model explains how male and female gametophyte identities are determined, it does not explain the hermaphrodite. One possibility is that in certain cells of the hermaphrodite, the activities of *FEM* and *TRA* are reversed, allowing *FEM* to be expressed in cells that will eventually differentiate as antheridia. Testing this and other possibilities will require the cloning of the sex-determining genes and assessing their temporal and spatial patterns of expression in the developing hermaphrodite.

The sex-determining pathway in *C. richardii* is remarkable in its resemblance to the GA signaling pathway in angiosperms (Sun, 2011), as illustrated in **Figure 3**. In *Arabidopsis*, GA is bound by its receptor GIBBERELLIN INSENSITIVE DWARF1 (GID1). The GA-GID1 complex triggers the rapid proteolysis of one or more DELLA proteins that are ultimately responsible for repressing GA responses. Proteolysis of DELLA requires

GID1 and the specific F-box protein SLEEPY1 (SLY1), which promotes poly-ubiquitination of DELLA by the SCR<sup>SLY1/GID2</sup> complex and results in its degradation by the 26S proteasome. Since DELLA acts as a repressor of GA responses, its GA-induced degradation results in a GA response. While targets of DELLA repression have been identified (Fleet and Sun, 2005), in the case of barley seed germination (which requires GA), DELLA directly or indirectly represses GAMYB, a transcription factor that promotes  $\alpha$ -amylase expression in germinating barley seeds (Gubler et al., 1995, 1999). Based on the similarities between the GA signaling pathway in angiosperms and the sex determination pathway in *C. richardii*, it is tempting to speculate that the *HER* genes in *C. richardii* encode GID1 and SLY1, that *TRA* encodes a DELLA protein, and that *FEM* encodes a GAMYB-like protein.

### ANTHERIDIEN BIOSYNTHESIS IS SPLIT BETWEEN YOUNG AND OLDER GAMETOPHYTE IN *Lygodium japonicum*

*Lygodium japonicum* is another homosporous fern species with an antheridiogen response. This species has the distinct advantage of having its antheridiogens structurally well characterized. Two different GAs have been identified as antheridiogens in this species, including GA<sub>9</sub> methyl ester (Yamane et al., 1979) and GA<sub>73</sub> methyl ester (Yamane et al., 1988). GA<sub>73</sub> methyl ester is the most active antheridiogen and is able to induce antheridia formation at the incredibly low concentration of 10<sup>-15</sup> M. To test the hypothesis that antheridiogen is synthesized through the GA biosynthetic pathway, *L. japonicum* genes related to five different GA synthesis genes, including *ent-copalyl diphosphate/ent-kaurene synthase* (CPS/KS), *ent-kaurenoic acid oxidase* (KAO), *kaurene oxidase* (KO), *GA 20-oxidase* (GA20ox), and *GA3-oxidase* (GA3ox), were identified and their expression patterns in developing gametophytes investigated (Tanaka et al., 2014). Their expression patterns revealed that all but GA3ox were more highly expressed in older gametophytes that secrete antheridiogen, consistent with the expectation that antheridiogen biosynthesis genes are up-regulated in gametophytes that secrete it. GA3ox expression showed the opposite pattern of expression; i.e., it was more highly expressed in young gametophytes that did not secrete antheridiogen but were capable of responding to antheridiogen. To explore this further, the same authors assayed the effects of prohexadione, a GA3ox inhibitor, on antheridia formation in the presence of GA<sub>4</sub> (which has an OH group at the C3 position) or GA<sub>9</sub> methyl ester (which lacks the OH group at C3); both GA<sub>9</sub> and GA<sub>4</sub> induce antheridia formation by themselves. Whereas prohexadione plus GA<sub>9</sub> methyl ester inhibited antheridia formation, prohexadione plus GA<sub>4</sub> did not, demonstrating that C3 hydroxylation of antheridiogen is essential for inducing antheridia formation. In another series of experiments, the authors found that GA<sub>9</sub> methyl ester was converted to GA<sub>9</sub> in young gametophytes. Based on these and other results, a model was proposed whereby antheridiogen (GA<sub>9</sub> methyl ester) is synthesized via a GA biosynthetic pathway and secreted by older gametophytes. When it is taken up by younger gametophytes, the methyl ester is removed by a possible methyl esterase then hydroxylated at the C3 position by GA3ox to GA<sub>4</sub>, where it is perceived and transduced by the GA signaling pathway in young

gametophyte. Because GA<sub>9</sub> methyl ester is more hydrophobic and more efficiently taken up by gametophytes than GA<sub>9</sub>, splitting the GA biosynthetic pathway between young and older gametophytes was proposed to enhance the sensitivity of young gametophytes to the secreted antheridiogen by their neighbors and, at the same time, promote the activation of male traits once inside the young gametophyte (Tanaka et al., 2014).

In addition to characterizing antheridiogen biosynthesis in *L. japonicum*, Tanaka et al. (2014) also made two other important discoveries. They found that a *L. japonicum* DELLA protein was degraded in GA<sub>4</sub> and GA<sub>9</sub> methyl ester treated gametophytes, and that the *L. japonicum* GID1 and DELLA proteins could interact in a yeast–two hybrid assay, but only in the presence of GA<sub>4</sub> (and not GA<sub>4</sub> methyl ester or GA<sub>9</sub> methyl ester). All told, the results of these experiments were used to define a model of the antheridiogen response in *L. japonicum* that is remarkably similar to the pathways illustrated in Figure 3.

### FUTURE DIRECTIONS

The elucidation of the antheridiogen biosynthetic and signaling pathways in ferns has only just begun and many questions regarding sex determination and sexual reproduction remain, many of which can be resolved by cloning all of the sex determining genes. Some of these questions are: To what extent are other hormones involved in sex determination? Is the split GA biosynthetic pathway in *L. japonicum* typical of other ferns? What is the relationship between the antheridiogen response in the gametophyte to GA responses in the sporophyte? Knowing that some mutations in *C. richardii* (e.g., *her* mutations) have no effect on the sporophyte while other mutations (e.g., *tra* mutations) severely affect the sporophyte suggest that at least some, but not all, genes are necessary in both generations. Is antheridiogen also involved in the developmental decision to produce mega- and micro-sporangia in heterosporous ferns? From an evolutionary perspective, was the antheridiogen signaling and responses in the gametophyte co-opted during or important for the evolution of heterospory from homospory in ferns? Addressing these and other questions will lead to a more comprehensive understanding of sex determination in ferns, including an understanding of the molecular mechanisms at play.

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# The male germline of angiosperms: repertoire of an inconspicuous but important cell lineage

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The male germline of flowering plants constitutes a specialized lineage of diminutive cells initiated by an asymmetric division of the initial microspore cell that sequesters the generative cell from the pollen vegetative cell. The generative cell subsequently divides to form the two male gametes (non-motile sperm cells) that fuse with the two female gametophyte target cells (egg and central cells) to form the zygote and endosperm. Although these male gametes can be as little as 1/800th of the volume of their female counterpart, they encode a highly distinctive and rich transcriptome, translate proteins, and display a novel suite of gamete-distinctive control elements that create a unique chromatin environment in the male lineage. Sperm-expressed transcripts also include a high proportion of transposable element-related sequences that may be targets of non-coding RNA including miRNA and silencing elements from peripheral cells. The number of sperm-encoded transcripts is somewhat fewer than the number present in the egg cell, but are remarkably distinct compared to other cell types according to principal component and other analyses. The molecular role of the male germ lineage cells is just beginning to be understood and appears more complex than originally anticipated.

**Keywords:** angiosperm sperm cells, male chromatin modification, male gamete expression, male germ lineage, pollen

The male gametophyte (pollen) of angiosperms is among the most reduced independent multicellular organisms in biology. Pollen is comprised largely of a vegetative cell that forms a pollen tube, which conveys the non-motile sperm cells that it contains into the female gametophyte. The male germline arises from an eccentric division of the post-meiotic haploid microspore that cleaves a relatively small lenticular generative cell from its much larger brother vegetative cell. This sessile generative cell migrates into the vegetative pollen cell and is the founding cell of the male germ lineage. Ultimately the generative cell forms two sperm cells—either in the pollen grain or pollen tube depending on the plant—that constitute the male gametes of flowering plants. Remarkably, both of the male gametes are required in the process of double fertilization. Fusion of one sperm cell with the egg cell results in an embryo—which forms the next generation; whereas fusion of the other sperm cell with the central cell initiates the endosperm—a tissue that is typically a nutritive lineage for the embryo and contributes to its embryonic development. The endosperm and double fertilization are sufficiently unique that they are often used as defining features of angiosperms.

The male gamete has traditionally been the less understood partner in flowering plant reproduction. Although the first realization that flowering plants displayed sexuality began with the work of Camerarius (Zarsky and Tupy, 1995), the realization that pollen grains formed tubes that sought out and entered the ovule began with the work of Giovanni Battista Amici,

who proposed that the tube harbored a fertilizing essence that stimulated seed production (Amici, 1824). Wilhelm Hofmeister would later recognize the presence of nuclei in the pollen tubes and document the behavior of their nuclear contents harbored inside pollen (Hofmeister, 1849). Eduard Strasburger, approximately 35 years later published the first details on flowering plant fertilization in which he described nuclear interactions occurring between the male and female gametes constituting karyogamy (Strasburger, 1884). Interestingly, he initially misidentified the conspicuous tube nucleus as the stimulus for the development of the egg cell to form the embryo, but this was rapidly corrected when he observed that the nuclei of sperm were fusing with the egg nucleus. The significance of the second sperm cell was recognized when its nucleus was noted fusing with the polar nuclei of the central cell and initiating the development of the endosperm at the end of the last century, a defining event of double fertilization (Nawaschin, 1898).

The inconspicuous nature of the male generative cell and their subsequent pair of male gametes has led to an underestimation of their importance—which to some extent continues. A century later, some beginning biology texts did not recognize flowering plant sperm as cells, but only bare nuclei—an observation that was mistakenly cited as an important difference from animals. In the early days of molecular biology, these diminutive, non-motile sperm cells were surmised to be completely dependent on the pollen grain/tube nutritionally, and were consequently thought to be dependent on the pollen vegetative cell for all transcription, translation and gene expression (Mascarenhas, 1990).

Recognition that the generative and sperm cells were largely transcriptionally and translationally independent was first shown directly in 1993 (Zhang et al., 1993). It is now clear that flowering plant sperm cells have their own unique patterns of transcription (Gou et al., 2001; Engel et al., 2003), their own unique promoters (Xu et al., 1999; Engel et al., 2005), cell cycle control factors (Borg et al., 2011; Twell, 2011), and silencing elements (Haerizadeh et al., 2006). Furthermore, sperm cell-expressed genes may even control early embryogenetic effects. The zygotically-expressed paternal transcript of SSP (*SHORT SUSPENSOR*), as an activator of YODA, was recognized to initiate an asymmetric pattern of cell division of the zygote, which forms a strongly asymmetric and polarized two-celled proembryo that contains a small apical cell at the tip and a larger basal cell (Bayer et al., 2009). This represents the initial deciding point in which the fate of the embryo proper is separated from that of the larger suspensor, establishing the suspensor as a terminal lineage. The male gamete can thus influence gene expression from the first cell division of the next sporophyte generation.

In contrast, the importance of pollen genes reaches its peak during pollen tube competition with other tubes during which their behavioral priority is to preemptively deliver their contained male gametes into a small receptive region of the female gametophyte, targeting the female gametes. The sperm cells meanwhile must be synchronized with the female gametes with respect to cell cycle and receptivity. Double fertilization depends on participation of all gametes, and the fusion of each of the two male gametes with their respective female counterpart. Although the successful competition of the pollen tube is critical to determining the

eligibility of male gametes to participate in fertilization, it is only the sperm cell that participates in the subsequent transmission of its paternal genes into the next generation through fertilization. Male gametophytic cooperation results in multiple well-choreographed successes of the two contrasting transcriptomes of the sperm and pollen cells in order to achieve passage into succeeding generations. The male strategy of over-production of pollen at lower energetic cost than egg cells is a strategy of reproduction that is highly conserved between plants and animals (Richards, 1997). This lower investment cost and more abundant production of pollen allows greater variability in the male gametophyte in competing for the ability to fuse with the egg cell and the polar nuclei, but also places high demands on the quality of the gametes and their interactions with the female gametes to assure sustained intergenerational success over time.

## Initiation and Origin of the Male Germ Lineage in Flowering Plants Appears to be Entirely Post-meiotic

Unlike animals which have predestined cells functioning as the germ cell lineage, plant cells appear not to possess a standing population of germ cells during their somatic phase and the emergence of the germ lineage appears to be entirely positionally determined. The diplohaplontic nature of plants maintains obligate passage through single cells at both meiosis and syngamy, alternating with multicellular sporophytic and gametophytic generations. Male gamete formation during normal sexual reproduction in flowering plants occurs through a microsporocyte that undergoes meiosis to form four microspores. When the microspore divides, the generative cell is formed through cell wall formation that partitions the cell in a typically convex pattern, initiating an eccentrically positioned cell plate from the center of the mitotic axis and expanding centrifugally. The formation of the generative cell partitions the pollen into a small lenticular generative cell that occupies approximately 1/20 of the volume of the pollen and a much larger pollen vegetative cell (Russell et al., 1996; Russell and Strout, 2005). The generative cell may further partition cytoplasmic regions that become isolated from the nucleus during development and continues to become smaller during development.

The outer wall of the generative cell originates directly from the microspore intine, whereas the inner wall forms from an interior cellular partitioning of the microspore. This dividing cell wall is somewhat unusual in composition, as it is rich in callose (a  $\beta 1 \rightarrow 3$  glucan), which is removed from this region during later maturation. The generative cell migrates into the interior of the vegetative cell through a unique separation mechanism that is correlated with the disappearance of callose labeling on the newly formed crosswall, and an intensification of labeling in the area of separation (Russell et al., 1996). Upon completion of separation of the generative cell from the intine, the generative cell typically polarizes and occupies a unique “cell-within-a-cell” configuration, which precedes the formation of the sperm cells. The generative cell becomes physically associated with the vegetative nucleus, establishing the “male germ unit.” The generative cell

and later sperm cells generate cytoplasmically-derived vesicles that appear to reduce their cellular volume throughout development (Yu and Russell, 1992). At maturity, sperm cells may occupy far less than 1% of the volume of the pollen and are among the smallest cells in many flowering plants (Russell and Strout, 2005).

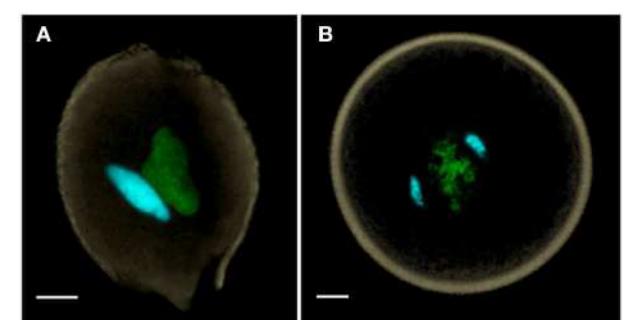
Asymmetry in the volume of the descendant cells appears to be required for the establishment of the male germ lineage. Equational divisions of the microspore giving rise to equal-sized cells result in the formation of two vegetative cells and no reproductive cells (Eady et al., 1995). Interestingly, the *sidecar* mutant can result in two equal-sized cells, initially retaining vegetative identity, but when one of these cells undergoes an asymmetric division, it forms a generative cell that divides to form two apparently completely normal sperm cells (Chen and McCormick, 1996). Dissimilar cell volumes presumably trigger the key transcription factors and activate the developmental program of the male germ lineage (Oh et al., 2011). Reactivation of the cell cycle in the generative cell appears to license the single mitotic division required to form the two sperm cells (Brownfield et al., 2009), whereas further cell cycle progression in the vegetative cell continues to be inhibited.

## Maturation in the Germline Entails Novel Structural, Physiological and Morphogenetic Features

The sperm cell surface does not have a traditional cell wall, which would impede fusion, but instead consists of a “periplasm” (McConchie et al., 1987), the nature of which appears to be similar to that of a brush-border. Freeze-substitution preparations have revealed this periplasmic region is characterized by the presence of insoluble polysaccharides, but these do not form discernible fibers, which confirms the absence of a traditional cell wall surrounding the sperm cells (Russell and Cass, 1981). Experiments using living tobacco pollen tubes at generative cell division revealed that newly-formed sperm cells could inadvertently fuse with one another; however, soon after division, the surface of the sperm cells had matured sufficiently that they no longer were able to fuse spontaneously on contact (Tian and Russell, 1998). Addition of a dilute solution of cellulose and pectinase could remove this inhibition, suggesting that multiple barriers to spontaneous fusion may exist. It is possible that carbohydrate moieties on the surface of the sperm cells may even assist in nullifying charge differentials on the surface of the gametes, thus contributing to overcoming the natural repulsion of negatively-charged membrane phospholipids during later fusion (Russell, 1992).

## Cellular Condition of Pollen, Cell Cycle Positioning of Gametes, and Gametic Cell Communication

Flowering plant pollen can be released at anthesis in two alternative conditions—one in which pollen is bicellular containing a generative cell—as in ~70% of angiosperms (Figure 1A), or one in which pollen is already tricellular, containing two sperm cells at



**FIGURE 1 | (A)** Bicellular pollen is exemplified by *Nicotiana benthamiana*, and **(B)** tricellular pollen by *Oryza sativa* (rice). Both of these anthesis pollen grains are labeled with DAPI, captured as a MIP using confocal laser scanning microscopy, and manually-segmented to portray generative and sperm nuclei in cyan, vegetative nucleus in green and pollen wall autofluorescence in beige. Scale bars = 5  $\mu$ m.

anthesis, as in the remaining ~30% of angiosperms (Figure 1B) (Brewbaker, 1967). The precocious formation of sperm cells prior to anthesis in tricellular pollen constitutes a heterochronic shift that is generally regarded as apomorphic (Williams et al., 2014). Although there are some species where anthers may even bear both bicellular and tricellular pollen within the same anther, these are rare. The cellular condition of pollen appears to be in evolutionary flux with abundant transitory examples of conversion and reversion of pollen cell types (Williams et al., 2014).

The majority of animals are known to fuse with the gametes in G1 (prior to S-phase in the cell cycle), but angiosperms may fuse in either G1 or G2 phase (Friedman, 1999). While gametic fusion in both G1 and G2 phases occur, no examples of fusion in S-phase are noted. Based on plants studied to date, tricellular pollen may be disseminated with gametes at G1, S or G2 phase, and bicellular pollen is disseminated with generative cells at G1 or G2 (Friedman, 1999). Defects in the control of the cell cycle are well-documented sources of cell identity defects and missed cell cycle cues are an important source of defective gamete behavior, many of which are informative with respect to the control of gamete maturation (Durbarry et al., 2005; Brownfield et al., 2009; Borg et al., 2011, 2014). Cell cycle synchrony appears to be required at the time of gamete fusion. Thus, missed cues can occur at multiple time points in the maturation of the gametes, and may occur late in the life of the gamete, as well. Tobacco sperm cells, which appear to fuse at G2, exemplify very late maturing gametes, as the sperm cells are discharged into the synergid in the G1 condition and fuse in G2. During the protracted time that sperm cells were observed in the receptive synergid, nuclear DNA quantity increased in both male and female gametes until they synchronized at G2-phase, when fusion would occur (Tian et al., 2005). Female gametes in unpollinated flowers would eventually enter G2 phase, but would be delayed by nearly a day and a half without pollination.

Some flowering plants appear to require new gene expression in order for pollen tubes to be able to detect female gametophyte signals and discharge their gametes, particularly with respect to *in vitro*-grown pollen. In *Arabidopsis*, for example, pollen tube

elongation within the style appears to be required to activate a number of genes necessary to control tube guidance and modulate reception in the female gametophyte (Higashiyama and Hamamura, 2008; Palanivelu and Johnson, 2010). As many plants require more than 6 h of pollen tube passage to reach the female gametophyte, it would not be surprising that late expressional changes may occur in the male gamete, especially in bicellular species. A stark contrast to this is rice (*Oryza sativa*), in which fertilization may be effected in <30 min. Comparisons of elongating rice pollen tubes with pollen grains reveal that the most major conspicuous change in rice pollen tubes is the intensification of metabolic response in secretory pathways with few other detectable changes in gene expression (Dai et al., 2006; Wei et al., 2010).

## Male Germ Lineage Transcripts and Products Reflect Rich, Complex and Dynamic Gene Expression

That gene expression in the male germ lineage would include a rich assemblage of transcriptional and translational products was evident from the early-1990s, when ESTs of sperm and generative cell cDNA libraries were first examined and sequenced (Zhang et al., 1993; Blomstedt et al., 1996). Among the earliest discovered novel proteins were those involved in chromatin changes in the male germ lineage, which included an unusually rich complement in substitution histones H2A, H2B, and H3 that displayed comparatively low homology in the highly conserved histone gene family (Ueda and Tanaka, 1995a,b). The lily generative cell lineage that Ueda and Tanaka studied had long been observed to have distinctive chromatin configurations in the nuclei of generative and sperm cells and the large size of the generative nuclei were attractive, particularly given the relative insensitivity of molecular methods at that time. Their studies revealed novel chromatin-related genes that encoded a number of variant histones, which were later observed in *Arabidopsis* (Okada et al., 2005b), soybean (Haerizadeh et al., 2009), rice and a number of other model angiosperms (Singh and Bhalla, 2007). These epigenetic factors continue to be a major theme in modern work as well.

Promoter analysis has revealed that there are male germline-selective promoters that are activated in the generative and sperm cells (Xu et al., 1999; Okada et al., 2005a). Whereas most promoters are positively controlled, there is also evidence for a complex silencing element that controls male germline expression through a repressor that is expressed in all but male germ cells (Haerizadeh et al., 2006). In the latter case, without the repressor protein, male germline genes are constitutively expressed. Conservation of DNA repair genes is suggested by the characterization of a lily generative cell homologe to the human excision repair gene ERCC1 (Xu et al., 1998). This supports that conservation of DNA repair enzymes may extend among distant groups of eukaryotes (Tuteja et al., 2001). The presence of a diversity of DNA repair enzymes in the male germ lineage is supported by evidence from in a number of expression libraries (Okada et al., 2006; Borges et al., 2008; Abiko et al., 2013b).

Activation of ubiquitin pathways contributes to increased rates of protein turnover and also represents evidence of protein dynamism. The ubiquitin pathway involves activating E1 enzymes, ubiquitin-conjugating E2 enzymes, and ligating E3 enzymes that link ubiquitin to proteins, thus targeting them for degradation in the proteosome pathway. Highly transcribed members of the ubiquitin pathway are common in transcriptomes of male germ-related lineages and may even be differentially enhanced in different male germ cells (Singh et al., 2002).

## Male Germline Cells and Pollen Display Unique Profiles, Distinct Gene Complements

Transcriptomic analyses of the developing cells of the male gametophyte of *Arabidopsis* using microarrays revealed complex patterns of gene regulation throughout pollen maturation, with a sharply decreasing number of active genes from the uninucleate microspore, to generative cell initiation in bicellular pollen, with still fewer genes expressed in tricellular pollen and post-anthesis pollen (Honys and Twell, 2004). The transcriptome of mature, anthesis pollen revealed a functional complement of genes highly upregulated in cell wall metabolism, cytoskeleton and cell signaling, but otherwise reflected a cell with a short remaining lifespan and an overall unsustainable metabolism—a transcriptomic composition reflective of its limited behavioral possibilities (Becker et al., 2003; Honys and Twell, 2003; Pina et al., 2005). This divergent and restricted expressional profile appears to be conserved in the anthesis pollen of multiple species of angiosperms examined to date, including soybean (Haerizadeh et al., 2009), rice (Wei et al., 2010), and tobacco (Hafidh et al., 2012).

As with all molecular and biochemical assays, sufficient high quality male gametes have to be available, combined with adequate detection sensitivity, to characterize the gametes. Two major protocols have emerged for the isolation of male gametes: (1) differential centrifugation, typically requiring the collection of cells from a continuous Percoll or discontinuous sucrose density gradient (Russell, 1991), and (2) fluorescence-activated cell sorting (FACS), using a sperm-selective promoter to drive the expression of a GFP reporter in order to label the targeted cells for isolation (Engel et al., 2003, 2005; Borges et al., 2008, 2012b). Both techniques produce samples that are sufficiently pure to separate enriched transcripts from the male germline cells with sufficient collections. Needless to say, vastly different quantities of pollen are available in wind-pollinated plants than in insect-, bird- or self-pollinated plants (Richards, 1997).

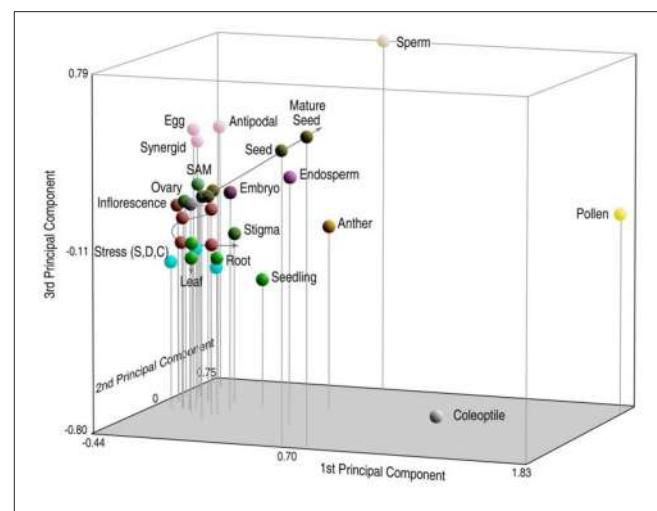
Transcriptomes of male gametes have clearly illustrated the divergent nature of gene expression in the male germ lineage compared to that of the vegetative pollen. Estimates of the number of genes present in the sperm, pollen and seedlings of *Arabidopsis* have yielded microarray presence calls of 27% for sperm cells (corresponding to 5829 genes), 33% for pollen (corresponding to 7177 genes), and 64% for seedlings (corresponding to 14,464 genes) using the MAS5 algorithm and gene counts normalized to the *Arabidopsis* genome (Borges et al., 2008).

Among transcriptional themes encoded by the sperm cells are DNA repair, ubiquitination, and cell cycle progression, which are common emerging themes. Gene expression estimates for rice, based on normalized triplicate microarray results using MAS5 unanimous presence calls yielded 10,732 sperm genes, 8101 pollen genes, and 15,449 seedling genes (Russell et al., 2012). The most highly represented functional categories in rice sperm cells involved metabolism, transcription and cell signaling. Additional functional categories up-regulated in sperm cells, as compared with other tissues, include transcription factors, cell signaling, protein modification, cellular identity and receptor-like molecules; these categories may each include some key players in functions unique to sperm cells.

The most sensitive and presumably accurate estimates for transcribed genes of the gametes and pollen are those available from RNA-Seq results (Anderson et al., 2013). Total reads using rice, indicated expression of up to ~25,000 genes in the sperm cell, ~29,000 genes in the pollen vegetative cell and ~27,000 genes in the egg cell, which were far higher than original microarray results. When gene counts were limited to sequences found in all three replicates of each cell, there were 16,985 genes detected in the sperm cell, 18,611 genes in the pollen vegetative cell and 21,172 genes in the egg cell (Anderson et al., 2013). Clearly the original expressional assays underestimated the breadth and depth of these cellular profiles, but they do not begin to answer the question of how many of these are translated and how many occupy essential roles. Based on microarray results, there is clearly a broad group of genes expressed in all of the cells that may reflect a “housekeeping” role, providing core metabolic functions.

To compare the relationship of genes transcribed in a gallery of different tissues, principal component analysis (PCA) was used to portray  $n$ -dimensional data sets on smaller 3D axes so that the components of greatest variation could be compared (Russell et al., 2012). Relative distances on the three-dimensional graph are proportionate to their degree of relatedness in  $n$ -dimensions. In Figure 2, rice sperm cells are compared with microarray expressional data from 31 different tissues, including sperm and pollen (representing the male gametophyte), 26 different vegetative and reproductive tissues of the sporophyte (representing different sporophytic phases of the life cycle, including different tissue conditions, organs, developmental conditions, and environmental responses) and three different tissues representing the female gametophyte. Interestingly, the high degree of divergence in expression between sperm and the pollen vegetative cell place them as anchor values on multiple axes and place them at a significant distance from sporophytic expression. These differences between tissues remain high, even when compared with the anther, which includes pollen and sperm as a subset (Russell et al., 2012). Overall expression also differs between the male germline and differentiated sporophytic tissues in other analyses as well (Borges et al., 2008; Abiko et al., 2013b).

The RNA-Seq data revealed expression profiles reflecting an upregulation of genes involved in chromatin conformation, indicating an unexpected degree of chromatin activation in the sperm cells. The transcriptomes of the egg and sperm reveal major differences in gene expression that will presumably be altered within the zygote. These differences represent the native state for



**FIGURE 2 | Principal components analysis (PCA) of probe set signal intensity on the rice transcriptome, reflecting gene expression patterns in 31 different tissues of rice.** Relative distances of sperm profiles from those of other cell and tissue types, including pollen, are indicative of highly divergent patterns of overall gene expression in the male germ lineage. Reproduced with permission of J.C. Wiley Press (Russell et al., 2008, 2012).

parent-of-origin gene expression and will be a baseline for further studies of the zygote during fertilization and early embryogenesis. Particularly pathways affecting epigenesis, methylation, hormonal control, cell cycle and specific gametic functions were examined in anticipation of their potential contributions to early zygotic and seed development (Anderson et al., 2013). Notably, three-quarters of the genes were differentially expressed between cell types.

## Proteomic Profiles of Egg and Sperm Lineages Have Many Common Elements, Few Specifically Divergent Proteins

Proteomic data on isolated generative cells and male gametes have been obtained for a limited number of species. In *Arabidopsis*, translation of a number of gene products have been confirmed, but not all transcripts are translated into proteins within the male germ lineage (Bayer et al., 2009). Lily was selected as a model bicellular pollen species in which protein expression profiles of GCs and SCs were examined by 2D-DIGE, displaying about 2500 protein spots with 226 displaying significant changes in expression; 124 were upregulated during SC development whereas 102 were downregulated (Zhao et al., 2013). Of the annotated proteins detected, 71% were involved in six main functional groups—metabolism, cell cycle, signaling, ubiquitin/proteasome pathway, chromatin remodeling, and stress response (Zhao et al., 2013).

In rice, 2138 proteins were detected in the egg cell and 2179 proteins in the sperm cells. A total of 102 proteins were preferentially expressed in the egg cell and 77 proteins were preferentially expressed in the sperm cells (Abiko et al., 2013a). Proteins selectively enriched in the egg cell proteome appeared to reflect a

functionally diverse collection of polypeptides. Proteins enriched in the sperm proteome appeared to reflect narrower motifs and were more centered to fusion-related functions (Boavida et al., 2013). Such selectively expressed transcripts encoded proteins including such motifs as protein modification, lipid-related proteins, and potential cell surface modifying proteins (Abiko et al., 2013a).

## Transposable Elements in Pollen and Male Germline

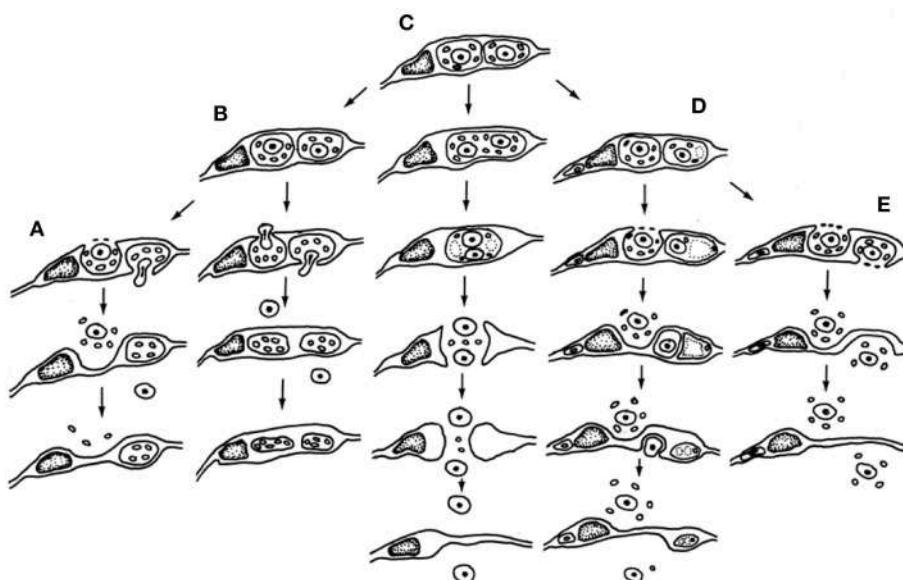
An enigmatic feature of the male germ transcriptome is the frequent occurrence of sequences that encode transposable elements (TEs), which may be present in remarkably differing quantities in different plants and different environmental conditions. The vegetative cell is known to be the site of considerable TE activity, which is prevalent enough that it has been directly observed using transposon displays in pollen (Turcich and Mascarenhas, 1994). LTR retrotransposons, which are particularly highly activated in the pollen, are believed to be strongly suppressed in the male germline (Slotkin et al., 2009). Sperm cells are regarded as displaying a high degree of DNA-level methylation, which is believed to play a central factor in suppression of TEs and follows three nucleotide motifs in flowering plants: CG, CHG, and CHH (where H may equal A, C, or T). Sequences to be silenced are typically encoded by RNA-directed DNA methylation through the action of small RNAs whereas CG and CHG motifs remain highly methylated in the male germ lineage, suppressing the activity of retrotransposons. The symmetrical nature of CG and CHG motifs confers similar methylation patterns in each DNA strand, resulting in similar epigenetic markings in their progeny, whereas the activation of DNA glycosylation, which demethylates DNA, is evident in the vegetative cell and activates DNA transposons at imprinted loci (Borges et al., 2012a). CHH motifs in microspore and sperm cells are believed to undergo a dramatic decrease in methylation in the male germ lineage. Although demethylation could compromise the repression of DNA transposons, methylation is not restored until the zygotic stage (Calarco et al., 2012). Upon fertilization, methylation is believed to be reactivated in the zygote by siRNA-based silencing elements from the pollen and the endosperm, which appear to control site-specific methylation. Chromatin-related changes in methylation are also a key consequence of combining the gametes, each of which have their own signature molecules (Slotkin et al., 2009). Activation of CHROMOMETHYLASE 3 (CMT3) appears to alter methylation at both CG and CHH sites, whereas the male germ lineage is relieved from TE suppression through the activity of (DRM2); this in turn is believed to release TEs from suppression by the histone methyltransferases KRYPTONITE (KYP/SUVH4) and SUVH5/6 (Calarco and Martienssen, 2011).

In the male gametes of *Arabidopsis* (Borges et al., 2008), lily (Okada et al., 2006), and *Plumbago* (Gou et al., 2009) relatively few TEs are transcribed, but in grasses such as maize (Engel et al., 2003) and rice (Russell et al., 2012), the high genomic content of TEs appears to be reflected in abundant transcripts. Sperm cell ESTs collected from maize pollen at anthesis had

approximately 9.46% annotated retrotransposons according to GenBank accessions, whereas DNA transposons represented only 0.06% of the transcripts. Although retrotransposon content may be proportionate to TE content in the maize genome, DNA transposons were scarce, suggesting that a combination of DNA-level methylation and chromatin modification may dominate the repression motifs in the epigenome of maize sperm, as is known to occur elsewhere (Borges and Martienssen, 2013). Further suppression of TEs through such short RNA species as siRNAs, are believed to convey a high degree of precision to the process of silencing (Creasey et al., 2014). Consistent with the conservative gender-based behavior seen in other eukaryotes, TE transcription does not seem prevalent in egg cells (Anderson et al., 2013). Female gametophytes of *Arabidopsis* displayed less than one-fifth the number of retrotransposons (1.69%) and more annotated DNA transposons (1.44%) than in maize (Yang et al., 2006). Differentially fewer female-transcribed TE motifs were also observed in rice (Anderson et al., 2013), suggesting that a generally lower number of TE transcripts are present. Such reduced TE activity may be a consistent feature among female germ lineages. Whether this reflects diminished transcription of TE genes or greater success in suppression of TE transcripts is not immediately evident. In either case, TE abundance is clearly less in the female gametes than in the male gametes (Russell et al., 2012; Anderson et al., 2013).

## Contribution of the Sperm Cells Upon Fertilization

The paternal nucleus represents half of the genomic complement of the zygote—thus an equal partner in the formation of the embryo and resulting sporophyte—but the quantity of transcripts encoded and expressed greatly differ, which by definition forms a starting point for gametic parent-of-origin effects (Luo et al., 2014). Size differences between the male and female gametes would nearly ensure a greater quantity of female gamete-expressed transcripts in the zygote, but this input alone does not eliminate potential impacts of a wide variety of epigenetic factors that may alter the number of functional transcripts delivered, expressed or sequestered. Cytoplasmic ratios between male and female gametes may be <1:50 in the zygote and <1:800 between the male gamete and central cell cytoplasm that constitutes the endosperm (Russell, 1987). A relatively low ratio of paternal-to-maternal cytoplasmic volume allows minimal opportunity for the transmission of sperm-delivered heritable organelles and likely this is a common event during fertilization. Such interactions may be entirely eliminated in species such as barley, in which the cytoplasm is excluded and the sperm cell cytoplasm appears to remain amid degenerating pollen and synergid cytoplasm outside of the egg cell (Mogensen, 1988). Other species may transmit varying amounts of paternal cytoplasm (Russell et al., 1990). **Figure 3** displays five different patterns of male cytoplasmic transmission that have been reported in the literature to date, and which lead to a predicted relaxed control of heritable organelle contributions (Birky, 1983). Yet, paternal organelles occur in the sperm cells of tobacco, that are detected within the zygote



**FIGURE 3 | Participation of male germline cytoplasmic organelles shows incorporation, partial incorporation, or exclusion of heritable cytoplasmic organelles (often restricted to mitochondria because of prior plastid exclusion or elimination from the generative cell).** Five models have been described and documented using electron microscopy. **(A–D)** Models favoring uniparental maternal inheritance. **(A)** In barley, paternal organelles are excluded from egg but not central cell (Mogensen,

1988); **(B)** In cotton, paternal organelles are excluded from both embryo and endosperm (Jensen, 1964); **(C)** binucleate model of (Wilms, 1981) in spinach; **(D)** in *Populus*, organelles are excluded by extra-cytoplasmic body production (Russell et al., 1990). **(E)** Composite model of biparental cytoplasmic transmission, based on *Petunia* (Van Went, 1970) and *Plumbago* (Russell, 1983). Reproduced with permission of Springer-Verlag (Russell et al., 1990).

and early embryo genetically (Yu et al., 1992, 1994) and there is molecular evidence of transmission of nuclear transcripts, as well (Ning et al., 2006). Paternal transcripts of SSP (*Short Suspensor*) are known to be transmitted into the zygote of *Arabidopsis*, translated, and their products expressed in the fertilized egg cell. As the SSP protein activates expression of YODA, this male contributed protein sequence establishes the asymmetry of the two-celled proembryo (Bayer et al., 2009). The diversity and distinctiveness of paternal transcripts in the sperm cells seems to suggest a role in shaping parental elements of the transcriptome of the zygote early in development (Russell et al., 2012). That the dimorphic sperm cells of *Plumbago zeylanica* (Russell, 1984) have different preferential fates during fertilization (Russell, 1985) further suggests that their differentially expressed transcriptional complements may target female cells and contribute to their different fates (Gou et al., 2009).

The paternal genome of animals is often silenced prior to the maternal to zygotic transition (MZT), thus the onset of zygotic expression in animals coincides with a suppression of messages from the egg cell and the onset of expression from both sets of chromosomes (Baroux et al., 2008). In plants, however, maternal and paternal chromosomes appear to be equal contributors from the earliest stages of embryogenesis (Nodine and Bartel, 2012). The endosperm, which typically receives two copies of the maternal genome, often displays strong evidence of maternal imprinting in the endosperm, which coincides with rapid demethylation of DNA, and thus the activation of the endosperm lineage (Bauer and Fischer, 2011). However, changes in gene activation in the zygote proceed methodically, involving removal of some histone

variants, such as MGH3 substitution histones in the embryo lineage (Ingouff et al., 2010). In rice, only a handful of genes in the embryo displayed imprinting and in all three cases these were maternal. In contrast, the endosperm had just over 2% of its genes discernibly imprinted. In the endosperm, however, unlike the embryos, the maternal-to-paternal imprinted genes neared a 2:1 ratio of contributed genomes of the two polar nuclei relative to the sperm nucleus (Luo et al., 2011).

## Male Expression and Evolutionary Selection

The degree to which male germ cells undergo effective evolutionary selection can be judged by the degree to which altered nucleotides in their genes are replaced with nucleotides encoding the same amino acid sequence, indicative of purifying selection, as opposed to random replacement. Haplloid regimes, which are by definition not masked by dominant genes, are particularly adaptable to selection. For example, mosses displaying purifying selection may be very effectively selected in strongly conserved phenotypes with highly expressed protein-coding regions (Szövényi et al., 2014). In pollen vegetative cells and pollen tubes, 6–11% of important genes display purifying selection with purifying selection in pollen far exceeding that in seedlings (Arunkumar et al., 2013). Adaptations that favor pollen tube competition appear to be strongly selected in the population. In contrast, genes expressed in sperm cells display fewer sites that are under strong purifying selection than either seedlings

or pollen (Arunkumar et al., 2013). Although genes expressed in gametes and synergids show high rates of protein evolution, a greater proportion of adaptive amino acid substitutions are the result of increased levels of purifying selection in pollen and pollen tube-specific genes. Prezygotic sexual selection involving interactions such as pollen tube competition may therefore be more successful than gametes at positive trait selection (Gossmann et al., 2013). Sperm-expressed mutants involved in pollen tube guidance, however, such as *hap2* may display unusually strong positive selection, as gametic interactions may result in increasingly complex patterns of communication designed to optimize success in later seed production (Beale and Johnson, 2013).

Among model systems for reproduction, *P. zeylanica* is one of the most remarkable because it has dimorphic sperm cells in which the fusion fate of the sperm is known from inception. The sperm cell that is associated with the vegetative nucleus ( $S_{vn}$ ) is known to preferentially fuse with the central cell forming the endosperm, whereas the other sperm cell ( $S_{ua}$ ) preferentially fuses with the egg cell to produce the embryo (Russell, 1985). In order to characterize the sperm transcriptome of these

two cell types in the absence of working transformation system (Wei et al., 2006), it was necessary to collect sperm cells using a micromanipulator (Zhang et al., 1998). Using collections of 12,000 sperm cells of each morphotype, representative sperm cell cDNA libraries and custom microarrays were constructed, ESTs characterized, and each sperm cell's functional profiles were compared. Surprisingly, the functional profile of the sperm cells appeared to coincide closely with their putative fusion product (Gou et al., 2009). Thus, the functional profile of the  $S_{vn}$  appeared similar to an expected endosperm-enriched profile, whereas that of the  $S_{ua}$  appeared more similar to an embryo profile. This appears to represent an instance where the precocious development of the embryo may be accelerated by providing targeted paternal genes to be activated upon double fertilization. With modern increases in the molecular sensitivity of characterization techniques and use of greater resolution techniques such as RNA-Seq, the accuracy of this prediction could be examined and potentially tested, providing essentially transcriptomic coverage during early embryogenesis to test the role of male gamete transcriptomes in early post-fertilization development.

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# Organization and regulation of the actin cytoskeleton in the pollen tube

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Proper organization of the actin cytoskeleton is crucial for pollen tube growth. However, the precise mechanisms by which the actin cytoskeleton regulates pollen tube growth remain to be further elucidated. The functions of the actin cytoskeleton are dictated by its spatial organization and dynamics. However, early observations of the distribution of actin filaments at the pollen tube apex were quite perplexing, resulting in decades of controversial debate. Fortunately, due to improvements in fixation regimens for staining actin filaments in fixed pollen tubes, as well as the adoption of appropriate markers for visualizing actin filaments in living pollen tubes, this issue has been resolved and has given rise to the consensus view of the spatial distribution of actin filaments throughout the entire pollen tube. Importantly, recent descriptions of the dynamics of individual actin filaments in the apical region have expanded our understanding of the function of actin in regulation of pollen tube growth. Furthermore, careful documentation of the function and mode of action of several actin-binding proteins expressed in pollen have provided novel insights into the regulation of actin spatial distribution and dynamics. In the current review, we summarize our understanding of the organization, dynamics, and regulation of the actin cytoskeleton in the pollen tube.

**Keywords:** actin, pollen tube, actin-binding protein, formin, villin, ADF, fimbrin

## INTRODUCTION

Pollen represents a critical stage of the plant life cycle and is essential for the production of seeds in flowering plants (McCormick, 2013). Upon landing on the surface of the stigma, pollen begins to hydrate and germinate, protruding outgrowth to form a tubular structure that extends rapidly in the style. This structure provides the passage for two non-motile sperm cells to be delivered to the female gametophyte and finally effect the double fertilization (Hepler et al., 2001; Lord and Russell, 2002; Cheung and Wu, 2008). Pollen tube growth is very rapid; the growth rate for maize pollen tubes in the style can reach up to 1 cm/h (Bedinger, 1992). During the journey of fertilization, the pollen tube normally traverses a distance 1000s of times the diameter of its grain. However, growth of the pollen tube is restricted to the tip region, which is therefore called “tip growth”. This type of tip growth found in the pollen tube is shared by several other cell types, including root hairs in plants, protonemal cells in moss, hyphae in fungi, and neurites in animals (Cheung and Wu, 2008; Rounds and Bezanilla, 2013). Among these systems, pollen tube growth is particularly analogous to neurite growth. Surprisingly, however, despite its rigid cell wall, the pollen tube extends very fast, e.g., the lily pollen tubes even grow one order of magnitude faster than neurite (Hepler et al., 2001). The rapidity of growth implies that the underlying cellular activities may be amplified in the pollen tube. Pollen is an excellent cellular model for study of tip growth, as it is easy to culture, and most of the features associated with *in vivo* growth of pollen tubes are also observed *in vitro*. Additionally, essential mutations associated with pollen function can be maintained under a heterozygous state, which

makes pollen tube a very nice genetic system to study polarized cell growth. For these reasons, over the past several decades, the pollen tube has served a very important model cellular system for intensive study of the mechanisms underlying polarized cell growth.

The actin cytoskeleton has been shown to be crucial for pollen tube growth (Taylor and Hepler, 1997; Gibbon et al., 1999; Hepler et al., 2001; Vidali et al., 2001; Smith and Oppenheimer, 2005; Hussey et al., 2006; Chen et al., 2009; Fu, 2010; Staiger et al., 2010). The precise molecular mechanisms underlying the function of the actin cytoskeleton in the pollen tube, however, remain poorly understood. Different models have been proposed regarding the function of the actin cytoskeleton during pollen tube growth. One of the more common ideas is that the actin cytoskeleton drives the intracellular transport system that carries Golgi-derived vesicles containing the materials necessary for cell wall synthesis and membrane fusion to the tip (Pierson and Cresti, 1992; Hepler et al., 2001; Vidali and Hepler, 2001). The actin cytoskeleton has also been viewed as a structural element that supports the turgor pressure needed to drive and maintain rapid pollen tube growth (Picton and Steer, 1982; Steer and Steer, 1989; Derksen et al., 1995). Additionally, actin polymerization itself has also been shown to be important for pollen tube growth (Gibbon et al., 1999; Vidali et al., 2001). Irrespective of the particular mechanism underlying the function of the actin cytoskeleton in polarized growth, it is important to precisely describe its spatial distribution and dynamics in the pollen tube. Since, the spatial distribution and dynamics of the actin cytoskeleton are modified by the presence of various actin-binding proteins (ABPs) in cells (Staiger et al., 2010; Huang

et al., 2014), it is also important to characterize the function and mode of action of these ABPs. Indeed, recent characterization of the mode of action of several pollen-expressed ABPs has provided exceptional insights into the regulation of actin organization and dynamics in the pollen tube. Thus, the purpose of this review is to summarize our current understanding of the organization and dynamics of the actin cytoskeleton, as well as its regulation, in the pollen tube.

## SPATIAL DISTRIBUTION OF ACTIN FILAMENTS IN THE POLLEN TUBE

The distributions of the actin cytoskeleton in fixed pollen tubes from different species have been characterized using immunostaining with anti-actin antibodies and staining with fluorescent phalloidin (Tang et al., 1989; Rutten and Derksen, 1990; Gibbon et al., 1999; Geitmann et al., 2000; Li et al., 2001; Ye et al., 2009). Historically, reaching a consensus with respect to the distribution of actin filaments in the apical and subapical regions has been quite problematic. Different results have been reported regarding the distribution of actin filaments in the apical and subapical regions (Derksen et al., 1995; Miller et al., 1996). The variation could be due to differences between species or due to the use of different staining methods. The variation in actin structures in the apical region of pollen tubes stained using different methods most likely results from alterations in the fixation step, in which apical actin filaments may not be well-preserved. This problem is presumably due to two factors: one is that the pollen tube grows too rapidly and cannot be fixed instantaneously, and another is that apical actin filaments are highly dynamic and fragile. The original observations from experiments using conventional fixation procedures showed that dense actin filaments are present in the tip (Tiwari and Polito, 1988; Tang et al., 1989; Derksen et al., 1995). However, injection of rhodamine phalloidin into pollen tubes failed to label actin filaments in the extreme apex (Miller et al., 1996), and several other studies showed that the extreme apex is depleted of actin filaments in fixed pollen tubes (Li et al., 2001; Raudaskoski et al., 2001; Vidali et al., 2001). Later, using improved fixation regimens more likely to better preserve the cellular structures, such as rapid freeze fixation, the distribution of the actin cytoskeleton in the apical region was reproducibly revealed in the pollen tube (Lovy-Wheeler et al., 2005). This actin distribution pattern was further confirmed by the results of actin filament labeling using live-cell actin markers (Kost et al., 1998; Fu et al., 2001; Qu et al., 2013; see also the description below).

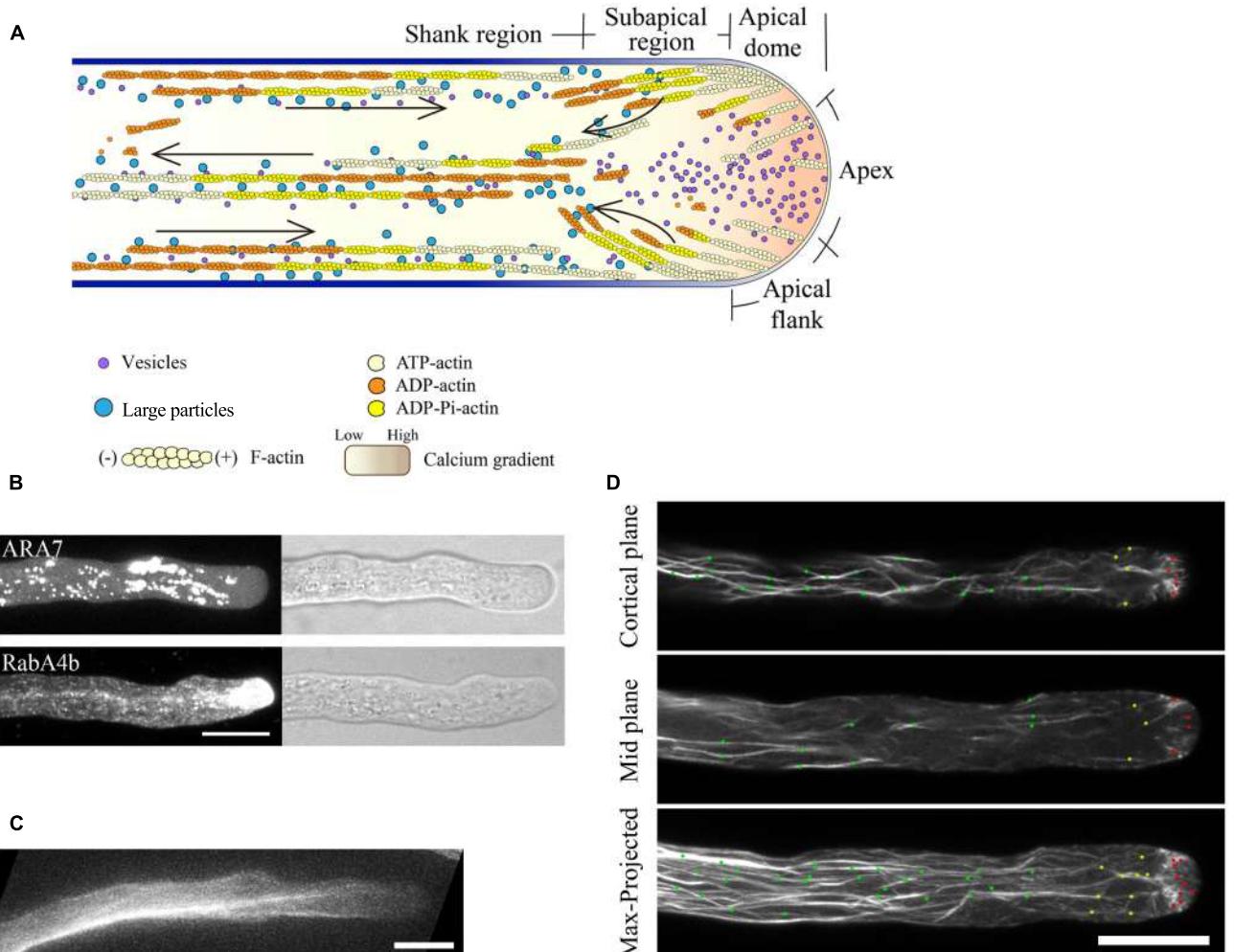
Thus, the current consensus view of the spatial distribution of actin filaments is that they are arrayed into at least three distinct structures in the pollen tube, consistent with the zonation of cytoplasm (**Figure 1A**; Lovy-Wheeler et al., 2005; Ren and Xiang, 2007; Chen et al., 2009; Staiger et al., 2010). In the shank, actin filaments are arranged axially into bundles with uniform polarity, which allows the transport of organelles or vesicles from the base to the tip along the cell cortex. At the subapex, actin filaments form regular structures referred to as the collar (Gibbon et al., 1999; Fu et al., 2001), fringe (Lovy-Wheeler et al., 2005), mesh (Geitmann et al., 2000; Chen et al., 2002), or funnel (Vidali et al., 2001) in pollen tubes from different species. In this region,

cytoplasmic streaming reverses direction and turns back toward the base along the axial actin cables in the center of the tube, giving rise to the reverse-fountain cytoplasmic streaming pattern (Hepler et al., 2001; Ye et al., 2009). Though large organelles do not enter the apical region, small vesicles enter into and become accumulated in the apical region (**Figures 1A,B**). In the apical region, actin filaments are less abundant, but are highly dynamic. The dynamics of the tip-localized population of actin filaments have been investigated in tobacco, lily, and *Arabidopsis* pollen tubes (Fu et al., 2001; Qu et al., 2013; Rounds et al., 2014 see also the detailed description below), which has expanded our understanding of the function of actin filaments. However, the means by which those actin filaments precisely regulate underlying cellular events, like vesicle targeting and fusion, remains to be explored.

## ACTIN MARKERS USED TO DECORATE ACTIN FILAMENTS IN GROWING POLLEN TUBES

Staining of actin filaments in fixed pollen tubes has yielded a great deal of useful information regarding the spatial distribution of actin filaments in the pollen tube (Gibbon et al., 1999; Geitmann et al., 2000; Lovy-Wheeler et al., 2005). However, the use of fixed tissues provides only a static image and does not reveal how the actin cytoskeleton is remodeled during pollen tube growth. Therefore, development of markers to label actin filaments in living pollen tubes was much needed, in order to allow tracing of actin filament dynamics during pollen tube growth. The introduction of green fluorescent protein (GFP) has revolutionized the way in which cellular dynamics are visualized (Brandizzi et al., 2002). Numerous useful GFP fusion markers have been developed in order to visualize cytoskeletal dynamics and have revolutionized research in the cytoskeleton field (Stringham et al., 2012). In plants, to date, GFP-actin has never been demonstrated to be a useful marker for decorating filamentous actin, presumably due to one or more of the following reasons. One possible issue is that the GFP tag alters the function of actin and prevents its incorporation into filamentous actin or alters the conformation of filamentous actin after its incorporation. Another possible reason is that filamentous actin only represents a small population of the total actin in plant cells (Staiger and Blanchoin, 2006); therefore, the filamentous signal might be masked by the overwhelming amount of monomeric signal.

Tagging of some ABPs or the actin-binding domains derived from them with GFP has provided a non-invasive way to image actin filaments in plants. Several of these actin markers have been used to image actin filaments in growing pollen tubes (**Table 1**). The earliest actin marker used for decorating actin filaments in pollen tubes was GFP-mTalin (**Table 1**; Kost et al., 1998). However, nowadays, GFP-mTalin is rarely used because it causes excessive filament bundling (Ketelaar et al., 2004b). GFP-ADF was shown to decorate actin filaments and most prominently at the subapical region of the pollen tube (**Table 1**; Chen et al., 2002; Cheung et al., 2008, 2010), and LIM-GFP and GFP-fimbrin/ABD2-GFP decorate longitudinal actin cables in the shanks of pollen tubes (**Table 1**; **Figure 1C**; Wilsen et al., 2006; Cheung et al., 2008). However, these actin markers each have distinct disadvantages



**FIGURE 1 | Spatial distribution of actin filaments in the pollen tube.**

(A) Schematic showing the spatial distribution of actin filaments in the pollen tube. At the apex, actin filaments are less abundant. In the subapical region, actin filaments form the regular actin collar structure. In the shank region, actin filaments are axially packed into cables, termed longitudinal actin cables. These actin structures are believed to perform distinct functions. Longitudinal actin cables provide the molecular tracks for movement of large organelles and vesicles from the base to the tip. The organelles and vesicles reverse direction at the subapical region and return to the base via the middle of the pollen tube, giving rise to the reverse-fountain cytoplasmic streaming pattern. These large organelles never enter the tip, resulting in the formation of the optical smooth zone at the tip referred to as the “clear zone”. However, this

region is filled with small vesicles. Therefore, actin filaments at the apical region are believed to be important for vesicle targeting and fusion events. The black arrows indicate the direction of cytoplasmic streaming. (B) Spatial distribution of ARA7-positive vesicles and RabA4b-positive vesicles in pollen tubes. ARA7-positive vesicles do not invade the apical region, whereas RabA4b-positive vesicles enter the apical region. Scale bar = 10  $\mu$ m.

(C) ABD2-GFP decorates longitudinal actin cables. Scale bar = 10  $\mu$ m. (D) Actin filaments in *Arabidopsis* pollen tubes were revealed by decoration with Lifeact-mEGFP. Images showing the cortical plane, the middle plane, and a projection of a representative pollen tube are presented. Actin filaments in the shank region, subapical region, and apical region are indicated by green dots, yellow dots, and red dots, respectively. Scale bar = 10  $\mu$ m.

in revealing actin structures in the pollen tube; for example, GFP-fimbrin/ABD2-GFP does not label actin filaments well in the apical and subapical regions (Figure 1C; Cheung et al., 2008). Despite these issues, these markers are useful for labeling different aspects of the actin cytoskeleton. Thus, the use of different actin markers can be effective for study of the distribution and changes in the actin cytoskeleton in the pollen tube. However, the ideal actin marker would be able to detect all arrays of actin filaments present in the growing pollen tube, and it would be even better if it had minimal effect on normal actin dynamics. In this regard, Lifeact-mEGFP has become the actin marker of choice

in the pollen tube. Lifeact-mEGFP contains an actin-binding site consisting of 17 amino acids derived from yeast ABP-140 fused with mEGFP. This protein decorates actin filaments in animal cells (Riedl et al., 2008) and has been used to detect actin filaments in growing tobacco and lily pollen tubes (Vidali et al., 2009; Dong et al., 2012). Recently, it has been employed to detect actin filaments in *Arabidopsis* pollen tubes (Qu et al., 2013; Zhu et al., 2013; Qin et al., 2014). Lifeact-mEGFP reveals actin structures nicely within different regions of the *Arabidopsis* pollen tube (Figure 1D; Qu et al., 2013), and results using this marker are reminiscent of results from actin staining of fixed pollen tubes

**Table 1 | Actin markers used to decorate actin filaments in living pollen tubes.**

| Actin markers        | Pollen tubes                          | Reference  |
|----------------------|---------------------------------------|--|
| GFP-mTalin           | Tobacco and <i>Arabidopsis</i>        | Kost et al. (1998), Fu et al. (2001), Ketelaar et al. (2004b), Wilsen et al. (2006), Wang et al. (2008a), Zhang et al. (2009, 2010b), Gui et al. (2014)              |
| GFP-FIMBRIN/ABD2-GFP | Tobacco and lily                      | Wilsen et al. (2006), Liao et al. (2010)   |
| GFP-ADF              | Tobacco and lily                      | Chen et al. (2002), Cheung and Wu (2004), Wilsen et al. (2006), Cheung et al. (2008, 2010)   |
| LIM-GFP              | Tobacco                               | Cheung et al. (2008)   |
| Lifeact-mEGFP        | Tobacco, lily, and <i>Arabidopsis</i> | Vidali et al. (2009), Daher and Geitmann (2011), Dong et al. (2012), Qu et al. (2013), Zhu et al. (2013), Gui et al. (2014), Qin et al. (2014), Rounds et al. (2014) |

(Gibbon et al., 1999; Lovy-Wheeler et al., 2005). Therefore, Lifeact-mEGFP represents an ideal actin marker for visualization of the organization and tracing of the dynamics of actin filaments in the pollen tube (**Figure 1D**; see the following section). Certainly, careful analysis of the organization and dynamics of the actin cytoskeleton in the entire pollen tube using a combination of standard and new actin markers will be useful in the future.

### ACTIN FILAMENT DYNAMICS IN THE POLLEN TUBE

The actin cytoskeleton plays an integral role during pollen tube growth. It is well-appreciated that the relatively stable longitudinal actin cables drive intracellular movement in the shank to propel pollen tube growth (Hepler et al., 2001; Ye et al., 2009; Wu et al., 2010). Meanwhile, several studies have indicated that the dynamic state of actin filaments in the tip region is also crucial for growth (Gibbon et al., 1999; Vidali et al., 2001). Apical actin filaments are thought to play an important role in regulating the velocity and direction of pollen tube growth by controlling vesicle docking and fusion events (Gibbon et al., 1999; Fu et al., 2001; Vidali et al., 2001; Lee et al., 2008; Qu et al., 2013). However, the precise functioning of actin filaments within the apical region remains poorly understood. This is partly because we know very little about the precise organization of the apical actin filaments. Direct visualization of individual actin filaments and quantification of the associated parameters are needed to provide insights into the organizational nature of these apical actin filaments.

Previous studies have shown that pollen tube growth is more sensitive than cytoplasmic streaming to actin depolymerizing drugs (Gibbon et al., 1999; Vidali et al., 2001), implying that the actin structures at the tip are highly dynamic. Direct visualization of GFP-mTalin-decorated actin filaments showed that the tip-localized actin filaments, termed short actin bundles, are indeed highly dynamic, and that their dynamics are connected to the formation of actin structures in the subapical region (Fu et al., 2001; Hwang et al., 2005). Our recent visualization and quantification of individual actin filaments within the apical dome of the *Arabidopsis thaliana* pollen tubes has provided further insight into this system. Through the use of the advanced imaging technology of spinning disk confocal microscopy, we traced the dynamics of individual actin filaments and quantified their associated parameters, such as filament elongation and shortening rates, severing

frequency, and other factors (Qu et al., 2013). Our observations revealed that actin filaments are constantly generated from the apical membrane of the pollen tube (**Figure 2A**; Qu et al., 2013), and that this process is most likely mediated by formins, such as AtFH5 (Cheung et al., 2010). Actin filaments originating from the extreme apex are highly dynamic. They are either turned over locally or moved to the apical flank, presumably with the membrane flow (**Figure 2B**), partially explaining why actin filaments are less abundant at the extreme apex. Our results also provide convincing evidence that exocytosis occurs at the extreme apex, supporting previous findings (Lee et al., 2008; Wang et al., 2013).

Actin filaments elongate very rapidly within the apical region (**Table 2**; Qu et al., 2013), consistent with measurements indicating that a high concentration of actin/profilin complex is present in pollen cells (Vidali and Hepler, 1997; Gibbon et al., 1999; Snowman et al., 2002). Actin filaments are also severed frequently within the apical region (**Figure 2B**; Qu et al., 2013), similar to findings showing that actin filaments in the cortical region of etiolated hypocotyl cells and BY-2 suspension cells are primarily eliminated by the filament severing activity (Staiger et al., 2009; Smertenko et al., 2010). Besides driving the local turnover of apical actin filaments, this severing activity may facilitate the departure of the severed actin filaments from the apical region, providing a pool of actin filaments that can be used for the construction of actin structures in the subapical region (**Figure 2A**; Qu et al., 2013). Thus, to some extent, actin filaments in the apical and subapical regions appear to be inherently connected, consistent with previous findings (Fu et al., 2001; Hwang et al., 2005; Cheung et al., 2010). Based on these observations, we present a simple model describing the organization and dynamics of actin filaments within the apical dome of the pollen tube (**Figure 2C**; Qu et al., 2013). A highly dynamic pool of actin filaments are constantly generated from the apical membrane. They are either turned over locally by filament severing and depolymerization activities, or they can move from the extreme apex to the apical flank, leading to decreased abundance of actin filaments in the apical region of the pollen tube (**Figure 2C**).

The dynamics of actin filaments within the shank region were also traced and quantified. By comparison, maximal filament length substantially increased, and severing frequency substantially decreased in the shank region compared to that

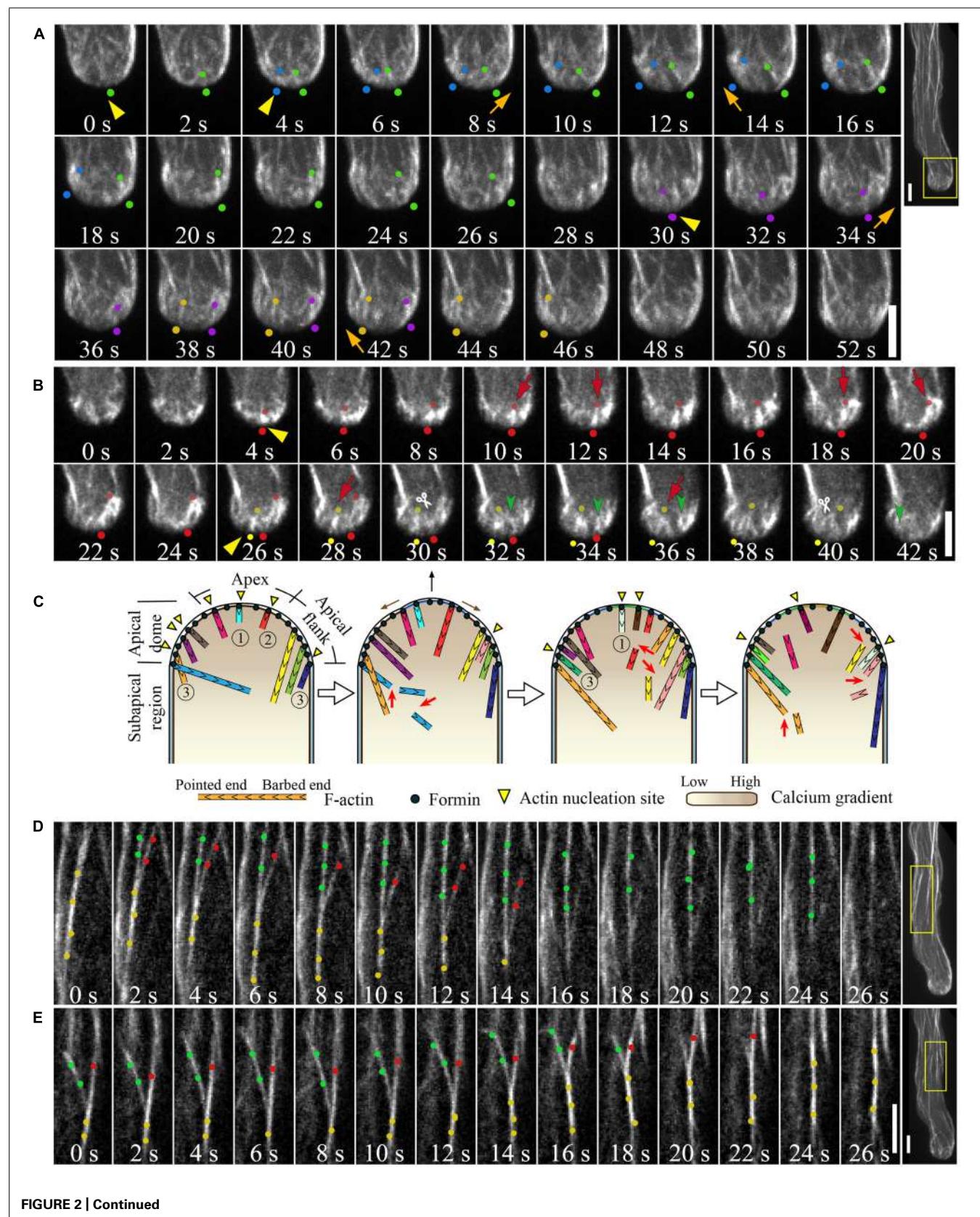


FIGURE 2 | Continued

**FIGURE 2 | Continued**

**Actin dynamics in the pollen tube.** **(A)** Actin filaments are constantly generated from the apical membrane within the apical dome. The images presented are maximally projected time-lapse images. Emerging individual actin filaments are marked by two dots of the same color. Yellow triangles indicate the origination of actin polymerization events, and movement of the filaments from the apex to the apical flank is indicated by orange arrows. Images are a higher magnification of the boxed region of the whole pollen tube shown in the far right panel. Scale bars = 4  $\mu\text{m}$ . **(B)** Corresponding single optical slices of images shown in **(A)** allowing clear visualization of single actin filament dynamics. Actin filaments are highlighted by two dots of the same color. Red arrows indicate filament elongation events, green arrows indicate filament shrinking events, and white scissors indicate severing events. Scale bar = 4  $\mu\text{m}$ . **(C)** Schematic describing the dynamics of actin filaments within the apical dome. Figure adapted from Qu et al. (2013). With the permission from American Society of Plant Biologists ([www.plantcell.org](http://www.plantcell.org)). For a detailed description, see the associated text and Qu et al. (2013). 1, 2, and 3 mark actin filaments that were nucleated from the membrane at the extreme apex, that moved from apex to the apical flank, and that were nucleated from the membrane at the apical flank, respectively. **(D,E)** Dynamic formation of actin bundles in the shank region. **(D)** Filament debundling events. Yellow dots highlight actin bundles that split into two bundles highlighted with red dots and green dots. The bundle marked by red dots is subjected to severing (indicated by scissors) and depolymerization. Images are a higher magnification of the boxed region shown in the far right panel. **(E)** Bundling event. Actin filaments marked by green dots and red dots were brought together via “zipping” to form the larger bundle indicated by yellow dots. Scale bars = 4  $\mu\text{m}$ .

in the apical region (Table 2), suggesting that shank-oriented actin filaments are relatively stable. This finding is consistent with previous observations showing that cytoplasmic streaming is more resistant than pollen tube growth to actin depolymerizing drugs (Gibbon et al., 1999; Vidali et al., 2001). Given that most actin filaments are packed into bundles, presumably by “zippering” individual actin filaments together via actin bundling factors, such as villin, fimbrin, and others, actin bundling activities were analyzed in *Arabidopsis* pollen tubes (Figures 2D,E). Two metrics describing bundle dynamics during live-cell imaging were analyzed: the bundling and debundling frequencies (Table 2). Bundling was observed at a frequency of  $2.3 \times 10^{-4}$  events/ $\mu\text{m}^2/\text{s}$ , whereas unbundling occurred at a frequency of  $5.4 \times 10^{-5}$  events/ $\mu\text{m}^2/\text{s}$  (Zheng et al., 2013). By comparison, the bundling frequency in hypocotyl epidermal cells was measured to be  $6.9 \times 10^{-5}$  events/ $\mu\text{m}^2/\text{s}$  (Hoffmann et al., 2014). These findings may explain, to some extent, why most actin filaments exist

in longitudinal bundles in the shank of pollen tubes. Future documentation of the role and mechanism of action of several major actin bundling factors in these processes will shed light on the regulation of the equilibrium between individual actin filaments and bundles.

## REGULATION OF ACTIN DYNAMICS IN THE POLLEN TUBE: THE ROLES AND MECHANISMS OF ACTION OF SEVERAL POLLEN-EXPRESSED ABPs

Considering the fact that the organization and dynamics of the actin cytoskeleton are directly regulated by various ABPs, studying their functions and mechanisms of action should provide insights into the regulation of the organization and dynamics of the actin cytoskeleton in the pollen tube. Indeed, genetic manipulation of pollen-expressed ABPs has increasingly enriched our knowledge in this area. Given that actin filaments in the apical and subapical regions are highly dynamic and inherently connected (Fu et al., 2001; Hwang et al., 2005; Cheung et al., 2010; Qu et al., 2013) and that shank-oriented longitudinal actin cables are relatively stable and, to some extent, functionally distinct, in the next sections, we will review the current state of knowledge regarding several ABPs that have been implicated in regulation of actin structures either in the apical and subapical regions or in the shank region.

## SEVERAL ABPs HAVE BEEN IMPLICATED IN REGULATION OF THE CONSTRUCTION AND REMODELING OF ACTIN STRUCTURES IN THE APICAL AND SUBAPICAL REGIONS

It is now generally accepted that a population of highly dynamic actin filaments are present in the apical region of the pollen tube. However, the organizational nature of the actin structures within the apical region remains poorly understood. Recent characterization of several ABPs has provided unique insights into this question and has also substantially expanded our understanding of the functions of these actin filaments, leading us to consider how they regulate cellular processes, such as exocytosis.

### FORMIN NUCLEATES ACTIN FILAMENTS FROM THE APICAL MEMBRANE

Live-cell imaging of actin filament dynamics has shown that actin filaments are constantly generated from the apical plasma membrane (Figure 2A), suggesting that membrane-anchored actin

**Table 2 | Dynamic parameters associated with actin filaments in different regions of the pollen tube.**

|   | Apical region       | Subapical region    | Shank               |
|---|---------------------|---------------------|---------------------|
| Maximal filament length ( $\mu\text{m}$ )                                   | $2.5 \pm 0.2^1$     | $3.277 \pm 0.322^2$ | $4.63 \pm 0.25^2$   |
| Filament lifetime (s)   | $20.2 \pm 2.9^1$    | $25.4 \pm 1.73^2$   | $25.7 \pm 1.2^2$    |
| Severing frequency (breaks/ $\mu\text{m}/\text{s}$ )                        | $0.034 \pm 0.009^1$ | $0.024 \pm 0.005^2$ | $0.020 \pm 0.002^2$ |
| Elongation rate ( $\mu\text{m}/\text{s}$ )                                  | $0.25 \pm 0.02^1$   | $0.245 \pm 0.02^2$  | $0.430 \pm 0.021^2$ |
| Depolymerization rate ( $\mu\text{m}/\text{s}$ )                            | $0.22 \pm 0.01^1$   | $0.204 \pm 0.01^2$  | $0.334 \pm 0.022^2$ |
| Bundling frequency (events/ $\mu\text{m}^2/\text{s}$ , $\times 10^{-4}$ )   | —                   | —                   | $2.3 \pm 0.54^2$    |
| Debundling frequency (events/ $\mu\text{m}^2/\text{s}$ , $\times 10^{-4}$ ) | —                   | —                   | $5.4 \pm 0.65^2$    |

The quantitative parameters associated with the dynamics of individual actin filaments were reported in Qu et al. (2013)<sup>1</sup> and Zheng et al. (2013)<sup>2</sup>.

nucleation factors may be required for this role. The Arp2/3 complex and formin proteins are arguably the best characterized actin nucleation factors in plants (Deeks et al., 2002; Blanchard and Staiger, 2010; Zheng et al., 2012; Yanagisawa et al., 2013). Historically, the Arp2/3 complex was assumed to play a role in regulating the nucleation of actin filaments in the apical region of the pollen tube (Mathur and Hulskamp, 2001). However, considering the observations that actin filaments grow outward quite linearly from the apical membrane and that loss of function of the Arp2/3 complex yields no obvious phenotype in pollen (unpublished observation), it is very unlikely that the Arp2/3 complex acts as the major nucleation factor in that region. In contrast, the formins might be more reasonable candidates for actin nucleation factors in this region. Overexpression of *Arabidopsis* Formin1 (AtFH1) has been shown to result in excessive formation of actin cables and to induce membrane curvature at the pollen tube tip (Cheung and Wu, 2004), implicating this formin in the generation of actin filaments from the apical membrane. Quite recently, it was demonstrated that AtFH5 is a major regulator of the nucleation of actin filaments growing from the apical membrane of the pollen tube (Cheung et al., 2010). AtFH5 localized at the apical membrane, and knockdown of AtFH5 diminished the abundance of actin structures in the apical and subapical regions of pollen tubes (Cheung et al., 2010), suggesting that apical membrane-anchored AtFH5 nucleates actin assembly for the construction of actin structures in the apical and subapical regions. In support of this hypothesis, biochemical data revealed that AtFH5 is a bona fide actin nucleation factor and is capable of nucleating actin assembly from actin monomers or actin monomers bound to profilin (Ingouff et al., 2005). Further studies are needed to dissect how the activity of AtFH5 is regulated during pollen tube growth, as well as how it may coordinate its activity with that of other formins. Additionally, considering the fact that the formin proteins can utilize the profilin/actin complex for assembly and that the actin monomer pool in plant cells is predicted to be buffered by profilin (Staiger and Blanchard, 2006), future analysis of the relationship between AtFH5 and profilin in the pollen tube is also necessary.

### CALCIUM-DEPENDENT FILAMENT SEVERING PROTEINS DRIVE THE TURNOVER OF APICAL ACTIN FILAMENTS

Functional characterization of villin proteins has recently provided unique insights into how the rapid turnover of actin filaments in the apical region of the pollen tube is achieved. Considering the fact that a tip-focused calcium gradient is present in the pollen tube with calcium concentrations that can reach 1–3  $\mu\text{M}$  in the apical region (Pierson et al., 1994; Holdaway-Clarke et al., 1997; Messerli et al., 2000), villin, a calcium-responsive actin depolymerization promoting factor, was suggested to be an important player (Hepler et al., 2001; Vidali and Hepler, 2001; Yokota et al., 2005; Staiger et al., 2010). Villin was originally isolated from lily (*Lilium longiflorum*) pollen biochemically (Nakayasu et al., 1998; Yokota et al., 1998) and was shown to reduce the length of actin filaments in the presence of calcium/calmodulin (Yokota et al., 2005). Since then, several members of the villin/gelsolin/fragmin superfamily of proteins have been implicated in regulation of actin dynamics in the pollen

tube (Xiang et al., 2007; Wang et al., 2008b). Importantly, by taking advantage of the power of *A. thaliana* genetic approaches, characterization of villins in *Arabidopsis* has provided exceptional insights into the roles of these proteins in regulating actin dynamics in the pollen tube and during its growth (Huang et al., 2014).

We have previously shown that pollen-expressed *Arabidopsis* villin2 (VLN2) and VLN5 are able to sever actin filaments in the presence of micromolar concentrations of free calcium (Zhang et al., 2010a; Bao et al., 2012). Remarkably, we also found that VLN5 promotes actin depolymerization in the presence of profilin under similar conditions (Zhang et al., 2010a), leading to the hypothesis that villins may regulate actin dynamics by promoting actin depolymerization in the apical region. Consistent with this idea, actin filaments accumulated in the apical region of *vln2 vln5* double mutant pollen tubes (Qu et al., 2013). Visualization of the dynamics of actin filaments at a single filament resolution showed that the average filament severing frequency decreased and the average maximum filament lifetime increased in the apical region of *vln2 vln5* double mutant pollen tubes (Qu et al., 2013). These results suggest that villins promote actin turnover via their calcium-dependent filament severing activity at pollen tube tips.

Consistent with the idea that actin filaments nucleated from the apical membrane are required for the construction of actin structures at the subapical region (Cheung et al., 2010), the accumulation of actin filaments in the apical region of *vln2 vln5* double mutant pollen tubes was accompanied by disorganization of actin filaments at the subapical region (Qu et al., 2013). Certainly, as villins are known to be versatile actin regulatory proteins (Huang et al., 2014), the filament bundling and stabilizing activity of VLN2 and VLN5 may also contribute to the construction of actin structures in the subapical region of the pollen tube. In support of this idea, actin filaments in the subapical region of *vln2 vln5* double mutant pollen tubes are more wavy and are thinner than those in their wild-type counterparts (Qu et al., 2013). Additionally, villin-mediated filament severing activity has also been implicated in regulation of the construction of the subapical region by eliminating actin filaments that do not align longitudinally (Qu et al., 2013).

Several recently characterized calcium-responsive filament severing proteins, such as MAP18 (Zhu et al., 2013) and MDP25 (Qin et al., 2014), may coordinate with villins to regulate actin dynamics in the pollen tube. Both MAP18 and MDP25 were shown to function as microtubule-associated proteins in vegetative cells (Wang et al., 2007; Li et al., 2011). Surprisingly, these proteins have been shown to act as regulators of actin dynamics in the pollen tube (Zhu et al., 2013; Qin et al., 2014). *In vitro* biochemical characterization revealed that both proteins are able to sever actin filaments in a calcium-dependent manner (Zhu et al., 2013; Qin et al., 2014). Direct visualization of the actin cytoskeleton showed that more actin bundles were present in the apical and subapical region of *map18* pollen tubes compared to the wild-type (Zhu et al., 2013), suggesting that MAP18 drives the turnover of actin filaments by severing. Similarly, filament severing frequency was decreased, and actin filaments were more abundant in the subapical region of *mdp25* pollen tubes (Qin et al., 2014). How these calcium-responsive filament severing proteins coordinate to regulate the

turnover of actin filaments at the pollen tube apex remains to be determined. Additionally, actin-depolymerizing factor (ADF) and actin-interacting protein (AIP1) have been implicated in the regulation of actin structures at the subapical region of the pollen tube (Chen et al., 2002; Lovy-Wheeler et al., 2006), although how they coordinate with calcium-responsive filament severing proteins to drive the turnover of actin filaments remains to be addressed.

### SEVERAL ABPs HAVE BEEN IMPLICATED IN THE GENERATION AND REMODELING OF LONGITUDINAL ACTIN CABLES IN THE SHANK

In the shank region, actin filaments are arranged into longitudinal cables (Lancelle and Hepler, 1992; Gibbon et al., 1999; Fu et al., 2001; Lovy-Wheeler et al., 2005). It is clear that these longitudinal actin cables regulate cytoplasmic streaming in pollen tubes by providing molecular tracks for myosins, similar to observations in other plant cells (Shimmen and Yokota, 2004). Previous results have shown that cytoplasmic streaming is relatively more resistant than pollen tube growth to actin depolymerizing drugs (Gibbon et al., 1999; Vidali et al., 2001), suggesting that longitudinal actin cables are relatively stable. However, the mechanisms by which these longitudinal actin cables are generated and maintained, as well as how they are remodeled, remain largely unknown. Characterization of several pollen-expressed ABPs, including several recently characterized in *Arabidopsis*, has shed new light on these mechanisms.

### FORMINS NUCLEATE ACTIN ASSEMBLY FOR THE CONSTRUCTION OF LONGITUDINAL ACTIN CABLES

Characterization of the pollen-specific Class I formin, AtFH3, has provided insight into the nucleation step required for the generation of longitudinal actin cables in the shank of the pollen tube (Ye et al., 2009). AtFH3 is a bona fide actin nucleation factor, capable of using the actin/profilin complex to nucleate actin assembly. It is also able to interact with the barbed end of actin filaments. Knockdown of *AtFH3* using RNAi affects the formation of longitudinal actin cables, resulting in depolarized growth of the pollen tube (Ye et al., 2009). Given that AtFH3 is an important regulator of actin nucleation, it will be very interesting to better understand exactly how its activity is regulated. Furthermore, characterization of how AtFH3 coordinates with other formins to nucleate actin assembly in the shank region should be a subject of future investigation.

### ADF/COFILIN REGULATES THE REMODELING OF LONGITUDINAL ACTIN CABLES

Actin-depolymerizing factor/cofilin is important for driving the rapid turnover of actin filaments in cells. Members of the ADF family have been implicated in regulation of actin turnover in pollen. For example, lily ADFs were shown to accumulate at the germination aperture during tube protrusion, but distribute evenly in the pollen tube (Smertenko et al., 2001). In contrast to this observation, tagging of tobacco pollen-specific ADF/cofilin (NtADF1) with GFP revealed that this protein decorates filamentous actin, particularly subapical actin structures and longitudinal actin cables in the shank (Chen et al., 2002). Overexpression of NtADF1 reduces the number of axially arranged fine actin cables

(Chen et al., 2002), implicating ADF as a driver in the turnover of longitudinal actin cables.

In order to probe the intracellular localization of pollen-specific *Arabidopsis* actin-depolymerizing factor 7 (ADF7) in the pollen tube, we generated several ADF7-EGFP fusion constructs containing EGFP inserted in different locations within the ADF7 molecule in hopes of minimizing the interference of the EGFP fusion on the function of ADF7. Among which, one ADF7-EGFP fusion protein (ADF7-EGFP<sub>V10</sub>) is fully functional (Zheng et al., 2013) and decorates filamentous actin throughout the pollen tube (Daher et al., 2011; Zheng et al., 2013). *In vitro* biochemical characterization showed that ADF7 is a typical ADF; it prefers ADP-loaded actin and inhibits nucleotide exchange, and it is able to promote actin depolymerization and sever actin filaments. However, by comparison, its actin depolymerizing and severing activity are lower than that of the vegetative ADF1 (Zheng et al., 2013). This observation is consistent with a previous report that the lily pollen ADF1 (LIADF1) has weak actin depolymerizing activity compared to the vegetative *Zea mays* ADF, ZmADF3 (Smertenko et al., 2001). These data suggest that reproductive ADFs may have evolved to play specific roles in the regulation of actin dynamics in the context of pollen.

Interestingly, ADF7-EGFP<sub>V10</sub> was found to fully retain both the monomer actin (G-actin) binding and filament severing activities and are fully functional *in vivo*, whereas another ADF7-EGFP fusion protein (ADF7-EGFP<sub>D75</sub>) retained G-actin binding, but was deficient in severing actin filaments, was non-functional in the pollen tube (Zheng et al., 2013). Results from analysis of these proteins suggested that the severing activity of ADF7 is crucial for its functions *in vivo*. Consistent with this hypothesis, specific abolishment of the severing activity of yeast cofilin has been shown to affect its *in vivo* function (Chen and Pollard, 2011). These data suggest that the associated severing activity is important for the function of ADF/cofilin protein family members *in vivo*. Additional observations showed that the turnover rate of actin filaments was decreased in *adf7* pollen tubes, consistent with a role in promotion of actin depolymerization. Consequently, the amount of filamentous actin and the extent of filament bundling were increased in *adf7* pollen tubes. These data suggest that ADF7 is an important player in driving the turnover of actin filaments in the shanks of pollen tubes (Zheng et al., 2013). Detailed documentation of the functional coordination of ADF7 with other ADF isoforms, such as ADF10 (Daher et al., 2011), will provide further insight into the regulation of actin turnover in the pollen tube. Additionally, determination of the functional relationship between ADF7 and other players, such as cyclase-associated protein (CAP1) (Chaudhry et al., 2007; Deeks et al., 2007) and AIP1 (Ketelaar et al., 2004a; Shi et al., 2013) will also shed light on the regulation of the dynamic turnover of longitudinal actin cables.

### ACTIN FILAMENT BUNDLING PROTEINS GENERATE LONGITUDINAL CABLES AND MAINTAIN THEIR LONGITUDINAL ARRANGEMENT

Several actin filament bundling proteins have been implicated in organization of actin filaments into bundles, as well as maintenance of the longitudinal arrangement of actin bundles in the shanks of pollen tubes. *In vitro* biochemical studies have shown that *Arabidopsis* FIMBRIN5 (FIM5) is a bona fide actin bundling

factor that stabilizes actin filaments (Wu et al., 2010). Loss of function of FIM5 affects pollen germination and polarized tube growth. FIM5 decorates actin filaments throughout the pollen tube, and loss of function of FIM5 results in disorganization of actin filaments in the pollen tube and alters the longitudinal arrangement of actin cables (Wu et al., 2010; Su et al., 2012). As a result, the pattern of cytoplasmic streaming is altered, exhibiting decreased velocity and altered direction (Wu et al., 2010). Unexpectedly, actin bundles were found to be thicker in *fim5* pollen tubes compared to wild-type tubes (Wu et al., 2010), suggesting that loss of function of FIM5 may upregulate the activity some other actin bundling factors. This question might be worthwhile to examine in the future. Furthermore, the activity of LI-FIM1 was shown to be sensitive to pH (Su et al., 2012), suggesting that fimbrin might act as a sensor that regulates actin dynamics in response to pH. Further study is needed to characterize the mechanisms by which fimbrin regulates actin dynamics in the pollen tube in response to oscillations in intracellular pH.

The bundling factor villin has also been implicated in regulation of longitudinal actin bundle formation in the shank of the pollen tube. Two pollen-expressed *Arabidopsis* villin isoforms, VLN2 and VLN5, were demonstrated to be bona fide actin filament bundling proteins (Zhang et al., 2010a; Bao et al., 2012). Though loss of function of VLN5 alone did not have an overt effect on the generation and formation of longitudinal actin cables (Zhang et al., 2010a), loss of function of both VLN2 and VLN5 decreased the amount of actin filaments, suggesting that these proteins stabilize actin filaments in the pollen tube. Additionally, actin cables became thinner and more disorganized in the shanks of *vln2 vln5* pollen tubes (Qu et al., 2013), suggesting that VLN2 and VLN5 function as actin bundling factors that regulate the formation of shank-oriented longitudinal actin bundles.

Several other actin filament bundling factors may also be involved in regulating the construction and maintenance of longitudinal actin cables, such as LIMs (Papuga et al., 2010) and the recently identified, novel, plant actin-crosslinking protein, CROLIN1 (Jia et al., 2013). For instance, LI-LIM1 was shown to promote actin filament bundling and stabilize actin filaments in the pollen tube (Wang et al., 2008a), suggesting that LI-LIM1 is involved in regulating the formation of longitudinal actin cables. Loss of function of CROLIN1 led to instability of actin filaments in the shanks of pollen tubes (Jia et al., 2013), implicating this protein in regulation of the construction of shank-oriented, longitudinal actin cables. However, the means by which CROLIN1 regulates the construction and dynamics of longitudinal actin cables needs to be carefully examined.

#### SCHEMATIC DESCRIBING THE REGULATION OF THE CONSTRUCTION AND REMODELING OF DISTINCT ACTIN STRUCTURES IN THE POLLEN TUBE

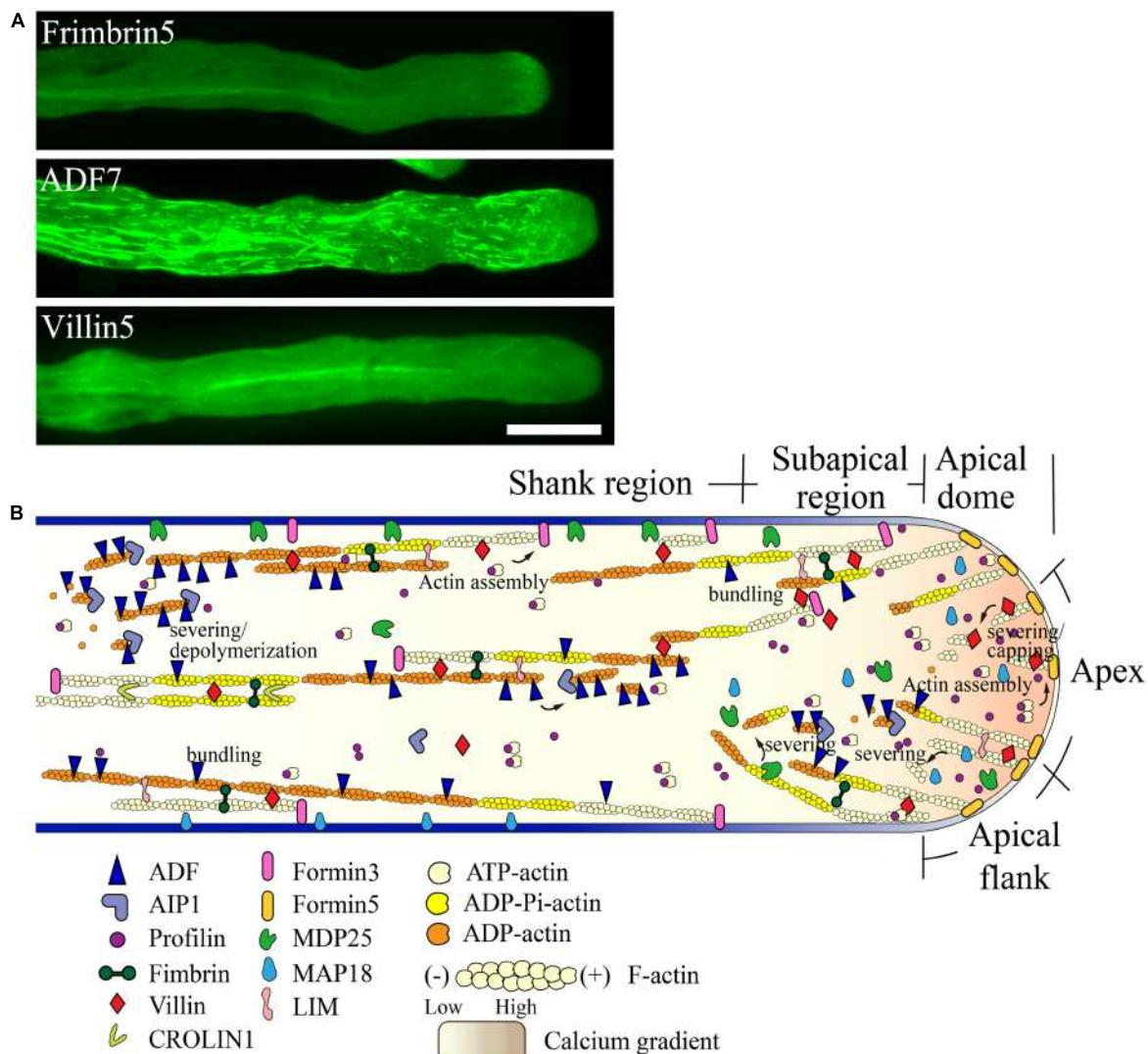
As described above, our knowledge regarding the organization and regulation of the actin cytoskeleton in the pollen tube has grown substantially. Functional characterization of several pollen-expressed ABPs has enriched our understanding of the relevant mechanisms. In particular, direct visualization and quantitative analysis of the dynamics of individual actin filaments in pollen tubes with loss of function of specific ABPs, as well as

careful comparisons with wild-type pollen tubes have yielded substantial insight. Based on these data, we propose a simple model describing the role of various ABPs in regulating the organization of the actin cytoskeleton in the pollen tube (**Figure 3**).

Two formins, AtFH5 and AtFH3, regulate the construction of actin structures in the apical and subapical regions and shank region, respectively. AtFH5 is localized on the apical membrane within the apical dome, where it nucleates actin filaments from the apical membrane that are used for the construction of actin structures in the apical and subapical regions (**Figure 3B**; Cheung et al., 2010). In contrast, AtFH3 nucleates actin filaments within the cytoplasm or from the membrane to generate longitudinal actin cables in the shank (**Figure 3B**; Ye et al., 2009). Certainly, transport of actin filaments from the apical and subapical regions to the shank could represent another potential mechanism leading to the construction of longitudinal actin cables. Additionally, other pollen-expressed formin proteins may play yet undiscovered roles in construction of distinct actin arrays in the pollen tube.

To build actin structures in the apical and subapical regions, AtFH5-generated actin filaments are instantaneously bundled by actin bundling factors, such as villins (Qu et al., 2013), fimbriins (Wu et al., 2010; Su et al., 2012), and/or LIMs (Papuga et al., 2010), allowing them to grow outward linearly from the membrane. These actin filaments are subjected to rapid turnover due to filament severing and depolymerizing activities. With respect to these activities, several calcium-dependent filament severing and depolymerizing proteins, including villins (Qu et al., 2013), MAP18 (Zhu et al., 2013), and MDP25 (Qin et al., 2014), are reasonable candidates for these roles. Together, these mechanisms presumably lead to the decreased abundance of actin filaments at the pollen tube apex. Certainly, a potential role for ADF and its cofactors in promoting the turnover of actin filaments in the apical region needs to be examined in the future.

Filament severing activity mediated by calcium-responsive severing proteins may allow the departure of actin filaments originating from the apical membrane away from the apical region. These filaments can then be used for the construction of subapical actin structures. This idea is partially supported by the observation that loss of function of villins affects the formation of actin structures at the subapex (Qu et al., 2013). Furthermore, actin filaments originating from the extreme apical membrane can shift toward the apical flank via membrane flow and can further elongate to directly participate in the construction of actin structures at the subapex. At the subapical region, villin plays a major role in the formation of regular actin collars through bundling and stabilization of longitudinally-aligned actin filaments (**Figure 3B**; Qu et al., 2013). Additionally, villin may facilitate the formation of regular actin collars by eliminating actin filaments that do not align longitudinally via its filament severing activity (**Figure 3B**; Qu et al., 2013). MDP25 may also have a similar function in severing of actin filaments that do not align longitudinally in the subapical region (Qin et al., 2014). Furthermore, previous studies suggest that ADF and AIP1 may also be involved in regulating the turnover of actin structures at the subapical region (Chen et al., 2002; Lovy-Wheeler et al., 2006).



**FIGURE 3 | Schematic describing the regulation of actin dynamics in the pollen tube based on the functional characterization of ABPs derived mainly from *Arabidopsis*. (A)** Intracellular localization of several ABPs in the pollen tube. For methods used to determine the localization of FIMBRIN5, ADF7, and VILLIN5, see descriptions in

previous studies (Wu et al., 2010; Qu et al., 2013; Zheng et al., 2013). Scale bar = 10  $\mu$ m. **(B)** Schematic describing the intracellular localization and function of various ABPs in the pollen tube. For detailed information regarding the intracellular localization and function of each ABP, see the description in the text.

In the shank, AtFH3-generated actin filaments (Ye et al., 2009) initially undergo dynamic turnover due to the activity of ADFs, such as ADF7 (Zheng et al., 2013), along with several ADF cofactors, like AIP1 (Ketelaar et al., 2004a; Shi et al., 2013) and CAP1 (Chaudhry et al., 2007; Deeks et al., 2007). Subsequently, actin bundling factors, such as fimbriins, villins, LIMs, and CROLIN1, may participate in packing these filaments into longitudinal actin cables in addition to maintaining their longitudinal arrangement and stabilizing them (Figure 3B).

## CONCLUDING REMARKS

There is no doubt that our knowledge regarding the organization, dynamics, and regulation of the actin cytoskeleton in the

pollen tube has grown substantially in recent years, although many issues remain to be resolved. Importantly, adoption of the *A. thaliana* pollen tube as a cellular system for the study of these processes has greatly facilitated progress in this field due to the powerful combination of *Arabidopsis* genomic and genetic approaches, as well as the introduction of complex spatiotemporal imaging technology and the development of appropriate actin markers that have allowed real-time visualization of individual actin filaments. Recent improvements in imaging technology have expanded our view of actin filament dynamics, as well as our understanding of the underlying organization of actin structures in the pollen tube. A detailed description of the dynamic properties of actin filaments in the apical region represents one of the exciting achievements resulting from these technologies (Qu

et al., 2013), and this data has greatly enhanced our understanding of the function of the actin cytoskeleton in regulating polarized pollen tube growth. Careful characterization of the mode of action of several pollen-expressed ABPs has provided additional insights into regulation of the actin cytoskeleton during pollen tube growth. As some ABPs act as direct sensors for various signals, these studies have also shed light on how various signals converge on these ABPs to control actin dynamics. For example,  $\text{Ca}^{2+}$  signaling has been shown to regulate actin dynamics by controlling the activity of villins in the pollen tube. An important future challenge will be to delineate the roles of various signaling transduction pathways in order to determine how various signals converge on ABPs to regulate actin dynamics in the pollen tube. Certainly, in this field, the eternal and most challenging question is still to understand precisely how the actin cytoskeleton functions to regulate the pollen tube growth.

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# The expression and roles of parent-of-origin genes in early embryogenesis of angiosperms

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## INTRODUCTION

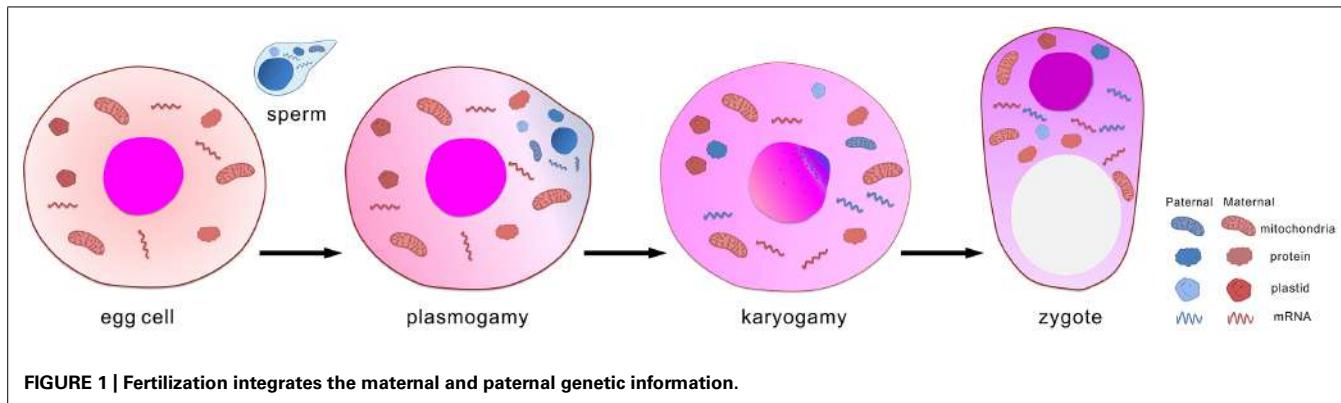
During sexual reproduction of flowering plants, male and female gametes are formed in the haploid gametophytic generation (Walbot and Evans, 2003; Chang et al., 2011). In angiosperm, the typical female gametophyte contains two kinds of female gametes, a haploid egg cell and a diploid central cell with two identical copies of the maternal genome. The male gametophyte is found in pollen, which carries one generative cell or two sperm cells. During pollen generation, sperm cells are transported through the pollen tube to the female gametophyte. Upon double fertilization, two sperm cells enter the embryo sac. One sperm cell fuses with the egg cell, and the other fuses with the central cell. This integration of the two gamete genomes results in the formation of a diploid embryo and a triploid endosperm, respectively. After fertilization, the embryo forms basic morphological and physiological structures (Le et al., 2007), during which the endosperm plays a nutritive role, similar to the placenta of mammals, to support embryonic development (Lopes and Larkins, 1993; Olsen, 2004). During this process, both paternal and maternal genetic information may contribute to the fertilization and development of the embryo, which leads to generation of the sporophyte. These parental information includes RNA that transcribed in sperm and/or egg cells, proteins that synthesized and deposited in gametes, paternal or maternal genome, and mitochondria and plastid genome. After fertilization they are brought into and integrated in zygote (Figure 1). Due to technical limitations the contribution of gamete-delivered proteins, mitochondria and plastid genome to zygote development and early embryogenesis are hardly investigated. Current studies as pioneer works mainly focus on *de novo* expression of imprinted genes and gamete-delivered transcripts.

Uniparental transcripts during embryogenesis may arise due to gamete delivery during fertilization or genomic imprinting. Such transcripts have been found in a number of plant species and appear critical for the early development of embryo or endosperm in seeds. Although the regulatory expression mechanism and function of these genes in embryogenesis require further elucidation, recent studies suggest stage-specific and highly dynamic features that might be essential for critical developmental events such as zygotic division and cell fate determination during embryogenesis. Here, we summarize the current work in this field and discuss future research directions.

**Keywords:** uniparental transcripts, gamete-delivered transcript, maternal control, paternal allele, genomic imprinting, embryo

The molecular mechanisms of fertilization and early embryogenesis, especially the role of parent-of-origin genes, have been well studied in animals. However, little is known about these processes in plants due to technical limitations. Gametogenesis, fertilization and embryogenesis occur deep in the plant saprophytic tissues, thus rendering it difficult to observe the developmental events and investigate the molecular mechanisms of these processes directly. Modern technological advances have allowed the isolation and analysis of gametes, zygotes, and early embryos in a wide variety of plants including maize, tobacco, *Arabidopsis*, rice, and wheat (Engel et al., 2003; Zhao et al., 2011; Nodine and Bartel, 2012; Anderson et al., 2013; Domoki et al., 2013). Therefore, great advances have been made toward understanding the role of uniparental transcripts in plant embryogenesis.

In animals, maternal allele products synthesized during gametogenesis exert control in all aspects of embryonic development prior to the global activation of the zygotic genome (Tadros and Lipshitz, 2009). However, in plants, the parental contribution in early embryogenesis has not yet been fully understood. Early reports indicated that the transcripts in early embryos were mainly originated from the maternally inherited alleles and the transcription of paternal alleles was delayed (Vieille-Calzada et al., 2000; Baroux et al., 2001; Golden et al., 2002; Grimanelli et al., 2005). Baroux et al. (2008) further suggested that early embryogenesis in plants was maternally controlled similar to that in animals, as early studies indicated that maternal transcripts could support embryonic development until the proembryo stage. At the same time, some other researches presented evidences of early activated paternal genome (Weijers et al., 2001; Scholten et al., 2002; Lukowitz et al., 2004; Sheldon et al., 2008). Recently, paternal transcripts were proved to be critical for the normal development of early



embryo (Ueda et al., 2011; Babu et al., 2013). More impressively, interleukin-1 receptor-associated kinase (IRAK)/Pelle-like kinase gene, *SHORT SUSPENSOR* (*SSP*) transcripts were found to be produced in mature pollen and were believed to be carried into the egg cell via fertilization in *Arabidopsis thaliana* (Bayer et al., 2009). *SSP* functioned during the asymmetric first division in the zygote, indicating that the paternal transcripts from sperm cells may be involved in many aspects of zygotic development and early embryogenesis in plants. Our previous work also confirmed that paternal transcripts in sperm cells could be found in zygotes soon after fertilization (Xin et al., 2011), suggesting the possibility that sperm-delivered paternal transcripts may be involved in zygotic development.

Meyer and Scholten (2007) reported the relative expression levels of parental transcripts in zygotes, suggesting equivalent parental contribution in maize zygotic development. Maternally expressed in embryo 1 (*meel1*) in maize was the first reported imprinted gene in a plant embryo, although its function is unclear (Jahnke and Scholten, 2009). Using deep sequencing in a genome-wide analysis, Autran et al. (2011) assessed the parental contributions in early embryogenesis and found that the maternal transcripts predominated at early embryonic stages in *Arabidopsis*. With development, the relative paternal contribution arose due to the gradual activation of the embryonic genome. Subsequently, Nodine and Bartel (2012) found that a majority of genes were expressed equally from both parents at the beginning of embryogenesis in *Arabidopsis*. Interestingly, some of these works focused on the quantitative ratio of maternal and paternal transcripts, some mainly analyzed the regulatory roles of these genes. It is not surprised to see various conclusions. Even more, a latest report indicated that the different results might be due to the different material (e.g., ecotypes) they used in their experiments (Del Toro-De León et al., 2014). Despite all these discussions, it is believed that some transcripts are derived primarily from one parent or from imprinted genes in embryos soon after fertilization (Autran et al., 2011; Nodine and Bartel, 2012). These studies indicate that the parent-of-origin gene transcripts indeed exist in the zygote or early embryo. Such transcripts could arise from both the gamete-delivered and *de novo* expression of imprinted genes. Each type of uniparental transcript may play specific roles in plant development, since they are regulated by different molecular mechanisms. This review highlights the

characteristics of uniparental transcripts during early embryogenesis.

## GAMETE-CARRIED MATERNAL OR PATERNAL TRANSCRIPTS INVOLVED IN EARLY EMBRYOGENESIS

### MATERNAL TRANSCRIPTS

The embryo originates from a fertilized egg cell, termed a zygote. Two sequential events occur during the integration of a sperm and an egg cell: plasmogamy and karyogamy. Not only do the two genomes integrate, but also various components of the cytoplasm mix during the fertilization process. For example, sperm mitochondria could be found in fertilized egg cells of tobacco (Yu and Russell, 1994) although mitochondria is usually inherited maternally.

During early embryogenesis in most animal species, maternal transcripts deposited in the egg cells are involved in various developmental processes before activation of the zygotic genome, such as formation of embryonic axes, cell differentiation, and morphogenesis (Johnston, 1995; Wylie et al., 1996; Nishida, 1997; Angerer and Angerer, 2000; Mohr et al., 2001; Pellettieri and Seydoux, 2002). Although various experimental data support the hypothesis that maternal control may also exist during early embryogenesis in plants (Baroux et al., 2008), little is known about transcripts stored in egg cells and their role in early embryogenesis (Xin et al., 2012).

Using microdissection, Sprunck et al. (2005) constructed a cDNA library from wheat egg cells, and a total of 404 clusters were found to function in metabolic activity, mRNA translation and protein turnover. Subsequently, another 226 expressed sequence tags (ESTs) were studied in wheat egg cells (Domoki et al., 2013). In a similar analysis carried out in tobacco, thousands of ESTs were detected, which may be involved in a variety of developmental processes (Ning et al., 2006; Zhao et al., 2011). In addition, microarray technology combined with laser-assisted microdissection (LAM) was used to analyze the expression profile in *Arabidopsis* egg cells (Wuest et al., 2010). Transcriptomic analysis of egg cells isolated by manual manipulation was performed in rice (Ohnishi et al., 2011; Abiko et al., 2013), and genome-wide deep sequencing was used to characterize the gene expression profile in rice egg cells (Anderson et al., 2013). The functional categories of approximately 27,000 genes detected proved to be comprehensive. However, a comparison of the egg-specific expression of transcriptomes in rice and

*Arabidopsis* revealed relatively different sets of genes in egg cells of rice and *Arabidopsis* (Ohnishi et al., 2011).

The role of mRNA stored in egg cells has been investigated. Although downregulation of RNA polymerase II by RNA interference (RNAi) impeded *de novo* transcription, the development of *Arabidopsis* embryos continued until the preglobular stage (Pillot et al., 2010). In tobacco, zygotic development continued without *de novo* transcription until 72 h after pollination (HAP). The cytological observation of developmental events in transcriptionally inhibited zygotes showed that maternal transcripts stored in egg cells were functionally competent in gamete fusion, zygote volume reduction, complete cell wall formation, large vacuole disappearance, and limited cell enlargement during early developmental stages. However, *de novo* transcripts would then seize control of embryogenesis to trigger subsequent developmental processes (Zhao et al., 2011).

Interestingly, small RNA-mediated transposon silencing is thought to be an essential regulatory mechanism in male and female gametes (Slotkin et al., 2009; Martínez and Slotkin, 2012). Anderson et al. (2013) evaluated the expression of genes involved in the miRNA and siRNA pathways in transcriptomes of rice gametes and showed that all important components involved in these pathways were active in egg cells rather than in sperm cells. Thus, transposon silencing is mediated by small RNAs produced in egg cells; moreover, it is regulated in the zygote by small RNAs inherited from the egg cells (Anderson et al., 2013).

Currently, the roles of female gamete transcripts in zygotes and early embryogenesis are unclear. Although *de novo* transcription in the zygotic genome is activated within hours after fertilization in maize, tobacco and *Arabidopsis*, the maternal transcripts deposited in the egg cells still play a key role in the initial stages of zygotic development (Meyer and Scholten, 2007; Zhao et al., 2011; Nodine and Bartel, 2012).

### PATERNAL TRANSCRIPTS

The sperm cell, the other contributor to zygote, has a simple structure including the karyoplasm and very little cytoplasm. Due to the condensed chromatin observed in sperm cells, it was generally thought that inactive male transcription made no contribution to early embryogenesis prior to zygotic genome activation. This view might be supported in animals, since almost all mRNAs in zygotes are inherited from egg cells (Ostermeier et al., 2004; Krawetz, 2005). However, the cytoplasm of sperm cells may play an important role during early embryogenesis after fertilization in plants, as the extracted sperm nuclei in maize was insufficient to achieve successful fertilization *in vitro* (Matthys-Rochon et al., 1994).

Recently, increasing evidence has confirmed the presence of a number of transcripts in the sperm cell, refuting the hypothesis that highly condensed chromatin in sperm cells impede activation of transcription. Various cDNA libraries have been constructed based on isolated sperm cells from rice (Gou et al., 2001), tobacco (Xin et al., 2011), maize (*Zea mays*; Engel et al., 2003), and *Plumbago* dimorphic (Gou et al., 2009). Additionally, genome-wide expression has been detected in different plants. Using microarray analysis, the transcriptomic profile in sperm cells was investigated in rice and *Arabidopsis* (Borges et al., 2008;

Russell et al., 2012; Abiko et al., 2013). The transcriptomes of rice sperm cells were studied by deep sequencing (Anderson et al., 2013), and ~25000 genes were analyzed. These studies revealed a diverse and broad constitution of mRNAs in sperm cells. Subsequently, sperm transcription profiles were compared among different plant species. Only 35 genes were found in common among 1,048 ESTs in tobacco (Xin et al., 2011), 5,829 genes in *Arabidopsis* (Borges et al., 2008), and 5,174 ESTs in maize (Engel et al., 2003). Analysis of these 35 genes suggested that active transcription in sperm cells is involved in many basic pathways and processes such as metabolism, transcription, translation, signal transduction and intercellular trafficking (Xin et al., 2011).

For years, plant scientists have questioned whether male transcripts are delivered to the zygote during the fertilization process, and if so, whether these sperm-carrying transcripts have a role in zygote development or early embryogenesis. In our previous work, we identified sperm-specific transcripts in zygotes at 96 HAP (Ning et al., 2006). Subsequently, two kinds of sperm transcripts with unknown function were revealed in zygotes ~10 h after fertilization (HAF). These results strongly suggested that paternal transcripts could be delivered into zygotes, where they might play a role in zygote activation and/or early embryogenesis (Xin et al., 2011). Similarly, Ohnishi et al. (2014) found abundant expression of the Os07g0182900 rice gene in sperm cells (Abiko et al., 2013), but not in unfertilized egg cells. The fact that its transcripts could be detected in the zygote ~10–20 min after fertilization indicated that transcripts in plant zygotes could be delivered from the sperm cells by plasmogamy. The Os07g0182900 gene encoding cytosine-5 DNA methyltransferase 1 (MET1) may be involved in the transition from the zygote to two-celled pre-embryo stage, as the process could be partially inhibited by a specific inhibitor of MET1 (Abiko et al., 2013). In *Arabidopsis*, the polarity of elongated zygotes contributed substantially to regular embryonic development. Corrected asymmetric cell division led to normal formation of the initial apical–basal axis and the embryo and suspensor ancestors in plants (Jeong et al., 2011; Zhang and Laux, 2011; Ueda and Laux, 2012). Sperm transcripts are now believed to be essential in this critical developmental process. Bayer et al. (2009) reported that transcripts of the IRAK/Pelle-like kinase gene, SSP, were produced in mature sperm cells and translated in zygotes after fertilization. Defective SSP influenced the elongation of the zygote and the formation of suspensor through the YODA-dependent MAPKKK signaling pathway. These examples suggest that sperm mRNAs might have vital functions in normal developmental embryogenesis.

### IMPRINTED GENES IN EARLY EMBRYOGENESIS

In mammals and flowering plants, genomic imprinting is a general epigenetic mechanism associated with the differential expression of parental alleles (Feil and Berger, 2007). The differential *de novo* transcription of parental alleles is caused by different epigenetic influences established in the germ line, rather than the nucleotide changes or uniparental transcripts caused by gamete delivery. Maternally expressed imprinted genes (MEGs) are expressed maternally but silenced paternally, whereas maternally expressed

imprinted genes (PEGs) are expressed paternally but silenced maternally.

### IMPRINTED GENES IN PLANT EMBRYOS

Imprinting is another cause of unequal contributions from parental transcripts in the early embryo. A minority of imprinted genes has been identified in endosperm using conventional methods, such as sequence homologies, small-scale transcriptional surveys, assays for reduced DNA methylation and mutant identification. The *mee1* gene in maize provided the evidence confirming the presence of imprinted genes in embryos (Raissig et al., 2011). The differential methylation status between paternal and maternal alleles regulates the maternal expression of *mee1* in the embryo and endosperm. Dynamic expression of *mee1* was found in the early embryo, but its function remains unclear (Jahnke and Scholten, 2009).

Recently, genome-wide approaches have been used to identify imprinted genes in *Arabidopsis*, maize and rice (Gehring et al., 2011; Hsieh et al., 2011; Luo et al., 2011; Waters et al., 2011; Wolff et al., 2011; Zhang et al., 2011). Several 100 endosperm-specific imprinted genes were newly detected in these species. However, the presence of imprinted genes in the embryo remains controversial. For example, Hsieh et al. (2011) identified 116 MEGs and 10 PEGs in *Arabidopsis* endosperm 7–8 days after pollination (DAP), while 37 MEGs and one PEG were found in the embryo during the same period. However, the imprinted genes in the embryo were considered to be false positives due to contamination with endosperm or maternal tissue (Hsieh et al., 2011). Similarly, Gehring et al. (2011) identified 165 MEGs and 43 PEGs in *Arabidopsis* endosperm at 6–7 DAP; additionally, 17 MEGs and one PEG were found in embryos during the same period. However, the imprinted genes in the embryo could have been due to endosperm contamination or biased expression dependent on an unchangeable allele (Gehring et al., 2011). In monocots, Luo et al. (2011) found 262 imprinted loci in rice endosperm at 5 DAF. An imprinted gene was detected in both the embryo and endosperm; however, this candidate requires further confirmation by confirming its expression in gametes (Luo et al., 2011). Waters et al. (2011) found 54 MEGs and 46 PEGs in maize endosperm at 14 DAP, with 29 MEGs and nine PEGs in embryos during the same period. However, these imprinted genes in embryos might be due to contamination, trafficking of transcripts produced in the endosperm to the embryo, or relatively stable transcripts inherited from the gametes (Waters et al., 2011).

Currently, genomic imprinting in *Arabidopsis* embryos has not been validated conclusively. Raissig et al. (2013) constructed cDNA libraries using 2 to 4-cells embryos and globular embryos isolated from the reciprocal cross of the Col-0 and the Ler accessions. Imprinted gene candidates were then chosen, and their relative expression levels between parental alleles were assessed by reverse transcription polymerase chain reaction (RT-PCR) and Sanger sequencing (Raissig et al., 2013). A total of 11 MEGs were expressed at the 2 to 4-cells and globular embryo stages, and one PEG was expressed at the 2 to 4-cells embryo stage. No transcripts in the one PEG or in nine of the MEGs were detected in the gametes, indicating that their imprinted expression in the embryo was derived from *de novo* transcription and was reliable.

To avoid contamination, strict procedures were adopted in constructing the cDNA libraries. In addition, an independent assay was used to confirm the genomic imprinting in embryos by fusing the promoters of seven MEGs and one PEG with the reporter gene β-glucuronidase (GUS). Promoter-GUS reporter lines (Col-0 background) were crossed reciprocally with wild-type plants (Col-0), and the analysis of stained F1 embryos showed that six MEG reporter lines were either imprinted fully or showed a strong bias for maternal expression (Raissig et al., 2013). Furthermore, Raissig et al. (2013) detected imprinted expression of all embryonic MEGs and the PEG in other samples, as early Col-0 × Cvi embryos (different accession, similar stage; Nodine and Bartel, 2012) and late torpedo-stage Col-0 × Ler embryos (same accessions, but later stage; Gehring et al., 2011). The results confirmed that the expression of most imprinted genes during early embryogenesis was maintained regardless of the different accessions or later developmental stage (Raissig et al., 2013). Therefore, these results indicated that genomic imprinting may not be restricted to the endosperm and may be more extensive in embryos than thought previously.

### FUNCTION OF IMPRINTED GENES INVOLVED IN EMBRYOGENESIS

In mammals, 100s of imprinted genes have been identified that are connected to the location of nutrient transfer from mother to offspring, embryogenesis, and postnatal development (Constâncio et al., 2004; Gregg et al., 2010). Abnormal imprinting can harm fetal growth, hormone systems after birth, and adult brain function. Whereas genome-wide approaches have revealed many imprinted genes involved in transcriptional regulation, chromatin modification, hormone signaling, ubiquitin degradation, small RNA pathways and metabolism (Gehring et al., 2011; Hsieh et al., 2011; Luo et al., 2011; Raissig et al., 2011, 2013) in plants, little is known regarding the involvement of imprinted genes in plant development (**Table 1**).

To date, only four imprinted genes in endosperm are known to be involved in embryogenesis (Raissig et al., 2011; Costa et al., 2012). In *Arabidopsis*, the FERTILIZATION-INDEPENDENT SEED (*FIS*) genes *MEA* (*FIS1*) and *FIS2* belong to the Polycomb group family (PcG). *MEDEA* (*MEA*) is expressed in both the embryo and endosperm; however, maternal imprinting has been confirmed only in the latter, and it remains to be determined whether *MEA* is imprinted in the embryo (Raissig et al., 2013). *FIS2* is a maternally imprinted gene, and its expression was detected both in the central cell before fertilization and endosperm after fertilization (Luo et al., 2000). Double fertilization products that contained maternal alleles of *mea* and *fis2* resulted in failure of endosperm cellularization. Moreover, embryogenesis ceased at the heart/torpedo stage, resulting in seed abortion (Luo et al., 2000). Another novel maternal imprinted gene *MPC* was found to be active in the central cell before fertilization and in the endosperm from fertilization to 4 DAP. Knockdown of *MPC* through RNAi resulted in defective seed development, with delayed embryogenesis and abnormal embryo and endosperm morphology (Tiwari et al., 2008). In maize, maternally expressed gene 1 (*Meg1*) encodes a new kind of signaling peptide located in endosperm nutrient transfer cells, where it regulates their establishment and differentiation. *Meg1* is the first identified imprinted gene in plants that

**Table 1 | Uniparental genes in sexual plant reproduction.**

|                    | Genes   | Epigenetic mark                          | Ecotype/inbred line for detection | Period of detection  | Mutant/RNAi phenotype | Reference   |
|--------------------|---|--|-----------------------------------|----------------------|-----------------------|---|
| <b>Endosperm</b>   |   |  |                                   |                      |                       |   |
| <i>Arabidopsis</i> | <i>MEA</i> (MEG) <sup>1,2</sup>                                   | H3K27me3<br>DNA-me<br>DNA-me             | Ler&RLD<br>Col-0, Ler, WS         | 6.78 DAP<br>6.78 DAP | Seed abortion<br>—    | Kinoshita et al. (1999),<br>Vieille-Calzada et al. (1999)<br>Kinoshita et al. (2004),<br>Julien et al. (2006)<br>Julien et al. (2006, 2008),<br>Luo et al. (2000) |
|                    | <i>FWA</i> (MEG)  | DNA-me                                   | C24&Col                           | 0.5-5 DAP            | Seed abortion         | Tiwari et al. (2008)  |
|                    | <i>FIS2</i> (MEG) <sup>1</sup>                                    | DNA-me                                   | Col&Ler<br>Col&C24                | 3.5,7 DAP<br>1-4 DAP | Abnormal seed<br>—    | Köhler et al. (2003, 2005),<br>Makarevich et al. (2008)   |
|                    | <i>MPC</i> (MEG) <sup>1</sup><br><i>PHE1</i> (PEG) <sup>1,2</sup> | DNA-me<br>H3K27me3<br>DNA-me<br>H3K27me3 | Col&Ler<br>Col&C24                | 5 DAP                | Endosperm defects     | Ingouff et al. (2005),<br>Fitz Gerald et al. (2009)   |
|                    | <i>FH5</i> (MEG) <sup>1,2</sup>                                   | H3K27me3                                 | Col&Ler                           | 3 DAP                | —                     | Shirzadi et al. (2011),<br>Wuest et al. (2010)  |
|                    | <i>AGL36</i> (MEG) <sup>1</sup>                                   | DNA-me                                   | Col-g&Ler                         | Torpedo-stage        | —                     | Gehring et al. (2009)   |
|                    | 3 MEGs&2 PEGs   | DNA-me                                   | Col&Ler                           | 7-8 DAP              | —                     | Hsieh et al. (2011)   |
|                    | 116 MEGs&10 PEGs  | —  | Col-0&Ler                         | 6 or 7 DAP           | —                     | Gehring et al. (2011)   |
|                    | 165 MEGs&43 PEGs  | —  | Col-0&Bur-0                       | 4DAP                 | —                     | Wolff et al. (2011)   |
|                    | 39 MEGs&27 PEGs   | —  |                                   |                      |                       |   |
| Maize              | <i>Fie1</i> (MEG) <sup>2</sup>                                    | DNA-me,<br>H3K27me3,                     | B73&Mo17;<br>SSS1&NSS1            | 2-15 DAP             | —                     | Danilevskaya et al. (2003),<br>Gutiérrez-Marcos et al.<br>(2006),<br>Hernon et al. (2007)   |
|                    | <i>Fie2</i> (MEG)   | H3/H4-Ac<br>DNA-me                       | B73&Mo17                          | 2.5 DAP              | —                     | Danilevskaya et al. (2003),<br>Gutiérrez-Marcos et al.<br>(2006),<br>Hernon et al. (2007),<br>Guo et al. (2003),<br>Haun and Springer (2008)                      |
|                    | <i>Nrp1</i> (MEG) <sup>2</sup>                                    | DNA-me,<br>H3K27me3,<br>H3/H4-Ac         | B73&Mo17;<br>SSS1&NSS1            | 10,14,21 DAP         | —                     | Gutiérrez-Marcos et al. (2003)  |
|                    | <i>Peg1</i> (PEG)   | —  | W22&Tx303                         | 12 DAP               | —                     |   |
|                    |   |  |                                   |                      |                       | (Continued)   |

**Table 1 | Continued**

| Genes                             | Epigenetic mark                 | Ecotype/inbred line for detection | Period of detection         | Mutant/RNAi phenotype       | Reference   |
|-----------------------------------|---------------------------------|-----------------------------------|-----------------------------|-----------------------------|---|
| <i>Meg 1</i> (MEG) <sup>1,2</sup> | DNA-me                          | F2, A69Y; VV23                    | 4 DAP                       | Reduced-size seeds          | Gutiérrez-Marcos et al. (2004), Costa et al. (2012) |
| <i>Mez1</i> (MEG)                 | DNA-me, H3K27me3, H3/H4-Ac      | B73&Mo17                          | 8-27 DAP                    | —                           | Haun et al. (2007), Haun and Springer (2008)        |
| <i>Mee 1</i> (MEG)                | DNA-me                          | UH005&UH301                       | 6 DAP                       | —                           | Jahneke and Scholten (2009)                         |
| $\alpha$ -Tubulin (MEG)           | DNA-me                          | W64A&A69Y                         | 20 DAP                      | —                           | Lund et al. (1995b)                                 |
| <i>Zein</i> (MEG)                 | DNA-me                          | W64A&A69Y                         | 19 DAP                      | —                           | Lund et al. (1995a)                                 |
| <i>R</i> gene (MEG)               | —                               | —                                 | —                           | —                           | Kermicle (1970), Ludwig et al. (1989)               |
| <i>Dzr-1</i> (MEG)                | —                               | BSSS53&Mo17                       | 15-27 DAP                   | —                           | Chaudhuri and Messing (1994)                        |
| 54 MEGs&16 PEGs                   | —                               | B73&Mo17                          | 14 DAP                      | —                           | Waters et al. (2011)                                |
| 93 MNCS&124 PNCS <sup>3</sup>     | —                               | B73&Mo17                          | 10DAP                       | —                           | Zhang et al. (2011)                                 |
| Rice                              | 177 MEGs&85 PEGs                | —                                 | Nip&93-11                   | 5 DAF                       | —   |
| <b>Embryo</b>                     |                                 |                                   |                             |                             |   |
| <i>Arabidopsis</i>                | 11 MEGs&1 PEGs <sup>1,2</sup>   | H3K27me3 <sup>4</sup>             | Cot-0&Ler                   | 2.5,4 DAP                   | Raisig et al. (2013)                                |
| Maize                             | <i>Mee 1</i> (MEG) <sup>2</sup> | DNA-me                            | UH005&UH301                 | 3,6,8 DAP                   | Jahneke and Scholten (2009)                         |
| <b>Gamete-carried transcripts</b> |                                 |                                   |                             |                             |   |
| <b>Zygote</b>                     |                                 |                                   |                             |                             |   |
| <i>Arabidopsis</i>                | SSP                             | Sperm-specific                    | Abnormal division of zygote | Abnormal division of zygote | Bayer et al. (2009)                                 |
| Rice                              | Oso7g0182900                    | Sperm-specific                    | Abnormal division of zygote | Abnormal division of zygote | Ohnishi et al. (2014)                               |
| Tobacco                           | <i>Ntsp002&amp;Ntsp0003</i>     | Sperm-specific                    | —                           | —                           | Xin et al. (2011)                                   |

The table lists the known information of imprinted and potentially imprinted genes discovered in plants to date. Phenotypes in seed development are also displayed. These data are partially adapted from Raisig et al. (2011). —, not shown or not known.

<sup>1</sup> Reporter activity of parent-of-origin-expression was identified (part of imprinted genes in *Arabidopsis* embryo).

<sup>2</sup> De novo transcription was identified (part of imprinted genes in *Arabidopsis* embryo).

<sup>3</sup> MNCS, maternal expressed transcripts; PNCS, paternal expressed transcripts. MNCS and PNCS include protein-coding genes and long non-coding RNAs.

<sup>4</sup> Epigenetic marks of a part of imprinted genes in *Arabidopsis* embryo.

participates in nutrient distribution to the embryo. Interestingly, in contrast to imprinted genes in mammals, *Meg1* promotes rather than restricts the transfer of nutrient flow from the mother to fetus (Costa et al., 2012).

The imprinted genes in the endosperm mentioned above have defined roles in the endosperm; however, the role of the imprinted genes in the embryo remain unknown. To identify the contribution of imprinted genes in the embryo during embryogenesis, T-DNA gene insertions were used to search for deviant phenotypes relative to embryonic development, but no obvious phenotypes were observed (Raissig et al., 2013). Interestingly, all the maternally imprinted genes in the *Arabidopsis* embryo were expressed in the seed coat, and some even showed a slightly biased expression toward the basal embryo and the suspensor (Raissig et al., 2013). Notably, some maternally imprinted genes were involved in metabolism (Raissig et al., 2013). Therefore, maternally imprinted genes in the embryo might function at the interface between the embryo and maternal tissue, possibly by linking seed coat metabolism and embryo metabolism, and rendering the genes in the embryo under maternal control (Raissig et al., 2013). This result may support the maternal–offspring **coadaptation** theory, which posits that maternally imprinted genes are critical for the events during mother–offspring interactions (Bateson, 1994; Wolf and Hager, 2006). Further research on the roles of imprinted genes in the embryo will lead to a better understanding of the function and evolution of genomic imprinting in plants.

#### REGULATION OF IMPRINTED GENES IN EMBRYOS

DNA methylation and histone modification are two distinct epigenetic mechanisms involved in the regulation of genomic imprinting in plants. The differential DNA methylation status of parental alleles in the endosperm is due mainly to genome-wide hypomethylation of maternal alleles in the central cell (Gehring et al., 2009, 2011). DNA glycosylase DEMETER (*DME*) with 5-methylcytosine excising activity (Kinoshita et al., 2004; Gehring et al., 2006) and the repression of *MET1* involved in maintaining DNA methylation (Jullien et al., 2008; Hsieh et al., 2011) are responsible for the DNA demethylation at CG sites. However, sometimes DNA methylation alone is not sufficient to establish different imprinting markers of some genes, and the Polycomb repressive complex 2 (PRC2) that catalyzes the trimethylation of histone H3 on lysine 27 (H3K27me3) is required (Baroux et al., 2006; Makarevich et al., 2008; Hennig and Derkacheva, 2009).

To investigate the epigenetic mechanism of genomic imprinting in the embryo, the fertilization-independent endosperm (*fie*) mutant was crossed reciprocally with wild-type plants, and *met1-3* mutants were used to pollinate wild-type plants (Raissig et al., 2013). F1 hybrid embryos were isolated, and mutant embryonic cDNA libraries were created. The detection of the allele-specific expression pattern of 11 embryonic MEGs in *Arabidopsis* demonstrated that imprinted expression of MEGs in embryos are not influenced by the paternal *met1-3* allele. However, disruption of the maternal FIE function changed the monoallelic expression of two MEGs and one PEG. Thus, the function of PRC2 may be comprehensive in regulating imprinted expression in both the embryo and endosperm (Raissig et al., 2013). Furthermore, the role of

asymmetric DNA methylation in the CHG context was negated in the establishment of imprinting in the embryo. Finally, Raissig et al. (2013) indicated that PRC2, but not MET1, played a role in regulating the imprinted genes in the embryo. This is consist with a previous conjecture that DNA methylation is unlikely to be a primary imprinting mark in maize embryos (Gutiérrez-Marcos et al., 2006; Jahnke and Scholten, 2009). Other undiscovered mechanisms may be involved in the establishment of genomic imprinting in the embryo.

#### PERSPECTIVES

Technological advances in genome-wide sequencing technology and acquisition of gametes, zygotes and early embryos will be important for elucidating the role of parent-of-origin genes during plant early embryogenesis. Such technological progress may lead to the identification of more uniparental transcripts in early embryos, whether gamete-delivered or imprinted gene-derived. Currently, in both gametes and early zygotes, it remains technically difficult to identify the origin of transcripts, which may be gamete-delivered during fertilization or transcribed *de novo* after fertilization. In addition, little is known about the functions of these parent-of-origin genes during fertilization and early embryogenesis. Obviously, no matter how parental transcripts may contribute to the transcriptome of early embryo, the function analysis of the parent-of-origin genes in specific developmental events at specific developmental stage will surely provide imperative knowledge to understand the parental effect in early seed formation.

Recently, the identification of imprinted genes in early embryos in *Arabidopsis* has questioned the concept that imprinted genes are restricted mainly to the endosperm. In light of so many candidate imprinted genes in the embryos of dicots and monocots, optimized methods are required to avoid contamination of maternal tissues and false positives or negatives in data collection and reliable analysis. Determination of the relevant functions of imprinted genes should shed light on our understanding of epigenetic mechanisms in promoting embryogenesis, embryo pattern formation, and cell fate determination during embryogenesis. With further technological advancement, the role of methylation in gene imprinting during embryogenesis might be further elucidated.

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# Possible roles for polycomb repressive complex 2 in cereal endosperm

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The polycomb repressive complex 2 (PRC2) is an evolutionarily conserved multimeric protein complex in both plants and animals. In contrast to animals, plants have evolved a range of different components of PRC2 and form diverse complexes that act in the control of key regulatory genes at many stages of development during the life cycle. A number of studies, particularly in the model species *Arabidopsis thaliana*, have highlighted the role of PRC2 and of epigenetic controls via parent-of-origin specific gene expression for endosperm development. However, recent research in cereal plants has revealed that although some components of PRC2 show evolutionary conservation with respect to parent-of-origin specific gene expression patterns, the identity of the imprinted genes encoding PRC2 components is not conserved. This disparity may reflect the facts that cereal plant genomes have undergone different patterns of duplication during evolution compared to *A. thaliana* and that the endosperm development program is not identical in monocots and eudicots. In this context, we focus this review on the expression of imprinted PRC2 genes and their roles in endosperm development in cereals.

**Keywords:** endosperm, epigenetics, cereal plants, polycomb, imprinting

## Introduction

The endosperm of plant seeds is the most important tissue in plants with regard to human life, because of its importance as a major source of dietary calories. Recent studies have highlighted the role played by polycomb repressive complex 2 (PRC2) as one of the controlling mechanisms of normal endosperm development (Kohler and Makarevich, 2006; Pien and Grossniklaus, 2007; Holec and Berger, 2012). PRC2 is an evolutionarily conserved, high molecular weight complex that was originally identified in *Drosophila* mutants because of its regulation of body-segmentation during embryogenesis (Pirrotta, 1995). Subsequently, PRC2 was shown to have methyltransferase activity for Lys27 of histone H3 (H3K27; Simon and Kingston, 2009). In *Arabidopsis thaliana*, the complex represses expression of target genes through epigenetic modification of the chromatin, and also controls parent-of-origin specific expression of downstream target genes and of the PRC2 component itself in the endosperm (Gehring, 2013). While most of our understanding of the role of PRC2 comes from studies in the model species *A. thaliana*, recent studies in cereal plants, such as maize, barley and rice, have also provided important insights.

In contrast to animal species, such as *Drosophila*, the components of the PRC2 complexes of plant species show considerable variation. Genome evolution in plants involved the generation of multi-gene families and also whole genome duplications, such as in *A. thaliana*, maize and

rice (Spillane et al., 2007; Dickinson et al., 2012). It has been hypothesized that whole genome-duplication may reduce evolutionary forces on duplicated genes, resulting in the accumulation of nucleotide substitutions in genes or gain-of-function changes in expression patterns (Ohno, 1970). Additionally, the relaxation of evolutionary constraints might allow transposon insertion at various sites in genes, leading to their silencing (Lynch and Conery, 2000; Rodin and Riggs, 2003). The latter has been postulated to act as a novel epigenetic control through the process of neofunctionalization (Dickinson et al., 2012; Yoshida and Kawabe, 2013). In this intriguing scenario, genes that show specific expression patterns in the endosperm may be associated with targeted genome-wide DNA demethylation in the central cell of the female gametophyte (Dickinson et al., 2012). Mechanisms for imprinted gene expression have been described in many reports (Gehring, 2013); however, questions regarding the biological relevance of genomic imprinting still remain to be answered. The increased understanding of the role of PRC2 in different plant species should be of value to addressing many of the unanswered questions.

## PRC2 in Cereal Plants

The PRC2 complex of animals has four major components: WD40 protein p55 (p55); Suppressor of Zeste 12 [Su(z)12]; Enhancer of Zeste [E(z)]; and extra sex combs (ESC; Schwartz and Pirrotta, 2013). These four components are conserved in *A. thaliana* and in cereal plants (Table 1). Although different combinations of the various subunits of PRC2 play distinct roles during development in *A. thaliana*, here we focus on the complex that determines endosperm fate. This complex has been termed FIS-class PRC2, and is encoded by the genes *Multicopy Suppressors of IRA 1 (MSI1)*, *Fertilization Independent Seed 2 (FIS2)*, *MEDEA (MEA)*, and *Fertilization Independent Endosperm (FIE)*, in *A. thaliana* (Kohler and Makarevich, 2006; Pien and Grossniklaus, 2007; Holec and Berger, 2012). To date,

the characteristics of this complex have not been fully elucidated in cereal plants.

### p55

The *Drosophila* p55 homolog in *A. thaliana*, *MSI1*, has been identified as a component of FIS-class PRC2 (Kohler et al., 2003; Guittot et al., 2004). *MSI1* is a WD40 repeat protein; a loss-of-function mutant of *MSI1* has been shown to display similar defects in cellularization and over-proliferation of endosperm as FIS-class PRC2 mutants. The *MSI1* homologs of maize (*Zea mays*) and rice (*Oryza sativa*) have been identified (Table 1) but have yet to be studied in detail (Hennig et al., 2005).

### *Su(z)12*

Three *Su(z)12* homologs have been identified in the barley (*Hordeum vulgare*) genome, and are termed *HvSu(z)12a*, *HvSu(z)12b*, and *HvSu(z)12c* (Kapazoglou et al., 2010). All three genes are included in the *Embryonic Flower 2 (EMF2)* clade by phylogenetic analysis (Kapazoglou et al., 2010). *HvSu(z)12b* transcripts have been detected in all tested tissues and found to increase during seed development. Expression of *HvSu(z)12c* is limited to the young shoots and the developing seed; *HvSu(z)12a* has not been detected in any tested tissue (Kapazoglou et al., 2010). The rice genome has two homologs of *Su(z)12*, named *OsEMF2a* and *OsEMF2b*, that are expressed in a wide range of tissues (Luo et al., 2009). Interestingly, eudicots such as *A. thaliana* have a single copy of *EMF2*, while monocots have two or three *EMF2*-like genes. This suggests that the *EMF2* gene family in the Poaceae (Gramineae) may have arisen from a recent duplication. No orthologs of *VRN2* or *FIS2* of *A. thaliana* have been identified in cereals (Luo et al., 2009).

### *E(z)*

Analyses of the barley genome have identified one *E(z)* homolog, termed *HvE(z)*, which is within the SWINGER (SWN) clade (Kapazoglou et al., 2010). Expression of *HvE(z)* occurs in both vegetative and reproductive tissues, and increases during seed development. The highest levels of *HvE(z)* expression have been found in young shoots (Kapazoglou et al., 2010). In maize, three *E(z)* homologs have been identified, namely, *Mez1*, *Mez2*, and *Mez3* (Springer et al., 2002; Haun et al., 2007). The *Mez1* sequence is similar to that of *CLF*, while *Mez2* and *Mez3* are more closely related to *SWN*. The *Mez2* and *Mez3* genes have high sequence identity, suggesting that they are duplicate genes formed during the paleotetraploid origin of maize (Springer et al., 2002). The three genes are widely expressed throughout the maize life cycle. *Mez1* shows maternal-specific gene expression (imprinted) in the endosperm, but shows bi-allelic (non-imprinted) expression patterns in the embryo (Haun et al., 2007). Three splicing variants are transcribed from the *Mez2* locus and show variations in their transcription among tissues (Springer et al., 2002). Analyses of sequence similarities indicate that the rice genome contains two homologs of *E(z)*, namely, *OsiEZ1(OsSET1)* and *OsCLF* (Thakur et al., 2003; Luo et al., 2009). These two rice genes are widely expressed in a range of tissues (Luo et al.,

**TABLE 1 | Components of Polycomb repressive complex 2 (PRC2).**

| Species            | PRC2 component |             |         |         |
|--------------------|----------------|-------------|---------|---------|
|                    | SET domain     | Zinc finger | WD40    | WD40    |
| <i>Drosophila</i>  | E(z)           | Su(z)12     | Esc     | p55     |
| <i>Arabidopsis</i> | MEA*           | EMF2        | FIE     | MSI1    |
|                    | CLF            | VRN2        |         |         |
|                    | SWN            | FIS2*       |         |         |
| Barley             | HvSWN          | HvEMF2a     | HvFIE   | ?       |
|                    | ?              | HvEMF2b     |         |         |
|                    |                | HvEMF2c     |         |         |
| Maize              | Mez1*          | ZmEMF2_1    | ZmFIE1* | ZmRBAP3 |
|                    | Mez2           | ZmEMF2_2    | ZmFIE2  |         |
|                    | Mez3           |             |         |         |
| Rice               | OsCLF          | OsEMF2a     | OsFIE1* | OsRBAP3 |
|                    | OsiEZ1(OsSET1) | OsEMF2b     | OsFIE2  |         |

\*Maternally expressed imprinted gene.

2009). Homologs of *E(z)* in cereal plants fall into the *CLF* and *SWN* clades. The *SWN* clade is specific to flowering plants, while the *CLF* clade also contains homologs from spikemosses (*Selaginella spp.*; Luo et al., 2009). The maize homologs of *E(z)* are more diverse than those of other cereal plants; it seems that the multiplication of homologous genes provided diversity of PRC2 functions in maize. The MEA protein is a core component of FIS-class PRC2, which is related to seed development in *A. thaliana*. However, no *MEA-like* gene has been identified in cereals (Luo et al., 2009).

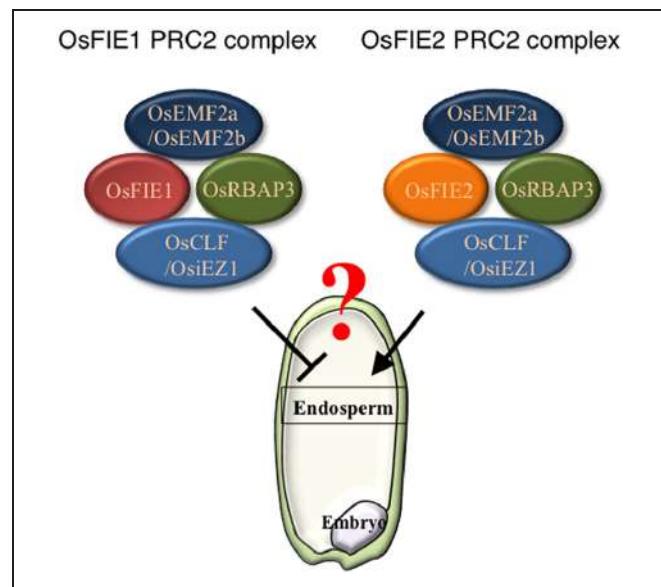
## ESC

Barley genome sequencing identified a single homolog of *ESC* (Kapazoglou et al., 2010); however, two duplicated genes for FIE-like proteins are present in both maize and rice genomes (Springer et al., 2002; Luo et al., 2009). In barley, *HvFIE* is widely expressed in vegetative and reproductive tissues. Similarly, *ZmFIE2* is expressed in a range of tissues in maize (Springer et al., 2002; Danilevskaya et al., 2003). These various genes are therefore the likely functional orthologs in cereals of *FIE* in *A. thaliana*. *ZmFIE1* in maize and *OsFIE1* in rice are predominantly expressed in the endosperm, and both display maternal-specific expression patterns (Danilevskaya et al., 2003; Gutierrez-Marcos et al., 2006). In maize, analysis using methylation sensitive restriction enzymes and PCR has shown that genome-wide DNA hypomethylation of the maternally derived genome occurs in the endosperm (Lauria et al., 2004). Related to this finding, differentially methylated regions (DMRs) have been identified that involve hypomethylation of the maternal allele of the *ZmFIE1* and *ZmFIE2* genes (Gutierrez-Marcos et al., 2006). The promoter region of *ZmFIE1* is demethylated in the central cell but not in the sperm cells; this asymmetric pattern of DNA methylation is inherited to the endosperm, where the maternally derived *ZmFIE1* is expressed while the paternally derived allele is silenced. The 5' region of *ZmFIE2* is hypomethylated in many tissues, but subjected to *de novo* DNA methylation only on the paternally derived allele in the endosperm after fertilization. These DMRs may be a mechanism for maternal specific gene expression during early endosperm development (Gutierrez-Marcos et al., 2006). Similarly, transcription of the paternal *OsFIE1* allele during early endosperm development is likely silenced by DNA methylation (Luo et al., 2009; Ishikawa et al., 2011; Zhang et al., 2012). The sequences and expression patterns of maize *ZmFIE1* and rice *OsFIE1* are very similar suggesting an orthologous relationship between these genes. In maize, *ZmFIE1* and *ZmFIE2* are located on different chromosomes (Springer et al., 2002), whereas rice *OsFIE1* and *OsFIE2* are located in the same genomic region on chromosome 8. Phylogenetic analysis of these maize and rice genes suggest that the two maize genomic regions arose from reciprocal deletion of one of the ancestral paralogs during maize genome evolution (Swigonova et al., 2004). The fact that rice *OsFIE1* and *OsFIE2* are closely positioned on the same chromosome suggests they arose through an intraspecies gene duplication event (Luo et al., 2009).

## Roles for PRC2 Complexes in Cereal Endosperm

In a comparison of gene expression patterns in two barley cultivars that have seeds of different sizes, differential expression of *HvFIE* and *HvE(z)* was shown to occur during seed development (Kapazoglou et al., 2010). *HvFIE* expression was found to increase immediately after fertilization in both cultivars, and then to decline in the cultivar producing larger seeds, but to increase in the cultivar with smaller seeds. The expression patterns of *HvFIE* are consistent with the predicted role of PRC2 in cereal plants, namely, the repression of endosperm development. *HvFIE* and *HvE(z)* expression can also be induced by the plant hormone abscisic acid (ABA), which is known to be involved in seed maturation, dormancy, and germination (Kapazoglou et al., 2010). These findings suggest that genes for PRC2 components can act at both earlier and later stages of endosperm development in barley; this may reflect the developmental program of endosperm of cereal species. Although the syncytial phase during early endosperm development is conserved in *A. thaliana* and cereal species, embryonic growth in *A. thaliana* later results in the consumption of the endosperm; by contrast, the endosperm persists in cereals (Sabelli and Larkins, 2009; Dante et al., 2014).

In *A. thaliana*, the imprinted genes *MEA* and *FIS2* encode PRC2 components and are involved in endosperm development through repression of the *AGL62* gene expression that controls the timing of cellularization (Kang et al., 2008; Hohenberger et al., 2012). In contrast to *A. thaliana*, *MEA* and



**FIGURE 1 | Polycomb repressive complex 2 (PRC2) components of *OsFIE1* and *OsFIE2* may have distinct roles in rice endosperm.** Based on recent findings, PRC2 complexes that contain *OsFIE1* and *OsFIE2* are likely to have distinct roles. In the endosperm, the *OsFIE1* protein is produced from the maternally derived allele and contributes to the *FIE1*-containing PRC2 (left). By contrast, *OsFIE2* protein derived from both maternal and paternal alleles is used to form *FIE2*-containing PRC2 (right).

*FIS2* orthologs have not been identified in barley, maize, or rice genomes. In rice, with the exception of *OsFIE1*, genes encoding PRC2 components are widely expressed in a range of tissues. *OsFIE1* shows specific expression in the endosperm and is the only imprinted PRC2 gene in rice endosperm (Luo et al., 2009); the gene is expected to be involved in multiple processes during endosperm development including cellularization. Plants homozygous for the *Osfie1* mutation do not display an obvious endosperm phenotype compared to wild type plants (Luo et al., 2009); by contrast, RNAi transgenic plant lines showed autonomous endosperm development (Li et al., 2014). This outcome may be due to off-target effects of the *OsFIE2* RNAi construct which silenced both *OsFIE1* and *OsFIE2* in the endosperm of the transgenic rice (Li et al., 2014). By contrast, the specific down-regulation of *OsFIE2* by RNAi results in the production of small seeds, which contain shrunken and defective endosperm and a relatively large embryo (Nallamilli et al., 2013). Although a sporophytic effect of the knock-down mutation, due to the dominant nature of RNAi construct, cannot be discounted in the latter experiment, this result suggests *OsFIE2* has a positive regulatory role in either early or late development of rice endosperm, in contrast to the role of FIS-class PRC2 in the endosperm of *A. thaliana*. It should be possible to more clearly determine the role of *OsFIE2* through use of the appropriate mutant alleles in combination with TALLEN or CRISPER/Cas technology (Kim and Kim, 2014). Such analyses would elucidate the role of *OsFIE2* in endosperm development, especially in relation to the timing of cellularization. There is evidence from interspecific and interploidy crosses in rice that the timing of cellularization and the eventual size of the endosperm are related (Ishikawa et al., 2011; Sekine et al., 2013). Therefore, investigation of cellularization in PRC2 mutants will be an essential approach to understanding the action of PRC2 in cereal endosperm.

Recently, an epigenetic allele of *Epi-df* was identified; this allele is a gain-of-function variant that likely resulted from hypomethylation of the 5' region of *OsFIE1* without any change in nucleotide sequence (Zhang et al., 2012). On the *Epi-df* mutant background, *OsFIE1* is ectopically expressed in vegetative tissues and the normally silent paternally derived allele is active in the endosperm (Zhang et al., 2012). The *Epi-df* plants show dwarfism and floral organ defects in a dominant fashion; the latter prevented investigation of the endosperm phenotype. By contrast, a recent study showed that expression of *OsFIE1* is correlated with the timing of cellularization (Folsom et al., 2014). Under moderately high temperature conditions, *OsFIE1* expression increases, and this elevated level of expression is correlated

with precocious endosperm cellularization (Folsom et al., 2014). Similarly, overexpression of *OsFIE1* causes decreased seed sizes and weights (Folsom et al., 2014). This is in contrast with the outcome of *OsFIE2* overexpression, which does not result in phenotypic changes in plants (Nallamilli et al., 2013). Overall, these findings suggest the possibility that *OsFIE1* and *OsFIE2* may have non-equivalent roles in endosperm development (Figure 1). Further analyses will be required to clarify precisely the roles of PRC2 in the cereal endosperm development.

## Conclusion

The data generated by cereal genome sequencing initiatives have enabled the identification of PRC2 genes in crop plant species. Detailed analyses of the expression of these genes have revealed remarkable differences in their behavior compared to orthologs in *A. thaliana*. Endosperm specific variants of the *Su(Z)12* homolog and *E(z)* homolog have been found, namely, *MEA* and *FIS2*; however, no variants of the *ESC* homolog are known in *A. thaliana*. By contrast, two *ESC* homologs *FIE1* and *FIE2* are present in maize and rice genomes. Although *FIE* is not consistently imprinted in *A. thaliana* (Yadegari et al., 2000), its homologs in maize and rice show maternal specific expression (Gutierrez-Marcos et al., 2006; Luo et al., 2009). In general, *ESC* and its homologs are WD40 repeat scaffolding proteins and do not seem to have any enzymatic activity. However, their animal counterparts have been shown to have binding activity for the N-terminal histone tail of H3 and to cause allosteric effects on the histone methyltransferase activity of EZH2; binding to chromatin residues associated with a repressive state of gene expression, such as H3K9me3, induces histone methyltransferase activity, while binding to chromatin residues associated with active transcription reduces its activity. Therefore, the protein–protein interactions of each PRC2 component are important determinants of the activity of the PRC2 complex. Further study of cereal PRC2 complexes will undoubtedly provide greater insights into their roles in endosperm development.

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# Using giant scarlet runner bean embryos to uncover regulatory networks controlling suspensor gene activity

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One of the major unsolved issues in plant development is understanding the regulatory networks that control the differential gene activity that is required for the specification and development of the two major embryonic regions, the embryo proper and suspensor. Historically, the giant embryo of scarlet runner bean (SRB), *Phaseolus coccineus*, has been used as a model system to investigate the physiological events that occur early in embryogenesis—focusing on the question of what role the suspensor region plays. A major feature distinguishing SRB embryos from those of other plants is a highly enlarged suspensor containing at least 200 cells that synthesize growth regulators required for subsequent embryonic development. Recent studies have exploited the giant size of the SRB embryo to micro-dissect the embryo proper and suspensor regions in order to use genomics-based approaches to identify regulatory genes that may be involved in controlling suspensor and embryo proper differentiation, as well as the cellular processes that may be unique to each embryonic region. Here we review the current genomics resources that make SRB embryos a compelling model system for studying the early events required to program embryo development.

**Keywords:** *Phaseolus coccineus*, scarlet runner bean, suspensor, gene regulatory network, *cis*-regulatory elements, transcriptome, comparative genomics

## WHY STUDY THE SUSPENSOR?

Embryogenesis in most higher plants begins with a double fertilization event, in which one sperm cell fertilizes the egg cell to form the zygote, and the other fertilizes the central cell to form the endosperm (Bleckmann et al., 2014). The zygote undergoes an asymmetric cell division, giving rise to a small, cytoplasm-rich apical cell and a large, vacuolated basal cell (West and Harada, 1993). The apical cell divides to form the embryo proper, which becomes the next generation plant, whereas the basal cell divides to form the suspensor, a terminally differentiated structure that transports nutrients to the embryo proper (Yeung, 1980; Nagl, 1990) and degenerates as the embryo matures (Yeung and Meinke, 1993). The uppermost cell of the suspensor, the hypophysis, contributes to the root meristem of the embryo (Dolan et al., 1993). While much is known about embryo proper development, comparatively little is known about the suspensor (Lau et al., 2012; Wendrich and Weijers, 2013). Genetic studies in *Arabidopsis* have illuminated some processes leading to suspensor differentiation. The molecular pathways involved in elongation of the zygote, the asymmetric division that forms the two-cell embryo, and apical and basal cell fate specification require (1) auxin signaling (Friml et al., 2003), (2) the YDA/MAPK signaling pathway (Bayer et al., 2009), and (3) the transcriptional networks involving RKD4 (Waki et al., 2011), WRKY2, WOX2, WOX8, and WOX9 (Ueda et al., 2011). However, genes in these pathways account for a very small percentage of the ~11,000 diverse mRNAs detected

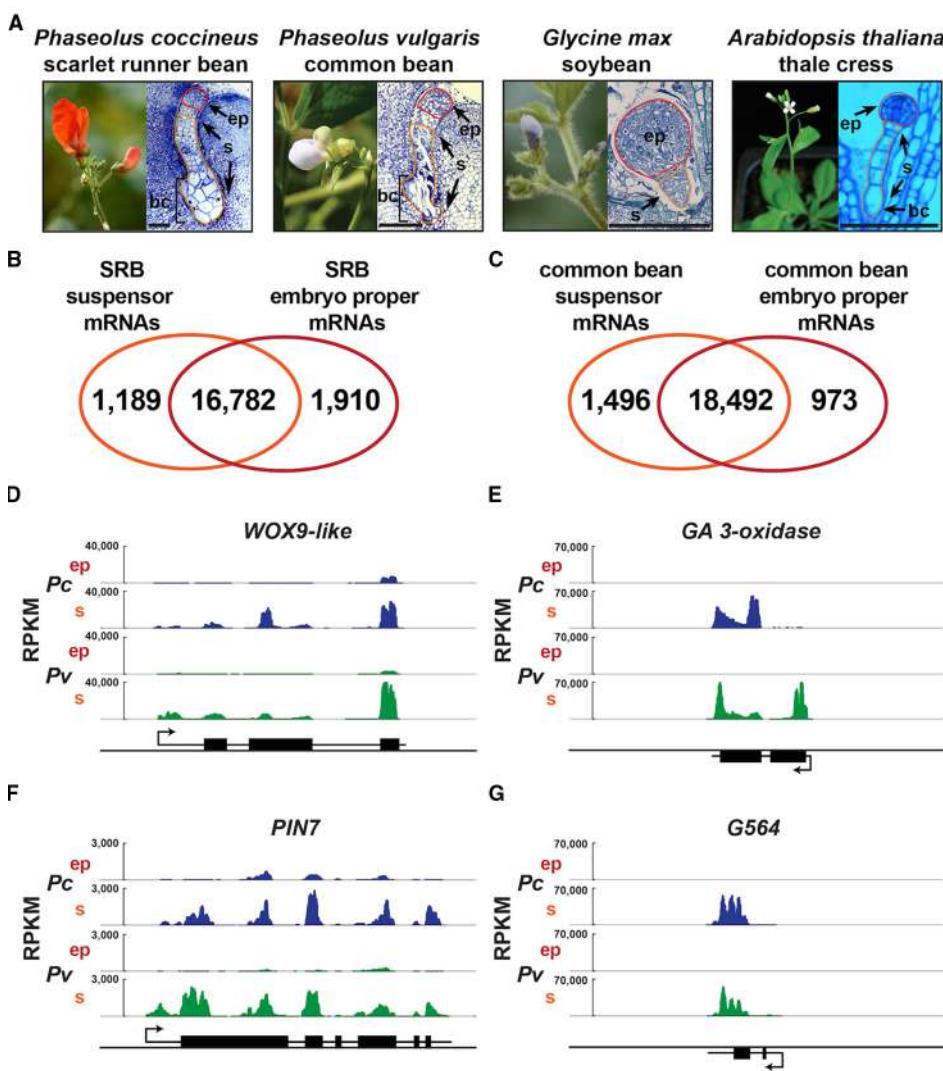
in the *Arabidopsis* suspensor (Belmonte et al., 2013), and the molecular mechanisms governing suspensor development and function remain largely elusive. In addition, little is known about (1) the regulatory networks controlling suspensor differentiation and development in species with diverse suspensor morphologies, (2) the mechanisms activating different gene sets in the embryo proper and suspensor after fertilization, and (3) the cellular processes governing suspensor degeneration in later embryo development.

## WHY USE SRB TO STUDY SUSPENSOR DIFFERENTIATION?

The physical features of the SRB suspensor (Figure 1A), including its massive size, enlarged basal cells, and polytene chromosomes (Nagl, 1962) provide a unique system to study the functional significance of this highly specialized suspensor, the cellular processes shared by all suspensors, and suspensor differentiation events. Additionally, SRB seeds are a protein-rich legume crop, closely related to soybean, common bean, and cowpea in the economically important *Phaseoleae* clade of legumes, and thus can serve as a model for legume seed development. Common bean (*Phaseolus vulgaris*), which is a major source of calories in many developing countries<sup>1</sup> and a \$1B crop in the United States<sup>2</sup>, and SRB are congeneric species that diverged less than eight million years ago (mya; Lavin et al., 2005) and can form

<sup>1</sup><http://faostat.fao.org>

<sup>2</sup><http://www.nass.usda.gov>



**FIGURE 1 |** **Suspensors with diverse morphologies and bean suspensor-specific gene activity.** (A) Scarlet runner bean, common bean, soybean, and *Arabidopsis* plants, plastic sections of globular-stage embryo of SRB, common bean and *Arabidopsis*, and paraffin section of globular-stage embryo of soybean. Common bean flower image was taken from <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC287537/> photographed by Valter Jacinto, and *Arabidopsis* flower image was taken from Kawashima and Goldberg (2010). (B,C) Venn diagrams representing the mRNAs detected in SRB (B) and

common bean (C) suspensor and embryo proper. RNA-Seq data for SRB and common bean are from GEO accession GSE57537. (D–G) Genome browser views of *WOX9-like*, *GA 3-oxidase*, *PIN7* and *G564* mRNA accumulation levels in SRB and common bean suspensor and embryo proper. Each panel depicts a 5 kb window including the gene structure. Black boxes represent exons. Black lines represent UTRs and introns. Arrows indicate the transcription start site. bc, basal cell(s); ep, embryo proper; RPKM, reads per kilobase per million; *Pc*, *P. coccineus*; *Pv*, *P. vulgaris*; s, suspensor. Scale bar: 100 µm.

successful hybrids (Lamprecht, 1941; Thomas, 1964), as was first reported by Mendel in 1865 (cited by Mok et al., 1986). SRB diverged ~19 mya from soybean (Lavin et al., 2005), the second largest crop in the United States (see text footnote 2). Taken together, SRB is an excellent plant in which to study suspensor development because of (1) its specialized structure, (2) its phylogenetic placement in the legume family, (3) a 40-year history of use as a model for embryo development (Yeung and Meinke, 1993; Kawashima and Goldberg, 2010), and (4) new genomic resources, including (i) the common bean genome sequence (Schmutz et al., 2014) and (ii) gene expression profiles for the SRB suspensor and embryo proper during early embryogenesis

(Le et al., 2007; Kawashima and Goldberg, 2010; GEO accession GSE57536).

## WHAT WAS LEARNED FROM USING SRB AS A MODEL FOR SUSPENSOR DEVELOPMENT FOR OVER 40 YEARS?

The first experimental studies of suspensor function were performed by Ian Sussex and collaborators using SRB because the large size of the SRB embryo allows hand-dissection of the suspensor and embryo proper, and facilitates the collection of large amounts of suspensor tissue for use in biochemical studies (Clutter and Sussex, 1968; Walbot et al., 1972; Sussex et al., 1973; Clutter et al., 1974; Lorenzi et al., 1978). Early SRB experiments

determined that the suspensor is required for the development of the embryo proper (Cionini et al., 1976; Yeung and Sussex, 1979), and that it is highly transcriptionally and translationally active (Walbot et al., 1972; Sussex et al., 1973; Clutter et al., 1974), in part due to its polytene chromosomes which can increase the DNA content of suspensor cells up to 8,192C (Nagl, 1962; Brady, 1973). There is a progressive increase in the level of polyteny from the chalazal pole of the suspensor to the large basal cells (**Figure 1A**; Brady, 1973). Although the biological function of polytene chromosomes and their puffs and loops in SRB suspensor cells (Nagl, 1970, 1974) is unclear, polyteny is a sign of terminally differentiated, highly specialized tissues such as *Drosophila* salivary glands (Heitz and Bauer, 1933).

Two specialized suspensor functions uncovered from early SRB studies are transport and hormone biosynthesis. The giant basal cells of the suspensor function as “transfer cells,” using their enlarged membrane surfaces and prominent ingrowths to absorb solutes from the surrounding seed tissues and transport them to the growing embryo proper (Gunning and Pate, 1969; Nagl, 1974, 1990; Yeung and Sussex, 1979; Yeung, 1980). The SRB suspensor not only acts as a conduit for nutrients, but also synthesizes growth regulators, e.g., gibberellic acid (GA) needed by the embryo proper in early development (Alpi et al., 1975). In fact, biochemical studies showed that SRB suspensors are a rich source of GAs (Alpi et al., 1975) and contain enzymes for synthesizing GAs (Ceccarelli et al., 1979, 1981). Classical approaches carried out 40 years ago revealed that the transport of nutrients and GA biosynthesis are essential processes carried out by the SRB suspensor for embryo development.

## HOW HAS GENOMICS BEEN USED TO DISSECT EARLY SRB SUSPENSOR DIFFERENTIATION AND DEVELOPMENT?

Because the SRB embryo is uniquely large, our laboratory was able to hand dissect globular-stage embryo-proper and suspensor regions and use pre-NextGen sequencing approaches—such as differential display, *in situ* hybridization, EST sequencing, and microarray analysis—to study the gene expression events that occur shortly after fertilization (Weterings et al., 2001; Le et al., 2007; Kawashima and Goldberg, 2010; Le, 2013). These experiments showed that the SRB embryo apical and basal regions transcribe different genes as early as the four-cell stage, suggesting that these regions are specified at the molecular level after division of the zygote (Weterings et al., 2001). At the globular stage there is a large overlap in genes expressed in the embryo proper and suspensor regions that are derived from the apical and basal cells, respectively (Le et al., 2007). Many suspensor-specific SRB genes were identified, however, including (1) all genes in the GA biosynthesis pathway, (2) a *WOX9-like* homeodomain transcription factor gene (*PcWox9-like*), and (3) *PcG564*, a gene of unknown function, among many others (Weterings et al., 2001; Le et al., 2007; Kawashima and Goldberg, 2010; Le, 2013; Henry, 2014). We confirmed these observations by using laser-capture micro-dissection (LCM) technology to collect SRB globular-stage embryo proper and suspensor regions with more precision (Le et al., 2007), RNA-Seq for transcriptome profiling (GEO accession GSE57536), and the common bean (*Phaseolus vulgaris*) as a reference genome (Schmutz et al., 2014; **Figure 1**). The genome

browser view illustrates the up-regulation of *PcGA 3-oxidase*, *PcG564*, and *PcWox9-like* genes in the SRB suspensor, in addition to the *PcPIN7* auxin transporter gene that has been shown by others to be up-regulated in the *Arabidopsis* suspensor and play an essential role in establishing apical-basal polarity (Friml et al., 2003; **Figures 1D–G**).

Knowing the spectrum of transcription factor genes that are active in the embryo proper and suspensor is a first step to building gene regulatory networks that program embryo development. One or more mRNAs unique to each embryo region could encode transcription factors that are directly linked to the processes by which these two regions of the embryo activate different gene sets shortly after fertilization and become specified for different developmental fates (Weterings et al., 2001). Our strategy of working backward from globular-stage gene activity to cell-fate specification is particularly amenable to the suspensor because its differentiation precedes that of the embryo proper, and the suspensor cells are direct clonal descendants of the basal cell of the two-cell embryo (Weterings et al., 2001; Kawashima and Goldberg, 2010). Thus, the factors that activate genes in the suspensor might be directly linked to the basal cell specification mechanism. For example the globular-stage expression pattern of the SRB *PcWOX9-like* gene is remarkably similar to its *Arabidopsis* counterparts *AtWOX8* and *AtWOX9* (Haecker et al., 2004). In *Arabidopsis*, *WOX8* mRNA accumulates in the zygote, and is then confined to the basal cell of the two-cell embryo and the globular-stage suspensor (Haecker et al., 2004). *AtWOX8* transcription is regulated, in part, by the *WRKY2* transcription factor (Ueda et al., 2011). Thus, the *WRKY2-WOX8* pathway functions in establishing zygote polarity by initiating a shift in organelle positions in the zygote enabling asymmetric division to occur (Ueda et al., 2011). Identifying the downstream target genes of *PcWOX9-like*, and other SRB suspensor-specific transcription factors, should facilitate building regulatory networks that program suspensor gene activity and uncovering the cellular events that are responsible for suspensor differentiation (Le et al., 2007).

## WHAT HAS BEEN LEARNED FROM USING COMPARATIVE GENOMICS TO IDENTIFY CONSERVED SUSPENSOR FUNCTIONS?

The suspensor is an evolutionarily conserved structure present in most seed-bearing plants and even some mosses, which diverged ~425 mya (Wardlaw, 1955; Kawashima and Goldberg, 2010). To understand more broadly the core functions carried out by all suspensors, the transcriptomes of suspensors from various species can be compared to identify conserved metabolic processes and transcription factors that may regulate conserved suspensor functions. We have previously reported that *PcG564* mRNA is also localized specifically in the basal region and suspensor of a transgenic globular-stage tobacco embryo transformed with an intact *PcG564* gene (Weterings et al., 2001). This shows that the suspensor transcriptional machinery regulating *PcG564* expression is conserved in plants that diverged ~150 mya (Paterson et al., 2004). It remains to be determined what other transcription factors are conserved in the suspensors of divergent species and what their downstream target genes are.

We have laid the foundation for a comparative genomics analysis of the SRB suspensor transcriptome with that of common bean, soybean, and *Arabidopsis*. Our laboratory has used LCM and RNA-Seq to profile the globular-stage suspensor and embryo proper transcriptomes of SRB and common bean (GEO accession GSE57537; **Figure 1**). WOX9-like, GA3-oxidase, PIN7 and G564 mRNAs are up-regulated similarly in both SRB and common bean suspensors (**Figures 1D–G**), demonstrating the conservation of gene activity and cellular functions carried out by giant bean suspensors. In collaboration with John Harada's laboratory at UC Davis, we have profiled the transcriptomes of the suspensor and embryo proper of soybean (*Glycine max*; GEO accession GSE57349) and *Arabidopsis* (Belmonte et al., 2013) embryos. Recently Slane et al. (2014) profiled *Arabidopsis* globular-stage embryo proper and suspensor nuclear transcriptomes using fluorescence-activated nuclear sorting (FANS). These datasets should illuminate several important questions regarding higher plant suspensors. What functions are conserved in the SRB and common bean giant suspensors? What are the functions of conserved transcription factors in legume suspensors? What functions are evolutionarily conserved in all suspensors regardless of size, morphology, or specialized function?

### WHAT UNIQUE PROCESSES OCCUR IN GIANT BEAN SUSPENSORS THAT DIFFER FROM LESS SPECIALIZED SUSPENSORS?

Suspensors display a wide range of morphological diversity in higher plants (Kawashima and Goldberg, 2010; **Figure 1A**). For example, closely related legume species, soybean and SRB, have distinct suspensors. The soybean suspensor is small, consisting of a few cells, whereas the SRB suspensor is huge containing ~200 cells (Sussex et al., 1973). The *Arabidopsis* suspensor, which is even smaller than that of soybean, is a single file of 7–10 cells. There may be several biological processes unique to giant bean suspensors and absent in smaller suspensors, such as those of *Arabidopsis* and soybean. One of the first characterized functions of the SRB suspensor, the synthesis of GA, may be unique to giant, highly specialized bean suspensors (Kawashima and Goldberg, 2010). In fact, GA also accumulates in the massive suspensor of the legume *Cytisus laburnum* (Picciarelli et al., 1984). While GA 3-oxidase mRNA (encoding the last enzyme in the GA biosynthesis pathway) accumulates to a high level in both SRB and common bean suspensors at the globular stage (**Figure 1E**; Solfanelli et al., 2005), mRNAs representing the *Arabidopsis* homologs of GA 3-oxidase do not accumulate in the suspensor; instead, they accumulate in the endosperm of globular-stage seeds (Belmonte et al., 2013). In dicots, the suspensor and the endosperm are both short-lived structures that degenerate once they have accomplished their function of nourishing the developing embryo proper. It has been suggested that in species with massive suspensors, such as SRB and common bean, the suspensor takes over endosperm functions, resulting in delayed endosperm cellularization and a decreased amount of endosperm (Tison, 1919; Schnarf, 1929; Newman, 1934; Lorenzi et al., 1978; Guignard, 1880). Although there are specific examples that do not support this hypothesis in all plants (Lersten, 1983), it may apply in some cases. Thus,

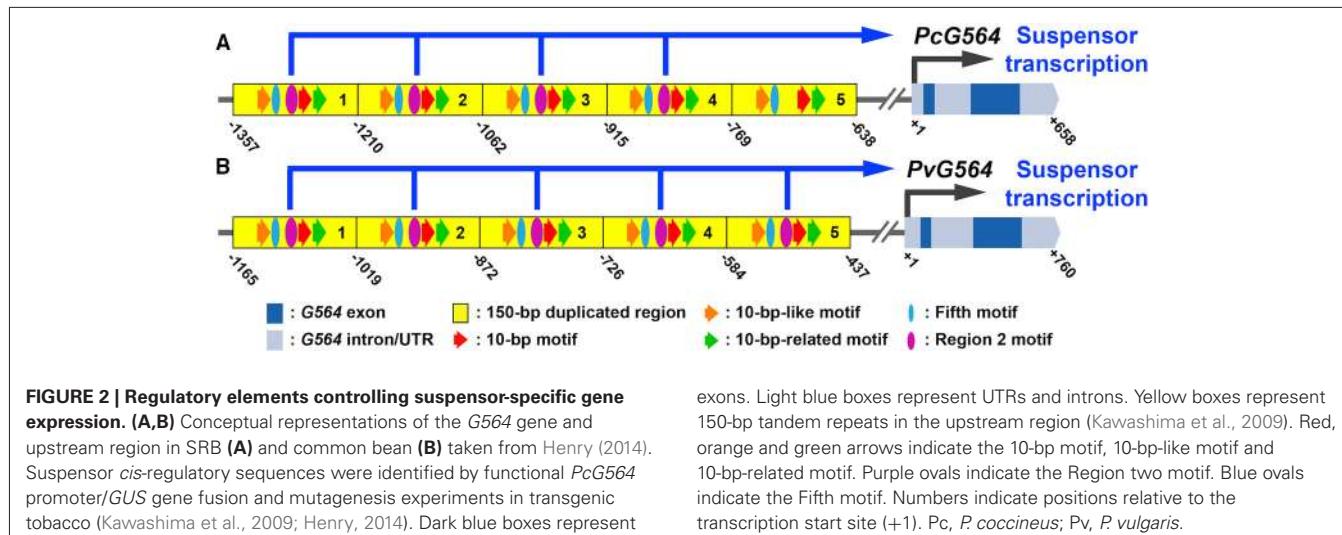
the endosperm GA biosynthesis gene regulatory network in *Arabidopsis* might have been co-opted by the giant bean suspensors, or vice versa. In *Arabidopsis* seeds, only the location of GA hormone synthesis has changed relative to giant bean seeds, not the developmental time at which hormone accumulation occurs. Perhaps the site of GA synthesis within the seed is not important, as long as the hormone is transported to the embryo proper at the globular stage of development.

Comparative studies of the gene regulatory networks controlling the development and differentiation of suspensors of divergent species will help to unlock the changes that occurred in evolution to produce morphologically and functionally distinct suspensors. A change in gene expression between species could be attributed to an alteration in a transcription factor protein, but more commonly it has been shown to result from changes in gene promoters (Pina et al., 2014). Identifying functional *cis*-regulatory elements and transcription factors that program suspensor gene activity, and comparison between different species will help to trace how novelties arose in gene regulatory networks, which may have led to the evolution of morphologically and functionally distinct suspensors across species.

### WHAT ARE THE *CIS*-REGULATORY ELEMENTS CONTAINED WITHIN THE GENOME THAT PROGRAM SUSPENSOR-SPECIFIC TRANSCRIPTION?

DNA sequence comparisons between related species have the potential to identify *cis*-regulatory elements that may regulate suspensor-specific gene transcription (Haeussler and Joly, 2011). However, wet-bench studies are required to determine whether predicted suspensor *cis*-regulatory elements are functional. Previously, we identified five *cis*-regulatory elements in the upstream region of the *PcG564* gene (**Figure 2A**) that activate transcription in transgenic tobacco and *Arabidopsis* suspensors (Kawashima et al., 2009; Henry, 2014). It remains unknown what other genes are regulated by *PcG564* suspensor *cis*-regulatory elements. The simplest hypothesis is that SRB suspensor up-regulated genes, such as *PcGA 20-oxidase* and *PcWOX9-like* (Le et al., 2007), are activated by the same suspensor *cis*-regulatory elements. Indeed, the *PcG564* suspensor *cis*-regulatory elements are found in the *PcGA 20-oxidase* and *PcWOX9-like* gene upstream regions (Kawashima et al., 2009; Henry, 2014), suggesting that these genes may comprise a suspensor gene regulatory network.

The common bean genome sequence (Schmutz et al., 2014) allows us to scan the upstream regions of all suspensor-specific genes for the presence of the five known suspensor *cis*-regulatory elements identified in *PcG564*. The common bean genome sequence can be used as a surrogate for the SRB genome because the two species diverged relatively recently (Lavin et al., 2005) and have similar gene expression profiles for the suspensor and embryo proper at the globular stage (**Figures 1B–G**). For example, G564 mRNA is up-regulated in the suspensor of both SRB and common bean relative to the embryo proper (**Figure 1G**), and the G564 upstream region is highly conserved in these two species (**Figure 2**; Henry, 2014). The *PcG564* and *PvG564* upstream regions contain five tandem repeats of 150-bp, and each repeat contains the five known suspensor *cis*-regulatory elements,



**FIGURE 2 | Regulatory elements controlling suspensor-specific gene expression.** **(A,B)** Conceptual representations of the *G564* gene and upstream region in SRB **(A)** and common bean **(B)** taken from Henry (2014). Suspensor *cis*-regulatory sequences were identified by functional *PcG564* promoter/*GUS* gene fusion and mutagenesis experiments in transgenic tobacco (Kawashima et al., 2009; Henry, 2014). Dark blue boxes represent

with the exception of the fifth repeat in *PcG564* (Figure 2). The suspensor *cis*-regulatory elements most likely function in *PvG564* because motif sequences and *G564* expression patterns are conserved in both bean species. The identities of the *trans*-acting factors that bind to the bean *G564* suspensor *cis*-regulatory elements remain a mystery. What other genes are regulated by the transcription factors that activate *G564*, what additional regulatory circuits control suspensor gene activity, and how these regulatory circuits are activated after fertilization remain unanswered questions.

## FUTURE PERSPECTIVES

The sequence of the common bean genome opens the door to *Phaseolus* suspensor gene regulatory network analysis on a genome-wide scale. Comparison of SRB and common bean suspensor transcriptomes with their embryo proper counterparts can identify suspensor-specific mRNAs that may be involved in processes specific to suspensor differentiation (Figure 1). The next major step is to identify suspensor-specific transcription factors, and determine their binding sites across the genome using, for example, ChIP-Seq. The power of the SRB system lies in its giant suspensor and polytene chromosomes, which can facilitate chromatin collection. Functional analysis of binding sites will also have to be carried out through promoter studies, as was done for *PcG564*, because transcription factor occupancy does not necessarily predict enhancer function *in vivo* (Peter and Davidson, 2009; Sanalkumar et al., 2014). The giant bean suspensor system has been resurrected, and should reveal new clues regarding processes that control suspensor differentiation and function in the near future.

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exons. Light blue boxes represent UTRs and introns. Yellow boxes represent 150-bp tandem repeats in the upstream region (Kawashima et al., 2009). Red, orange and green arrows indicate the 10-bp motif, 10-bp-like motif and 10-bp-related motif. Purple ovals indicate the Region two motif. Blue ovals indicate the Fifth motif. Numbers indicate positions relative to the transcription start site (+1). *Pc*, *P. coccineus*; *Pv*, *P. vulgaris*.

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# Evolutionary developmental genetics of fruit morphological variation within the Solanaceae

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Morphological variations of fruits such as shape and size, and color are a result of adaptive evolution. The evolution of morphological novelties is particularly intriguing. An understanding of these evolutionary processes calls for the elucidation of the developmental and genetic mechanisms that result in particular fruit morphological characteristics, which determine seed dispersal. The genetic and developmental basis for fruit morphological variation was established at a microevolutionary time scale. Here, we summarize the progress on the evolutionary developmental genetics of fruit size, shape and color in the Solanaceae. Studies suggest that the recruitment of a pre-existing gene and subsequent modification of its interaction and regulatory networks are frequently involved in the evolution of morphological diversity. The basic mechanisms underlying changes in plant morphology are alterations in gene expression and/or gene function. We also deliberate on the future direction in evolutionary developmental genetics of fruit morphological variation such as fruit type. These studies will provide insights into plant developmental processes and will help to improve the productivity and fruit quality of crops.

**Keywords:** domestication, evolutionary developmental genetics, fruit, gene expression, morphological novelty, natural variation, Solanaceae

## Introduction

Diversification of plant morphology occurred during evolution as a result of plant adaptation to changes in the environment. The origin of the fruit is an evolutionary adaptation that facilitates survival and distribution of progeny. For example, fruits protect the developing seeds from adverse environments and/or foraging by animals during premature stages, thus enhancing the survival rate. However, fruits that contain nutrients and minerals can become favorite foods for animals and humans as part of a balanced diet. The energetic cost of producing fruits are paid for through subsequent seed dispersal, e.g., birds, mammals, and humans disperse seeds to different habitats where they can propagate. In some cases, the origin of morphological novelties or particular structures associated with fruits play an essential role in seed dispersal by wind, water and animals. Diverse colors and flavors of mature fruits attract animals that eat the fruit and aid seed dispersal. Thus, the morphological variations of fruits have diversified considerably. Furthermore, humans have domesticated a wide range of plants as fruit crops with different sizes, shapes, colors, flavors, and textures.

The Solanaceae family has a rich diversity of fruit types and flower morphologies (Knapp, 2002; He et al., 2004). In addition, this family ranks as one of the most economically important

plant families among the angiosperms. Solanaceous fruits represent an important part of the human diet and common fruit crops in this family include tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and chili/pepper (*Capsicum* spp.). The fruits from some species of *Physalis* (such as *Physalis philadelphica* and *Physalis peruviana*) and *Lycium* (e.g., *Lycium barbarum* and *Lycium chinense*) have both curative and culinary usages. Moreover, the Solanaceae family contains many model species for the study of plant developmental processes, including tomato, potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*), and *Petunia hybrida*. Thus, this plant family has served as a model for linking genomics and biodiversity (Knapp et al., 2004). The Solanaceous fruits exhibit considerable morphological diversity (Knapp, 2002), including size, shape, and color, both within and between different species (Figure 1). How do such morphological variations arise, and what are the underlying genetic bases? In recent years, modern molecular biology and genomic tools have been used extensively to elucidate the mechanisms underlying the evolution and development of these fruit morphological variations. In this review, we summarize the known genetic control of Solanaceous fruit morphological variations, highlight the general mechanisms involved in the evolution of plant morphology, and discuss the future direction.

## Genetic Control of Fruit Size and Shape

Plant fruits exhibit considerable morphological diversity in size and shape. Fruit size and shape variation usually contribute to reproductive isolation of species and have clear evolutionary consequences in natural conditions. Moreover, fruit size is

a prime breeding target, and fruit shape is often altered following the size alteration, indicating that the two traits might share a common set of genetic controllers. Solanaceous crops display significant variation in fruit size and shape within and among populations (Figure 1). Thus, determination of the genetic basis of these fruit-associated trait variations is the most common type of application-oriented fundamental evolutionary study. Quantitative trait loci (QTLs) for variation of morphological traits between the Solanaceous crops and their closely related wild relatives are well-conserved (Doganlar et al., 2002; Ben Chaim et al., 2003; Frary et al., 2003; Zygier et al., 2005; Borovsky and Paran, 2011; Carvalho et al., 2014; Portis et al., 2014), but most of them have not yet been cloned. Multiple QTLs and/or genes regulating fruit size and shape are well-characterized in the Solanaceae (Table 1). The considerable progress in the genetic control of fruit size and shape in tomato was reviewed by van der Knaap et al. (2014). Therefore, in this section we briefly summarize the findings in tomato and focus on the new findings in other Solanaceous species. The identified “fruit morphological variations” QTLs/genes that encode regulators with diverse chemical attributes might form an interaction and regulatory network to control cell division activity/patterns or cell expansion. Therefore, any alteration in these regulators or their pathways may contribute to variations in fruit size and shape.

## Regulators of Cell Division Activity or Patterns

The two characterized genes that regulate fruit weight are *Fruit weight 2.2* (*FW2.2*) and *Fruit weight 3.2* (*FW3.2*). *FW2.2* is the first cloned QTL in plants (Frary et al., 2000). The allele that increases fruit weight causes an enlargement of the placenta and columella regions of the fruit, which control ~30% fruit size in tomato (Nesbitt and Tanksley, 2001; Cong et al., 2002). A mutation in the *FW2.2* promoter leads to heterochronic expression of the gene during fruit development, resulting in differences in fruit size between cultivated tomato and its wild relatives. *FW2.2* is a plasma membrane-anchored protein that is involved in the cell cycle pathway for the control of ovary size (Liu et al., 2003a). *FW3.2* encodes a cytochrome P450 homolog, i.e., the putative ortholog of *Arabidopsis KLUH* (Chakrabarti et al., 2013) and is therefore designated as *S. lycopersicum KLUH* (*SlKLUH*). A mutation located 512 bp upstream of the predicted start of *SlKLUH* transcription is responsible for a change in tomato fruit weight. The increase in fruit weight of *FW3.2* is primarily due to an increase in cell number in the pericarp and septum areas. The putative ortholog of *KLUH* in pepper is also associated with larger fruit suggesting a possible role of the cytochrome P450 family in parallel domestication processes in fruit and vegetable crops (Chakrabarti et al., 2013). The functional conservation of *FW3.2* in angiosperms and the underlying mechanisms require further investigation, whereas the role of *FW2.2* in organ size and cell division is highly conserved in most plant species examined. *FW2.2* was found to correspond to a major fruit weight QTL in eggplants (Doganlar et al., 2002). However, *FW2.2* does not play a significant role in controlling fruit size variations between wild and cultivated peppers because pepper fruit has little placental



**FIGURE 1 |** The diverse variations of fruit morphology in the Solanaceae family. (1–3), *Solanum melongena*; (4), *Solanum pimpinellifolium*; (5–8), *Solanum lycopersicum*; (9–14), Variants of *Capsicum annuum*; (15), *Physalis alkekengi*; (16), *Physalis floridana*; (17–19), *Physalis philadelphica*. The Chinese lantern in *Physalis* spp. was opened to show the berry inside. Bar = 1 cm.

**TABLE 1 | QTLs/Genes characterized for variation in fruit size and shape in the Solanaceae.**

| QTL/gene     | Allelic variation               | Protein   | Process affected                           | Species                            | Reference   |
|--------------|---------------------------------|---|--|------------------------------------|---|
| <i>fw2.2</i> | Promoter-Regulatory             | A cell number regulator (CNR) family protein              | Cell division/fruit size                   | Tomato eggplant<br><i>Physalis</i> | Frary et al. (2000), Cong et al. (2002), Doganlar et al. (2002), Li and He (2015)                                       |
| <i>fw3.2</i> | Promoter-regulatory             | A cytochrome P450 protein                                 | Cell division/fruit size                   | Tomato pepper                      | Chakrabarti et al. (2013)   |
| <i>ovate</i> | Premature stop                  | Ovate family proteins                                     | Cell division/fruit shape                  | Tomato eggplant pepper             | Liu et al. (2002), Spinner et al. (2010, 2013), Tsaballa et al. (2011), Drevensek et al. (2012), Gramazio et al. (2014) |
| <i>sun</i>   | Transposon insertion-regulatory | A member of the IQD family of calmodulin-binding proteins | Cell division/fruit shape                  | Tomato eggplant                    | Xiao et al. (2008), Jiang et al. (2009), Wu et al. (2011), Gramazio et al. (2014)                                       |
| <i>fas</i>   | Intron -regulatory              | A YABBY-like transcription factor                         | Cell division/locule number/shape and size | Tomato                             | Cong et al. (2008)  |
| <i>lc</i>    | SNPs in downstream-regulatory   | A putative ortholog of WUSCHEL                            | Cell division/locule number/shape and size | Tomato                             | Munoz et al. (2011)   |
| <i>POS1</i>  | Intron-regulatory               | A transcription factors with two CRF-AP2 domains          | Cell expansion/fruit size                  | <i>Physalis</i>                    | Wang et al. (2012, 2014)  |

tissue (Zygier et al., 2005). *FW2.2*-like genes have been renamed as the *Cell Number Regulator* (CNR) family (Dahan et al., 2010; Guo et al., 2010; Libault et al., 2010; Guo and Simmons, 2011; De Franceschi et al., 2013; Xu et al., 2013; Monforte et al., 2014). CNR family members are localized to the membrane to facilitate the transport of ions (Song et al., 2004; Nakagawa et al., 2007), but the mechanism of regulation of ion transport leading to changes in cell division is unknown. Recently, Li and He (2015) found that *Physalis floridana* *Cell Number Regulator 1* (*PfCNR1*) encodes a putative ortholog of *FW2.2*. The heterochronic expression levels of *PfCNR1* alleles in ovaries are negatively correlated with cell division activity and berry size variation between different *Physalis* species. *PfCNR1* was found to interact with *PfAG2*, an AGAMOUS (AG) homolog for ovary identity determination (Yanofsky et al., 1990). Moreover, *PfAG2* binds to the CArG-box in the *PfCYCD2;1* promoter to repress the expression of this gene. The work in *Physalis* suggests a novel mechanism mediated by an MADS-domain protein for a cell membrane-localized protein to control cell division suggesting a molecular link between ovary identity and growth in plants (Li and He, 2015).

Fruit elongation is an important feature that affects fruit shape. Elongation in the tomato fruit is controlled mainly by *OVATE* and *SUN*. *OVATE* encodes a member of the ovate family proteins (OFPs), and a mutation that results in a premature stop codon leads to the pear-shaped fruit in tomato (Liu et al., 2002). The *Arabidopsis* OFP members act as transcriptional repressors in controlling cell elongation, plant growth, and development (Wang et al., 2007, 2011). Yeast two-hybrid screens using the tomato *OVATE* as bait identified the TONNEAU1-recruiting motif (TRM) superfamily as prey. TONNEAUs (TON) and TON-TRM interaction play critical roles in preprophase band formation and microtubule array organization of plant cell division and cell elongation (Spinner et al., 2010, 2013; Drevensek et al., 2012). Thus, an interaction between *OVATE* and TRMs may provide a mechanistic link between fruit patterning and growth, nonetheless, this assumption needs substantiation.

OFPs are present in all major lineages of land plants (Liu et al., 2014); whether they shared a conserved role needs to be investigated. At least *Ovate*-like genes from pepper and eggplants are also involved in determining fruit shape (Tsaballa et al., 2011; Gramazio et al., 2014). *SUN* encodes a member of calmodulin-binding proteins (Xiao et al., 2008, 2009) and regulates vegetative growth and reproductive organ shape by changing cell division patterns (Wu et al., 2011). Wild-type *SUN* is only expressed 10 days post-anthesis fruit (van der Knaap et al., 2014); however, a transposition of unusual 24.7 kb duplication event mediated by the retrotransposon *Rider* causes mutations in some tomato cultivars (Jiang et al., 2009). This leads to greater expression in the entire floral and fruit development, and elongated fruit (Xiao et al., 2008, 2009). How *SUN* regulates cell division pattern remains unclear, but *SUN* ortholog also controls the fruit shape in eggplant (Gramazio et al., 2014) implicating a conserved developmental role of this gene family.

Alteration in the locule number frequently affects both fruit shape and size. For example, the wild species *Solanum pimpinellifolium* commonly contain two to four locules while tomato cultivars have more; and in extreme cases, more than eight locules have been observed (Munoz et al., 2011). Most phenotypic variation due to locule number variation is explained by *fasciated* (*fas*) and *locule number* (*lc*). *FAS* encodes a YABBY-like transcription factor *SIYABBY2* (Cong et al., 2008). The mutation *fas*, which resulted from a 294-kb inversion with one of the breakpoints in the first intron of *SIYABBY2*, led to the increases in locule number, and was a critical step in the production of extreme fruit size during tomato domestication (Cong et al., 2008). However, the details of how *SIYABBY2* impacts locule number in tomato are not well-understood. *LC* was identified to be associated with two single nucleotide polymorphisms (SNPs) located 1080 bp downstream of the putative tomato ortholog of *WUSCHEL* (*WUS*), a homeodomain transcription factor (Clark, 2001; Munoz et al., 2011). *LC* controls the number of carpel primordia and a mutation results in a fruit with more than the typical two to three locules.

Since increased expression of *AtWUS* leads to increased floral organ number in *Arabidopsis* (Clark, 2001), *SlWUS* is the most likely candidate to underlie *lc*. *AtWUS* positively regulates *AtAG* while *AtAG* down-regulates *AtWUS*; the down-regulation is mediated by two downstream CArG *cis*-regulatory elements bound by *AtAG* (Lenhard et al., 2001; Lohmann et al., 2001; Liu et al., 2011). In tomato, the two SNPs associated with *lc* are located in a putative CArG *cis*-regulatory element, but surprisingly, a considerable change in the expression of *SlWUS* compared with the wild type was not observed (Munoz et al., 2011). More evidence is needed to verify the role of *SlWUS* in the control of fruit size, or to link *lc* and *SlWUS*.

### Cell Expansion Regulators

The above-characterized genetic regulators mainly affect cell division activity or patterns; however, alteration in cell expansion also plays a role in the evolution of fruit size. Pericarp size, particularly pericarp thickness, is a strong determinant of Solanaceous fruit size. Pericarp thickness appears to be governed by endoreduplication (Chencl et al., 2005). Endoreduplication – arrest in mitotic activity accompanied by a concomitant increase in nuclear DNA levels during fruit development – is believed to drive cell expansion, and is mainly regulated by cell cycle genes (Chevalier et al., 2011). During tomato fruit development, endoreduplication acts as an important morphogenetic factor supporting cell growth and multiple physiological functions (Chevalier et al., 2014). Impairment in the expression of *WEE1*, which encodes the cell cycle-associated protein kinase in transgenic tomato plants, results in a reduction in plant and fruit size, because of decrease in cell size that correlates with a decrease in the DNA ploidy levels (Gonzalez et al., 2007). Downregulation of tomato *CELL CYCLE SWITCH A 52 kDa (SlCCS52A)* does not affect the number of pericarp cell layers, but results in the formation of significantly smaller fruit, along with a sharp reduction in the ploidy level and pericarp cell size (Mathieu-Rivet et al., 2010a,b). The auxin *Sl-IAA17* transcriptional repressor also controls tomato fruit size by regulating endoreduplication-related cell expansion (Su et al., 2014). The role of endoreduplication in increased cell expansion in fruit development is controversial (Chevalier et al., 2011; Nafati et al., 2011), and its role in natural variation of fruit size is not known. Recently, a key cell expansion regulator was characterized in *P. philadelphica* (tomatillo). The characterized gene is *Physalis Organ Size 1 (POS1)*, previously designated as *Pp30*, which encodes a putative transcription factor with two CRF (cytokinin response factor)-AP2 (APETALA2) domains, and positively controls floral and fruit organ sizes in tomatillos (Wang et al., 2012, 2014). The expression levels of the *POS1* gene were positively associated with size variation in tomatillo reproductive organs such flowers, berries and seeds. *POS1* knockdown resulted in smaller flowers and berries with smaller cells compared with their wild type counterparts. Conversely, *POS1* overexpression promoted organ size without increasing the cell number. The first introns of the *POS1* alleles from large, intermediate and small tomatillo groups contained one, two and three 37-bp repeats,

respectively. Furthermore, copy variation of repeats in the first intron of *POS1* alleles resulted in differential expression of this gene. Thus, the novel regulatory variation in *POS1* regulates reproductive organ size variation in tomatillos (Wang et al., 2014).

### Genetic Basis of Fruit Color Variation

Fruit color is essential for attracting animals and humans, and thus, facilitates seed dispersal. Color is determined by different proportion of surface pigments, such as carotenoids, chlorophyll, flavonoids, and anthocyanins (Liu et al., 2003b; Nashilevitz et al., 2010; Kachanovsky et al., 2012). The color of berries varies widely and can be red, purple, orange, yellow, or green (Figure 1). Brightly colored berries generally tend to be juicy and extremely soft, whereas, mature green berries are harder and have a woody texture (Symon, 1987). Phylogenetic reconstructions suggest that green fruits belong to the primitive clade, whereas brightly colored (red, orange, yellow) species are derived clades (Peralta and Spooner, 2001). Several genes were characterized in tomato, pepper, and eggplant. Carotenoid content is the primary determinant of fruit color that affects nutritional value and appearance. In the carotenoid pathway, color diversity depends on the quantity of pigment produced, and the point where the pathway is arrested. Many transcription factors participate in controlling this pathway. Rodriguez-Uribe et al. (2012) determined the carotenoid composition in a number of orange-colored pepper fruit, and compared it with transcript abundance for the carotenogenic enzymes, such as phytoene synthase (*Psy*), lycopene  $\beta$ -cyclase (*LcyB*),  $\beta$ -carotene hydroxylase (*CrtZ*), and capsanthin-capsorubin synthase (*Ccs*). A splicing mutation in the *Psy* gene 1 (*Psy1*) causes orange coloration in Habanero pepper fruits (Kim et al., 2010). A chimeric transcript containing *Psy1* and a potential mRNA is associated with *yellow flesh* color in tomato accession PI114490 (Kang et al., 2014). The single dominant tomato *LcyB* gene increases  $\beta$ -carotene in the fruit while *old-gold* (*og*), a recessive mutation of the *LcyB* abolishes  $\beta$ -carotene and increases lycopene (Ronen et al., 2000). *LcyB* is homologous to *Ccs*, an enzyme that produces red xanthophylls in pepper fruits (Ronen et al., 2000). A tandem repeat structure in the promoter region of *Ccs* causes the yellow fruit color in pepper (Li et al., 2013). *CrtZ* mutant results in accumulation of  $\beta$ -carotene and conversion of red to orange color in pepper fruit (Borovsky et al., 2013). Nowadays, many QTLs/genes were found to be involved in fruit coloration through affecting plastid characteristics. QTL *pc8.1*, affects carotenoid content in pepper fruit and is associated with variation in plastid compartment size (Brand et al., 2012). The variations in chromoplasts are associated with carotenoid compositional differences and fruit color of different pepper cultivars (Kilcrease et al., 2013). An *Arabidopsis pseudo response regulator 2-like (APRR2-like)* gene is linked to pigment accumulation in tomato and pepper fruits. Overexpressing this gene in tomato increased plastid number, area, and pigment content; thus, enhancing the levels of chlorophyll in immature unripe fruits and carotenoids in red ripe fruits (Pan et al.,

2013). The positions of ten genes in the carotenoid biosynthetic pathway of pepper were homologous with the positions of the same genes in tomato (Thorup et al., 2000). Amino acid substitutions in homologs of the STAY-GREEN protein of rice are responsible for green-flesh and chlorophyll retainer mutations of tomato and pepper (Barry et al., 2008). *CaGLK2*, a GOLDEN2-like transcription factor regulates natural variation of chlorophyll content and fruit color in pepper fruit (Brand et al., 2014). Virus-induced gene silencing (VIGS) of *SlMYB12* resulted in a decrease in the accumulation of naringenin chalcone, and pink-colored tomato fruit, suggesting an important role for this gene in regulating the flavonoid pathway in tomato fruit (Ballester et al., 2010). Major anthocyanin in eggplant peel was delphinidin-3-rutinoside while the predominant pigment in violet pepper was delphinidin-3-*trans*-coumaroylrutinoside-5-glucoside (Sadlova et al., 2006). It is possible that a conserved complex regulatory pathway controls Solanaceae fruit colors, but the complete genetic components in the carotenoid regulatory pathway have not yet been revealed, even in a Solanaceae species.

## The Evolution of the Fruit Morphological Novelty

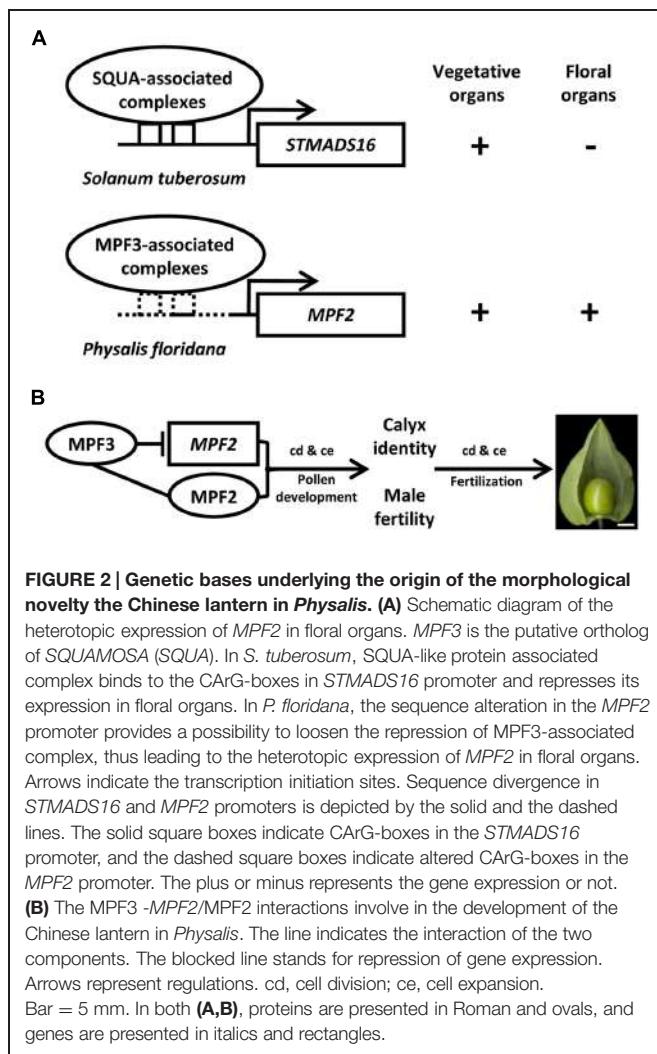
The origin of morphological novelties is a long-standing dispute in evolutionary biology. An understanding of this process demands the elucidation of the developmental and genetic mechanisms that produce such structures. Unlike *Solanum* and *Capsicum* species, *Physalis* has distinguished fruit morphology with a papery husk as the accessory trait of fruits (Whitson and Manos, 2005). The distinct trait of *Physalis* species is termed the Chinese lantern or the inflated calyx syndrome (ICS) since it is a derivative of the calyx (He and Saedler, 2005). Within the Solanaceae, only five genera (*Physalis*, *Withania*, *Przewalskia*, *Margaranthus*, and *Nicandra*) feature this morphological novelty. The nature of the Chinese lantern is an inflated fruiting calyx, and fertilization/hormonal signals trigger the formation of ICS in *Physalis* and *Withania* (He and Saedler, 2005, 2007; Khan et al., 2012). A series of microevolutionary time scale studies revealed that the origin of the Chinese lantern is associated with the heterotopic expression of the *Physalis* MADS-box gene 2 (*MPF2*) in floral organs. Moreover, its identity is determined by another *Physalis* MADS-box gene 3 (*MPF3*; He and Saedler, 2005; Zhao et al., 2013). Unlike its ortholog of *S. tuberosum* MADS-box gene 16 (*STMADS16*), *MPF2* is heterotopically expressed in the floral organs of *Physalis* (Figure 2A). The heterotopic expression of *MPF2* may result from the variation in the CArG-boxes in its promoter. The phenotypic variation of *MPF2* knockdowns further supports the role of *MPF2* in male fertility and fruiting calyx growth (He and Saedler, 2005). Thus, heterotopic expression of *MPF2* is the key to the origin of the Chinese lantern morphology. While *MPF3* is specifically expressed in floral tissues, this gene encodes a euAP1 MADS-domain protein, which is primarily localized to the nucleus, and it interacts with *MPF2* and some floral MADS-domain proteins to selectively bind the variants of CArG-boxes in the *MPF2* promoter (Figure 2A, Zhao

et al., 2013). Besides the role in calyx identity, *MPF3* regulates ICS formation and male fertility through interactions with the *MPF2* gene or *MPF2* protein (Figure 2B, Zhao et al., 2013). The ICS-determined genes also function in male fertility; either in pollen maturation or yields, and their encoding proteins also interact with floral MADS-proteins for stamen development (He et al., 2007). *MPF3* downregulation increases *MPF2* expression significantly in the calyces and androecium; however, the expression of *MPF3* is not affected in *MPF2*-downregulated flowers (Zhao et al., 2013). Therefore, the novel role of the *MPF3-MPF2* regulatory circuit in male fertility is integral to the origin of the Chinese lantern. Thus, any molecular interactions associated with *MPF2* and *MPF3* may contribute to ICS formation (He et al., 2007; Zhao et al., 2013). Dissecting the *double-layered-lantern mutant1 (doll1)*, a *P. floridana* *GLOBOSA (GLO)*-like MADS-box gene 1 (*PFGLO1*) genomic locus deletion mutant (Zhang et al., 2014a) further suggested a role of male fertility in the development of the Chinese lantern in *Physalis*. The corolla and androecium of *doll1* are respectively transformed into the calyces and gynoecium (Zhang et al., 2014a). On the other hand, downregulating *PFGLO2*, the paralog of *PFGLO1* impaired male fertility (Zhang et al., 2015). Further evolutionary analyses suggest that the evolution of ICS in Solanaceae is associated mainly with divergence related to *MPF2*-like genes, and alteration in *MPF2*-related molecular traits plays a crucial role (Hu and Saedler, 2007; Khan et al., 2009; Zhang et al., 2012).

## Future Research Highlights

### The Evolution of Fruit Type in Solanaceae

Fruit is the vehicle for seed dispersal, and the origin of the fruit is an evolutionary adaptation that facilitates survival and distribution of progeny. Thus, the evolution of fruit morphology is under strong selective pressures. Fruit size and shape are mostly related to domesticated crops; however, fruit type is a key adaptive feature to terrestrial habitats in natural conditions. Therefore, revealing the genetic basis of the alteration of fruit types should be a theme of future evolutionary research. Berry (fleshy fruit), capsule, drupe, dry indehiscent fruit, non-capsular dehiscent fruit, and mericarp are the six known types of Solanaceae fruits (Knapp, 2002; Olmstead et al., 2008). We mapped the six fruit types on the phylogenetic tree of the family Solanaceae (Figure 3). Berry and capsule are apparently predominant types of fruits. Capsules occur in the most basal clades and broadly distribute in basal taxa while the origin of berry happened in Cestreae but became predominant after the origin of Anthocercidae. Berry covered by ICS seems to have multiple independent origins in Physalinae, Withaninae, Nicandreae, and Hyoscyameae. Non-capsular dehiscent fruit occurs independently in Solanaceae, Physalinae and Hyoscyameae. Drupe and pyrene occur at least twice in Duckeodendreae, Goetzeoideae, and Lycieae. Dry indehiscent fruit is only observed in Sclerophylax. The genetic control of fruit type is not well-studied, and as the research is hampered by large evolutionary and genetic distance among plants with different fruit types. However, the evolutionary genetic control

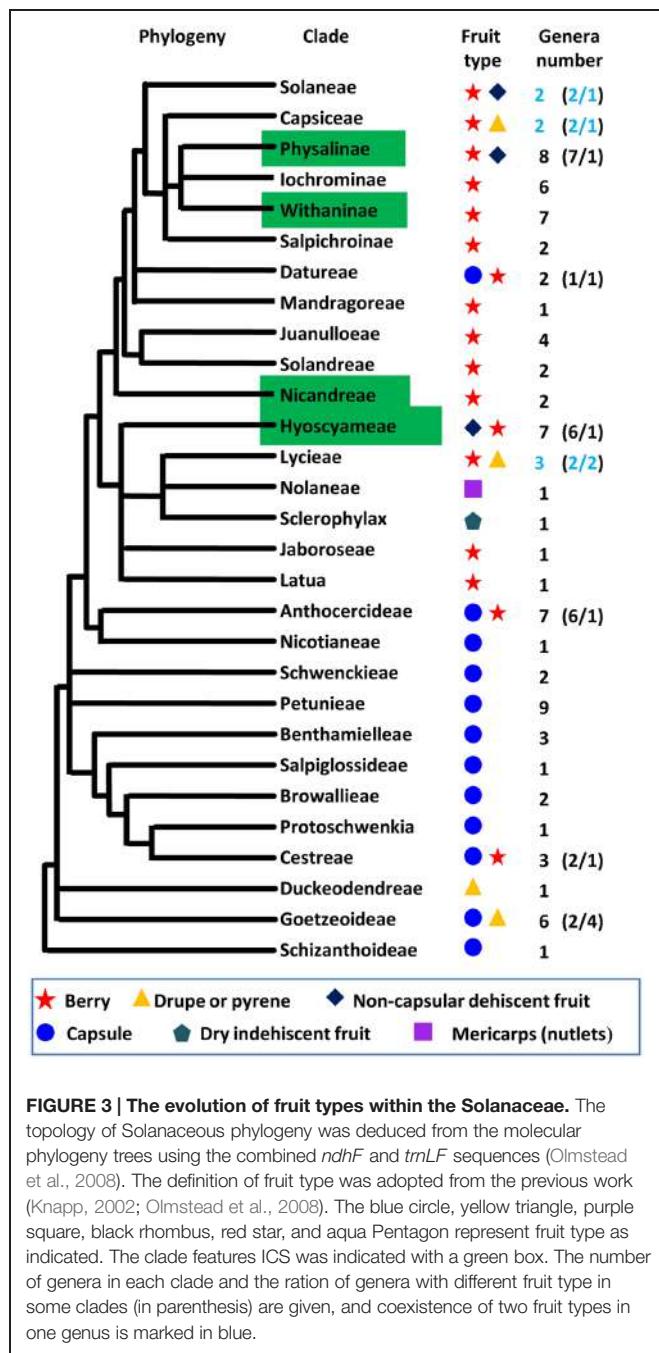


**FIGURE 2 |** Genetic bases underlying the origin of the morphological novelty the Chinese lantern in *Physalis*. (A) Schematic diagram of the heterotopic expression of *MPF2* in floral organs. *MPF3* is the putative ortholog of *SQUAMOSA* (*SQUA*). In *S. tuberosum*, *SQUA*-like protein associated complex binds to the CARG-boxes in *STMADS16* promoter and represses its expression in floral organs. In *P. floridana*, the sequence alteration in the *MPF2* promoter provides a possibility to loosen the repression of *MPF3*-associated complex, thus leading to the heterotopic expression of *MPF2* in floral organs. Arrows indicate the transcription initiation sites. Sequence divergence in *STMADS16* and *MPF2* promoters is depicted by the solid and the dashed lines. The solid square boxes indicate CARG-boxes in the *STMADS16* promoter, and the dashed square boxes indicate altered CARG-boxes in the *MPF2* promoter. The plus or minus represents the gene expression or not. (B) The *MPF3*-*MPF2*/MPF2 interactions involve in the development of the Chinese lantern in *Physalis*. The line indicates the interaction of the two components. The blocked line stands for repression of gene expression. Arrows represent regulations. cd, cell division; ce, cell expansion.

regarding the transition between the various fruit types will be an extremely interesting target. Thus, coexistence of two fruit types in some Solanaceous clades, particularly in a genus from each clade of Solanaceae, Capsiceae, and Lycieae (Figure 3) provides a good system for comparison to understand the genetic variation causing such morphological divergence.

### Natural Variation and Domestication

Natural variation of a trait, even the maintenance of a morphological novelty, is a consequence of adaptation to natural environments. The Solanaceae family displays considerable diversities at different levels and is therefore proposed as a good model to study the evolutionary mechanisms of biodiversity (Knapp et al., 2004). Several model plant species have been established in laboratory experiments, such as tomato, and this will facilitate the work in this family. However, diversity and natural variation are poorly evaluated within the phylogenetic context, and, therefore, the evolutionary mechanisms are not well-understood. In particular, the origin of morphological novelties and the evolution of fruit type (Figure 3) have long been overlooked. Besides the Chinese lantern, other novel morphological traits need to be identified.



**FIGURE 3 |** The evolution of fruit types within the Solanaceae. The topology of Solanaceous phylogeny was deduced from the molecular phylogeny trees using the combined *ndhF* and *tmlF* sequences (Olmstead et al., 2008). The definition of fruit type was adopted from the previous work (Knapp, 2002; Olmstead et al., 2008). The blue circle, yellow triangle, purple square, black rhombus, red star, and aqua pentagon represent fruit type as indicated. The clade features ICS was indicated with a green box. The number of genera in each clade and the ratio of genera with different fruit type in some clades (in parenthesis) are given, and coexistence of two fruit types in one genus is marked in blue.

In addition, the Solanaceae family contains fleshy fruits and vegetables such as tomato, eggplant, chili/pepper, and tomatillo that are eaten by humans, thus several species are domesticated crops and are bred for their diverse morphologies (Figure 1). How human selection affects the genomes of these species, compared with their closely related wild relatives and plants with other fruit types, and further creates ideal traits to meet people's demands is not well-known. Understanding the processes of how plants respond to alteration of natural and/or human environments are the most fundamental to understand the process of life and should, therefore, be a high priority for research.

## Enhancing the Work in the New Model

### *Physalis*

The fruit morphology in *Physalis* varies significantly (**Figure 1**). This genus displays the post-floral morphological novelty Chinese lantern, and the color, flavor, and size of the berries show an impressive variability. A few *Physalis* species, such as *P. peruviana*, *P. philadelphica*, *P. alkekengi*, and *P. angulata* are becoming new leading Solanaceous horticultural and medicinal crops. With efforts in our group in recent years, evolutionary developmental genetics of *Physalis* fruits (berry and ICS) are now understood. In particular, many molecular research tools have been established in *Physalis*, including gene isolation, gene expression detection, protein-protein interactions, transformation system, and VIGS approach (He and Saedler, 2005; He et al., 2007; Zhao et al., 2013; Wang et al., 2014; Zhang et al., 2014a,b; Li and He, 2015). In this respect, *Physalis* has, therefore, has been established as an emerging model plant for development, evolution and ecology. The genetic repertoire for berry and ICS development via genetic and genomic tools needs further investigation. Moreover, multiple experimental approaches will help understand the selective values of the Chinese lantern, and the evolutionary mechanisms of variations in berry size, color, and medicinal components of *Physalis*.

Instead of domesticated crops, more wild plants have to be included. Comparative analyses of Solanaceous crops and their wild relatives will bring new insights into growth, development, and evolution. Thus, comparative microevolutionary-scale studies between closely related genera/species at different levels, including the development, the cellular process, and genetic variation in a phylogenetic context, are major themes in evolutionary developmental genetics of fruit morphological variation.

## Conclusion

The evolution of morphological variation is a consequence of adaptive evolution. Advances in genetics and genomics provide genetic and molecular tools that have facilitated the map-based and candidate-gene-based cloning of several key genes in fruit development, creating new inroads into understanding the primary regulatory mechanisms underlying fruit morphological variation. Recruitment of a preexisting (regulatory) gene frequently occurs. The recruiting mechanisms include alteration

of gene expression and/or gene function through mutations in the regulatory and/or coding regions. The regulatory motifs are often demonstrated to be located in the promoter or the intron, and altering them may cause heterotopic (alteration of expression place), heterochronic (change of expression time), ectopic (a high expression level), or downregulated expression of a gene that appears to play a predominant role in the evolution of plant morphology. Species-specific evolution cannot be excluded; however, independently recruiting the same genetic variation, and regulatory networks could to some extent explain the multiple origins of a particular trait state. In addition, multiple losses of a trait state may occur because once the interacting and regulatory networks have been established for a trait, the evolutionary pattern of that trait may be determined by any secondary mutation in the trait biosynthetic pathways.

In the coming years, there will be considerable focus on isolating new developmental genes and bridging the gap between these genes and their functions. Understanding their recruiting mechanisms and interactions with environments to determine fruit morphological variation in a phylogenetic context are key scientific questions in evolutionary developmental biology. Translation of the information from a few model plants to the large portion of remaining non-model species should be improved. These results will provide fundamental insights into plant developmental processes as well as help to establish novel strategies to improve the productivity and fruit quality of crops.

## Author Contributions

CH designed the work; LW constructed **Figure 1** and **Table 1**; JZ drew **Figure 2**; JL and LW compiled the **Figure 3**; WL and CH wrote the manuscript; JZ and JL contributed critically to the editing the manuscript, and approved the manuscript.

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# Analysis of *Arabidopsis* floral transcriptome: detection of new florally expressed genes and expansion of Brassicaceae-specific gene families

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The flower is essential for sexual reproduction of flowering plants and has been extensively studied. However, it is still not clear how many genes are expressed in the flower. Here, we performed RNA-seq analysis as a highly sensitive approach to investigate the *Arabidopsis* floral transcriptome at three developmental stages. We provide evidence that at least 23,961 genes are active in the *Arabidopsis* flower, including 8512 genes that have not been reported as florally expressed previously. We compared gene expression at different stages and found that many genes encoding transcription factors are preferentially expressed in early flower development. Other genes with expression at distinct developmental stages included *DUF577* in meiotic cells and *DUF220*, *DUF1216*, and *Oleosin* in stage 12 flowers. *DUF1216* and *DUF577* are Brassicaceae specific, and together with other families experienced expansion within the Brassicaceae lineage, suggesting novel/greater roles in Brassicaceae floral development than other plants. The large dataset from this study can serve as a resource for expression analysis of genes involved in flower development in *Arabidopsis* and for comparison with other species. Together, this work provides clues regarding molecular networks underlying flower development.

**Keywords:** *Arabidopsis thaliana*, RNA-Seq, differentially expressed genes, floral development, gene families

## INTRODUCTION

Flower is one of the most complex structures of the angiosperms (flowering plants), and is thought to make great contribution to sexual reproduction in either developmental or evolutionary aspects (Alvarez-Buylla et al., 2010). The basic floral architecture is highly conserved among the core eudicots, including *Arabidopsis thaliana*, which is an important model plant for studying flower development. Over the past three decades, extensive molecular genetic analyses have identified a large number of key floral regulators controlling flower development (O'Maoileidigh et al., 2014), making it one of the best-understood aspects of plant development. However, the present knowledge in understanding gene regulatory network in flower development is incomplete, such as information on genes with low expression levels.

Genome-wide approaches have become valuable tools in characterizing gene expression and in elucidating the genetic networks of flower development at a global level. In the past, large-scale analyses of transcript enrichment among *Arabidopsis* floral organs largely depends on hybridization, such as cDNA and oligonucleotide arrays (Hennig et al., 2004; Wellmer et al., 2004, 2006;

Zhang et al., 2005; Alves-Ferreira et al., 2007; Benedito et al., 2008) and represents a major step in the spatial characterization of floral transcriptome, resulting in identifying many genes important for flower development (Alvarez-Buylla et al., 2010; Irish, 2010). However, array analyses and other hybridization-based approaches have several limitations, including knowledge of genes for probe design, non-specific hybridization, and difficulty in detecting low level expression (Marioni et al., 2008). On the other hand, more recently developed RNA sequencing (RNA-seq) technologies can overcome such limitations of hybridization-based approaches and other conventional large-scale gene expression analysis methods (Marioni et al., 2008; Xiong et al., 2010). It also has great sensitivity, allowing the detection of transcripts with lower expression levels, such as those of many transcription factors (Marioni et al., 2008; Chen et al., 2010). In the last few years, RNA-seq has been extensively applied in the characterization of transcriptome regarding developmental stage, organ, even specific cell types or single cell level, from yeast to human, including several plant species (Jiao et al., 2009; Zhang et al., 2010; Yang et al., 2011). To date, RNA-seq has been used for cell-specific analysis of actively translated mRNAs

associated with polyribosomes in developing flowers, providing insights and resources to further study flower development (Jiao and Meyerowitz, 2010).

To further explore the *Arabidopsis* flower transcriptome, we employed RNA-seq for three developmental periods. We detected 8512 additional genes that are not present on previously used microarray experiments, and provide evidence that at least 23,961 genes are truly expressed in the *Arabidopsis* flower. We also identified differentially and specifically expressed genes and gene families during flower development.

## MATERIALS AND METHODS

### SEQUENCING DATASETS

The inflorescent meristem (IM), stage 1–9 flowers (F1–9) and stage 12 flowers (F12) samples for RNA-seq were collected in our lab, and the three samples were subjected to 50 bp single-end sequencing on a SOLiD 3 platform; details for the methods were recently described in a study for alternative splicing (Wang et al., 2014a). All sequenced short reads were submitted to NCBI Short Read Archive under accession number SRP035230. The datasets for seeding and stage 4 flowers were from previous studies, which generated 36-bp and 42-bp long reads, respectively, using the Illumina genome analyzer (Filichkin et al., 2010; Jiao and Meyerowitz, 2010). The meiocyte datasets were from our previous study, which included two runs (36 and 50 bp) using Life Technologies' SOLiD sequencing platform (Yang et al., 2011).

### ALIGNMENT OF SEQUENCING READS

Sequence reads from the three sample plus the three floral samples were mapped using PerM (Chen et al., 2009) to the *Arabidopsis* genome (release 9) from the *Arabidopsis* Information Resource (TAIR) database (TAIR9; [www.arabidopsis.org](http://www.arabidopsis.org)) allowing 5, 4, and 3 mismatches per 50, 42, and 36-bp read, respectively.

### DIGITAL GENE EXPRESSION AND EXPRESSION ARRAYS

For the RNA-seq experiments, we used at least 10 reads mapped to a gene as the threshold for being expressed. The raw digital gene expression counts were normalized using the reads per kilo-base of mRNA length per million of mapped reads (RPKM) method. The equation was used:

$$\text{RPKM} = \frac{10^9 * C}{N * L}$$

Where  $C$  is the uniquely mapped counts determined from mapping results,  $L$  is the length of the cDNA for the longest splice variant for a particular gene model and  $N$  is the total reads that were mapped to the genome. Log2-transformation of this normalized value was performed as in other analyses.

To test differential expression with mapping data DEGseq was used (Wang et al., 2010). Fisher's Exact Test ( $P < 0.01$ ) method was selected. Microarray results were obtained from a previous study (Zhang et al., 2005). The Microarray experiments have a background value, which was 5 (log value of base 2) as previously described (Zhang et al., 2005) for the evaluation of "expressed" or "unexpressed" genes. Identification of differentially expressed genes according to the microarray data also used the Fisher's Exact Test method.

### Z-SCORE

Calculation of the Z-score was based on the log2-transformed RPKM-normalized transcript levels as follows:

$$Z = (X - \mu)/\sigma$$

$X$  is the RPKM of a gene for a specific tissue/developmental stage.  $\mu$  is the mean RPKM of a gene across all tissues/developmental stages and  $\sigma$  is the RPKM standard deviation of a gene across all tissues/developmental stages. All calculations and plotting were performed by Perl and excel, respectively.

### GENE FAMILY AND FUNCTIONAL ANNOTATION

The protein domain annotations were obtained from the Pfam database (<http://pfam.sanger.ac.uk>) (Punta et al., 2011). *Arabidopsis* protein sequences were then searched against protein family models in the Pfam-A database, resulting in 21102 *Arabidopsis* proteins identified as having at least one Pfam domain. Transcription factor family annotations were from The Database of *Arabidopsis* Transcription Factors (<http://datf.cbi.pku.edu.cn/>) (Guo et al., 2005), which contains 1922 transcription factors in *Arabidopsis*. Gene ontology (GO) enrichment analysis was performed with the agriGO browser (<http://bioinfo.cau.edu.cn/agriGO/>) (Du et al., 2010) using Singular Enrichment Analysis.

Multiple sequence alignment was performed in MUSCLE (<http://www.drive5.com/muscle/>) using the default parameters. Maximum likelihood (ML) trees were constructed by FastTree ([www.microbesonline.org/fasttree](http://www.microbesonline.org/fasttree)) with the approximate likelihood ratio test method.

## RESULTS AND DISCUSSION

### GLOBAL GENE EXPRESSION OF FLOWER TRANSCRIPTOMES IN ARABIDOPSIS

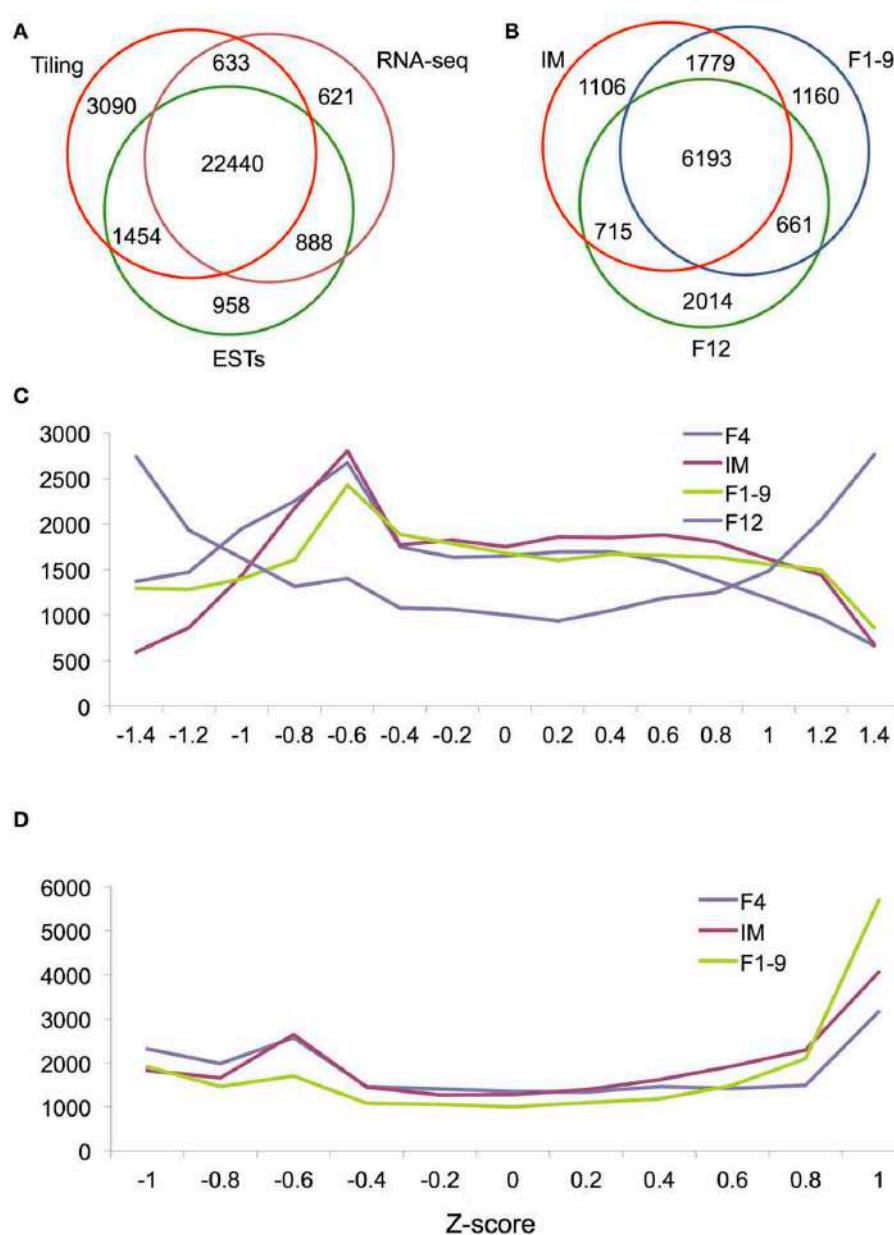
To obtain more insights about the overall transcriptome landscape during flower development, we analyzed RNA-seq datasets of the *Arabidopsis* flower at three developmental stages recently generated in our laboratory; these datasets were analyzed for alternative splicing in a separate study (Wang et al., 2014a): inflorescent meristem (IM), stage 1–9 flowers (F1–9) and stage 12 flowers (F12), detecting 21,181 (IM), 22,137 (F1–9), and 22,827 (F12) reliably expressed genes (Table S1, Figure S1). A recent report summarized that a total of 126 *Arabidopsis* genes have been demonstrated genetically to have a role during flower development (Alvarez-Buylla et al., 2010), 122 of which were also detected as expressed in our dataset (Table S2), indicating that our data were very reliable, and can be used for further analysis. To compare gene expression during flower development, besides the three datasets described above, we also included data for *Arabidopsis* male meiocytes that we had generated previously (Yang et al., 2011), and two other public datasets of *Arabidopsis* seedlings and stage 4 flowers (Filichkin et al., 2010; Jiao and Meyerowitz, 2010), the latter of which were from isolated polysomic.

To further explore how many genes are truly expressed in *Arabidopsis* flower, we searched The *Arabidopsis* Information Resource (TAIR) database and obtained a total of 24,570 genes,

which are supported by at least one EST. Then, we searched the present tiling array database to find available probes for 30,228 genes. 4734 genes were found to be tiling array-specific compared with ESTs and RNA-seq data. Among them, 2634 and 276 are transposons and pseudogenes, respectively. The other 1824 genes seem to be expressed at very low levels. The average value of the 4734 genes is 5.4, which is regarded as a threshold in this study for the evaluation of “expressed” or “unexpressed” genes. Based on this criterion, we believe that tiling array can detect at least 27,617 genes. As described previously, RNA-seq detected 24,769 genes in flowers. Comparison of the detected genes among EST,

tiling array and RNA-seq found that 22,440 genes were detected by three data sets and 1521 genes were detected by RNA-seq and either ESTs/tiling array (**Figure 1A**). The results suggest that at least 23,961 genes are reliably detected as expressed in the *Arabidopsis* flower. In addition, 621 genes were only detected by RNA-seq; most of these are low abundance genes that are nearly undetectable by arrays and the others are likely to be stage-specific genes.

Characterization of stage or cell-specific genes provides a foundation for unraveling their molecular mechanisms. Previous studies in multiple plants demonstrated that each stage or tissue



**FIGURE 1 | Global gene expression during flower development. (A)** A Venn diagram showing the overlap in detected genes between three technologies: the tiling array, RNA-seq and ESTs. **(B)** A Venn diagram showing the overlap between IM, F1-9, and F12 for genes

called as DEG by RNA-seq. Histograms of relative expression levels (measured by Z-scores) in four **(C)** and three **(D)** organs. For easy visualization, we plotted Z-score on the x-axis and gene numbers on the y-axis.

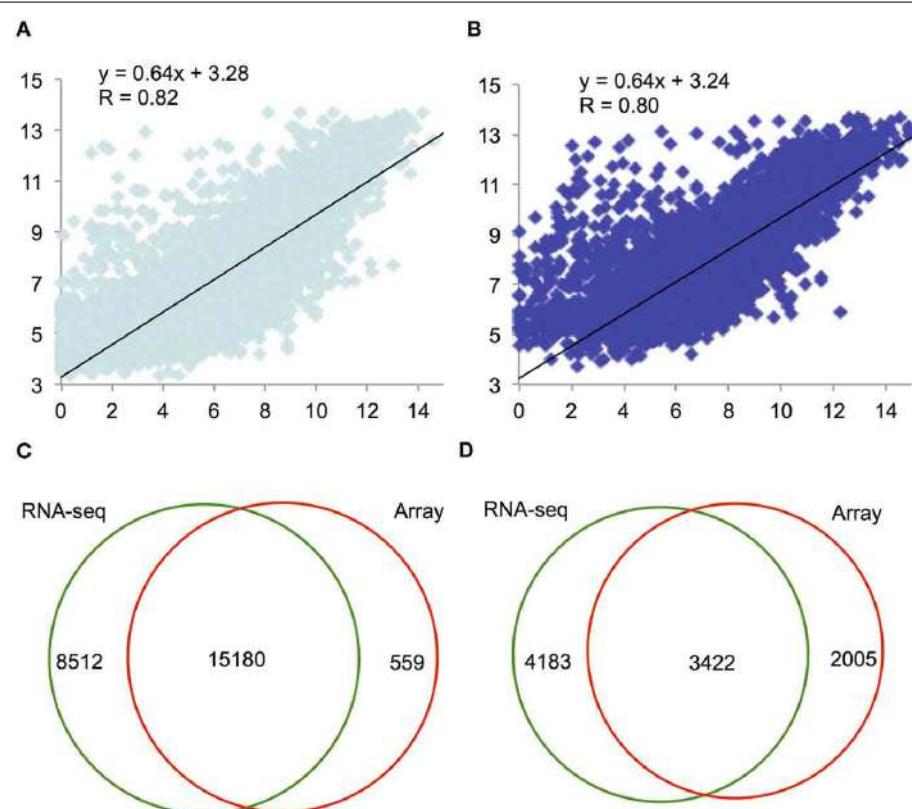
has specific transcripts (Jiao et al., 2009; Jiao and Meyerowitz, 2010; Yang et al., 2011; Liu et al., 2014). To better establish the genome-wide gene expression pattern of flower development, we conducted a Z-score analysis to assess the extent of differential gene expression for florally expressed genes. Results showed that the Z-score distribution of gene expression in F12 was dramatically different from that for early flower development (F1–9, F4, and IM) (Figure 1C), suggesting that nearly mature flowers require many more specifically or differentially expressed genes than early flowers. In contrast, Z-score distributions were very similar between F1–9, F4, and IM (Figure 1D), further supporting the idea that the developmental programs of these stage/organ are similar.

#### DETECTION OF EXPRESSION OF 8512 GENES IN THE ARABIDOPSIS FLOWER NOT REPORTED FROM MICROARRAY ANALYSIS

We first compared the F1–9 and F12 RNA-seq data with the Affymetrix ATH1 array data at similar stages (Zhang et al., 2005). We compared the number of sequencing reads mapped to each gene with the corresponding (normalized) absolute intensities from the array (Figures 2A,B), and found that the correlations between the two platforms were high, with Spearman

correlation coefficients of 0.82 (F1–9; Figure 2A) and 0.80 (F12; Figure 2B). Thus, comparison of RNA-seq and microarray identified 15,180 overlapping genes with relatively high expression levels (Figure 2C), covering 96% of genes detected using microarray. In addition, our RNA-seq identified additional 8512 genes that were undetected by microarray (Zhang et al., 2005), whose expression levels were obviously lower; the average expression level is 56.12 (F1–9) and 57.82 (F12) RPKM (Figure S2b), compared to the average expression level about 250 for the 15,180 genes. Also, the curvature of the comparison toward the microarray axis suggested that microarray possibly underestimated the expression level of genes relative to RNA-seq (Figures 2A,B). Together, these results suggest that RNA-seq has a great advantage over microarray in detecting low-abundance transcripts, consistent with previous reports (Marioni et al., 2008; Yang et al., 2011).

To investigate further regarding the 8512 genes, we analyzed the enrichment of protein family (PFAM) domains (gene families) among these genes, and identified several enriched gene families that were not reported previously as enriched, including *F-box*, *NB-ARC*, *C1\_3*, *PPR*, *LRR\_1*, *Myb*, *bHLH*, and *AP2* gene families (Table 1). Previously, many *F-box* genes were reported as unexpressed or undetectable by microarray analysis (Schmid



**FIGURE 2 |** A scatter plot of relative expression values obtained by RNA-seq and microarray for F1-9 and F12. **(A)** Comparison of expression levels between RNA-seq and microarray for F1-9, RNA-seq and microarray; gene expression levels transformed with log2 were plotted. **(B)** Comparison of expression between RNA-seq

and microarray for F12. **(C)** A Venn diagram illustrating genes detected by RNA-seq (left) and microarray (right) analyses. **(D)** A Venn diagram presenting the overlap of differentially expressed genes between F1-9 and F12 from RNA-seq (left) and from microarray analysis (right).

**Table 1 | Enriched protein family (Pfam) among 8512 genes that are undetected by microarray analysis.**

| Family | Total | RNA-seq | Percent | Family        | Total | RNA-seq | Percent |
|--------|-------|---------|---------|---------------|-------|---------|---------|
| NB-ARC | 167   | 108     | 0.65    | bHLH          | 134   | 59      | 0.44    |
| FBD    | 115   | 71      | 0.62    | AP2           | 145   | 62      | 0.43    |
| TIR    | 132   | 80      | 0.61    | UDPGT         | 115   | 49      | 0.43    |
| SCRL   | 25    | 14      | 0.56    | peroxidase    | 82    | 33      | 0.40    |
| U-box  | 61    | 31      | 0.51    | Myb           | 256   | 103     | 0.40    |
| MATH   | 60    | 30      | 0.50    | Malectin_like | 78    | 31      | 0.40    |
| DUF26  | 97    | 47      | 0.48    | LRR_1         | 391   | 154     | 0.39    |
| Auxin  | 79    | 38      | 0.48    | PMEI          | 122   | 46      | 0.38    |
| C1_3   | 146   | 70      | 0.48    | SLR1-BP       | 41    | 15      | 0.37    |
| DUF295 | 78    | 36      | 0.46    | p450          | 249   | 91      | 0.37    |
| PPR_1  | 301   | 138     | 0.46    | zf-rbx1       | 174   | 63      | 0.36    |
| PPR    | 465   | 211     | 0.45    | ABC_tran      | 117   | 42      | 0.36    |
| NAC    | 113   | 51      | 0.45    | Kelch_1       | 110   | 38      | 0.35    |
| F-box  | 522   | 233     | 0.45    | Ank_2         | 106   | 36      | 0.34    |
| FBA_1  | 176   | 78      | 0.44    | zf-C3HC4      | 306   | 96      | 0.31    |

et al., 2005), further suggesting that microarray is not as sensitive as RNA-seq for detecting low-abundance transcripts. In addition, we also detected some enriched gene families that belongs to the highly expressed genes; for instance, Plant self-incompatibility response (SCRL) and S locus-related glycoprotein 1 binding pollen coat protein (SLR1-BP) are specifically enriched in F12 (**Table 1** and **Table S3**), suggesting a potential role at this stage. In contrast, 559 genes detected by microarray were not found by RNA-seq, possibly due to difference in growth conditions.

We further employed a widely used Fisher's Exact Test method to identify differentially expressed genes (DEGs) between F1–9 and F12 in RNA-seq and microarray data. Altogether, 7605 and 5327 DEGs were identified in each dataset. Among them, 3422 genes were detected by both platforms (**Figure 2D**), 1272 and 84 DEGs were only detected by RNA-seq and microarray, respectively (**Figure 2D**), and consistent with the fact that RNA-seq is more sensitive for detection and comparison of gene expression. Taken together, these results indicate that deep sequencing can greatly increase the sensitivity of transcriptome analysis.

## IDENTIFICATION OF STAGE-DIFFERENTIALLY EXPRESSED GENES DURING FLOWER DEVELOPMENT

Floral organ identity and cell fate determination are highly regulated by the temporal and spatial gene expression, with each organ or cell type having distinct transcriptomes (Jiao et al., 2009; Yang et al., 2011; Wang et al., 2014b). To investigate DEGs between one of the floral stages with seedlings, we compared the flower transcriptomes of IM, F1–9, or F12 with that of seedlings, and identified IM with 9793 DEGs, F1–9 with 9583 DEGs, and F12 with 9340 DEGs (**Table 2**). Furthermore, the intersection between these three sets contained 6193 genes (**Figure 1B**), indicating these three samples are quite similar regarding differentially gene expression compared with seedlings. GO annotation showed that these genes were enriched for categories such as "histone modification" and "methylation" ( $p = 3.2\text{E-}11$  and  $4.7\text{E-}9$ ), suggesting

**Table 2 | Differentially expressed genes (DEGs) for each floral sample compared with seedling.**

|           | Seedling | IM   | F4   | F1–9 | F12  | Meiocytes |
|-----------|----------|------|------|------|------|-----------|
| Seedling  |          | 9793 | 8703 | 9583 | 9340 | 4966      |
| IM        | 6627     |      | 3747 | 6866 | 6943 | 3900      |
| F4        | 5812     | 5354 |      | 6016 | 6460 | 3449      |
| F1–9      | 6866     | 7228 | 4834 |      | 7697 | 3773      |
| F12       | 5987     | 8960 | 6724 | 8109 |      | 3820      |
| Meiocytes | 5110     | 7167 | 6154 | 6628 | 6401 |           |

these genes are involved in the establishment of transcription regulation during flower development. Likewise, 2014 genes were specifically expressed in F12 and showed significant enrichment for genes in reproduction ( $p = 2.2\text{E-}47$ ), flower development ( $p = 2.9\text{E-}22$ ) and post-embryonic development ( $p = 1.3\text{E-}194$ ), which might suggest that genes expressed during gametophyte development can function in later stages.

To further examine the combined set (13,628 genes) of the above floral DEGs, we compared these genes with 4505 genes identified as potential targets of the SEP3 and/or AP1 proteins by ChIP-seq (Immink et al., 2009; Kaufmann et al., 2010). The results showed that 2506 genes overlapped between the floral DEGs and the SEP3/AP1 targets with significance (Fisher's test,  $p = 7.03\text{e-}08$ ). It is likely that some of these genes are involved in the regulation of flower development, but the role of these genes in flower development needs to be determined using molecular genetic analyses.

We then analyzed the enrichment of protein domains as defined in the PFAM database, and found several enriched domains ( $P \leq 0.01$ ; **Table 3**), including ATPase, Helicase\_C, DEAD box, WD40, SET, and PHD domains, suggesting that chromatin associated transcriptional regulation might be one of the major features underlying flower development. In addition, proteins with "UCH," "hydrolase," and "IQ" domains were also significantly over-represented, although their functions in flower development are largely unknown. Interestingly, we also identified "PPR," "Mito\_carr" and "Miro" domains as significantly enriched; members of these genes are involved in gene expression and other functions in mitochondria and plastid, suggesting that such organellar functions might be important for flower development.

## DISTINCT ENRICHMENT OF TRANSCRIPTION FACTORS IN EARLY FLOWER DEVELOPMENT

Identification of transcription factors (TFs) expressed in a specific stage provides a foundation for understanding the transcriptional regulatory networks underlying the development, structure and function of the stage. To investigate the expressed TFs during flower development, we examined the TFs among IM, F1–9, and F12 and identified a total of 1667 transcription factors, 927 of which showed differential expression compared with the seedling (**Figure 3A**). Among the 927 TFs, 70% showed highest expression in IM (designated as D1), whereas 14 and 16% showed highest expression in F1–9 (designated as D2) and F12 (designated as D3), respectively (**Figure 3B**).

**Table 3 | Significance of enriched Pfam domains in differentially expressed genes during flower development.**

| Pfam domain | Total | Num. | Percent | P-value  | Pfam domain  | Total | Num. | Percent | P-value |
|-------------|-------|------|---------|----------|--------------|-------|------|---------|---------|
| Helicase_C  | 149   | 133  | 0.89    | 4.00E-07 | Proteasome   | 24    | 23   | 0.96    | 0.02    |
| WD40        | 234   | 184  | 0.79    | 1.00E-06 | Cyclin_C     | 30    | 28   | 0.93    | 0.02    |
| DEAD        | 114   | 97   | 0.85    | 5.00E-05 | HATPase_c    | 35    | 30   | 0.86    | 0.02    |
| RRM_1       | 245   | 171  | 0.70    | 0.0003   | AAA_5        | 45    | 37   | 0.82    | 0.02    |
| Kinesin     | 61    | 55   | 0.90    | 0.001    | Mito_carr    | 59    | 46   | 0.78    | 0.02    |
| PHD         | 52    | 47   | 0.90    | 0.002    | Galactosyl_T | 21    | 20   | 0.95    | 0.03    |
| SNF2_N      | 45    | 40   | 0.89    | 0.005    | HA2          | 21    | 20   | 0.95    | 0.03    |
| PPR_1       | 301   | 188  | 0.62    | 0.006    | Cyclin_N     | 52    | 40   | 0.77    | 0.03    |
| IQ          | 56    | 46   | 0.82    | 0.008    | Hydrolase    | 60    | 45   | 0.75    | 0.03    |
| PPR         | 465   | 275  | 0.59    | 0.008    | Miro         | 114   | 76   | 0.67    | 0.03    |
| AAA         | 144   | 98   | 0.68    | 0.009    | OB_NTP_bind  | 20    | 19   | 0.95    | 0.04    |
| LSM         | 26    | 26   | 1.00    | 0.01     | Cpn60_TCP1   | 23    | 21   | 0.91    | 0.04    |
| UCH         | 45    | 39   | 0.87    | 0.01     | KH_1         | 25    | 22   | 0.88    | 0.04    |
| ResIII      | 48    | 40   | 0.83    | 0.01     | SET          | 46    | 35   | 0.76    | 0.04    |
| RuvB_N      | 46    | 38   | 0.83    | 0.01     | Histone      | 67    | 48   | 0.72    | 0.04    |

D1 mainly contained members of the homeobox domain (HB), MADS, MYB, AP2, and NAC families, suggesting that floral meristem development largely requires those transcription factor (**Figure 3B**). For example, *homeobox* genes encode transcription factors that contain a classic DNA binding domain with about 60 amino acids and regulate gene expression via Polycomb-dependent modulation of chromatin structure, thereby controlling development in animals, fungi and plants (Zhong and Holland, 2011). Several known members of HB (**Figure 3C**) identified in IM support that early floral development requires active HB genes, consistent with the finding that epigenetic reprogramming of gene expression is important for the establishment of initial floral identity (Mukherjee et al., 2009). The co-expressed pattern between HB genes and chromatin factors in IM is in agreement with previous studies that a number of floral genes with similar expression patterns and/or associated with each other regulate the expression of downstream genes to ensure proper flower development (Kaufmann et al., 2009, 2010; Deng et al., 2011).

D2 included *MADS-box*, *MYB*, *AS2*, *C2H2*, *bZIP*, *ABI3*, and *bHLH* families (**Figure 3C**). *MADS-box* genes encode not only key repressors or activators for flowering transition, but also master regulators of reproductive organ identities (Alvarez-Buylla et al., 2010). Our data detected expression of most *MADS-box* genes known to be involved in flower development (**Figure 3C**), such as *FLC*, *SHP1/2*, *AP3*, *AG*, *AGL11/15/77*, *TT16*, *SEP1-3*, *STK*, *AT5G49420*; *AG*, *AP3*, and *SEP1-3* are genes for the ABCE model, consistent with their known function in floral organ identity (Smacznia et al., 2012). In addition, genes coding for transcription factors important for microsporogenesis were also uncovered, such as *AMS*, *MS1*, *MYB35*, and *MYB99* (Chang et al., 2011), as well as *MMD1* required for meiosis (Yang et al., 2003).

D3 was enriched in *MADS*, *MYB*, *AP2*, *C2H2*, *C2C2-CO-like*, *NAC*, *AUX-IAA-ARF*, and *bHLH* families (**Figure 3C**). Previous studies showed that auxin-dependent transcriptional regulation requires the auxin/indole-3-acetic acid (Aux/IAA) and auxin response factor (ARF) families of TFs and formation of

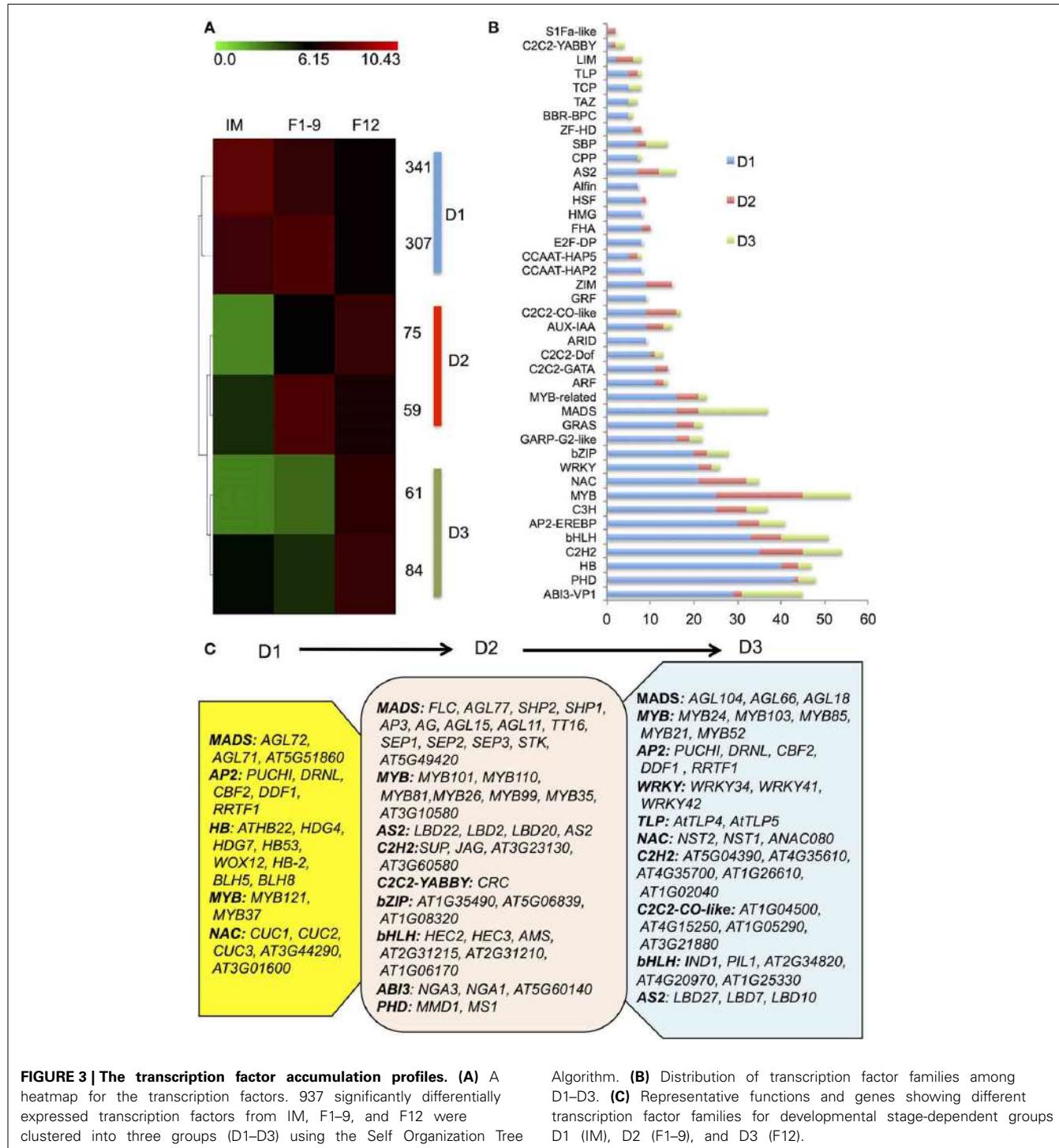
Aux/IAA-ARFs heterodimers represses auxin signaling (Reed, 2001), which has been demonstrated to participate in pollen development, pollination and fertilization (Sundberg and Ostergaard, 2009), as well as female gametophyte specification (Pagnussat et al., 2009). Indeed, our data identified several known and unknown ARFs and IAA factors in G3, suggesting that the Aux/IAA-ARF regulatory pathway is vital for late reproductive development. However, the function of other enriched TFs in flowers is still largely unknown. Together, these results demonstrate that flower development at different stages requires common and distinct transcription factor families.

#### IDENTIFICATION OF SPECIFIC GENE FAMILIES AT DISTINCT STAGES OF FLOWER DEVELOPMENT

We sought to identify stage-specific genes, which were defined as those genes that were differentially expressed (>4-fold change) at one stage over all other stages studied here using DEG seq. The largest numbers of stage specific genes were identified in the seedling, F12 and meiocytes (1083, 552, and 652 genes, respectively; **Table 4**). Given the lack of correlation in overall gene expression between the floral transcriptome (F12) and the other stages sampled (**Figure 2C**), it was not surprising to identify this stage as having the largest number of organ-specific genes. These genes are strong candidates for determining the specific functional components of the nearly mature flower.

Interestingly, the F1–9 flower-specific genes with 8-fold changes had 26 genes, including 9 transposons and 5 snoRNAs (**Table S4**), consistent with the previous finding that transposons and small RNAs were enriched among genes expressed in male meiocytes (Chen et al., 2010; Yang et al., 2011). There are also 12 coding genes, one of which (AT5G09780) codes for a transcription factor of the B3 family and two (AT1G48700 and AT4G03050) are for iron binding proteins.

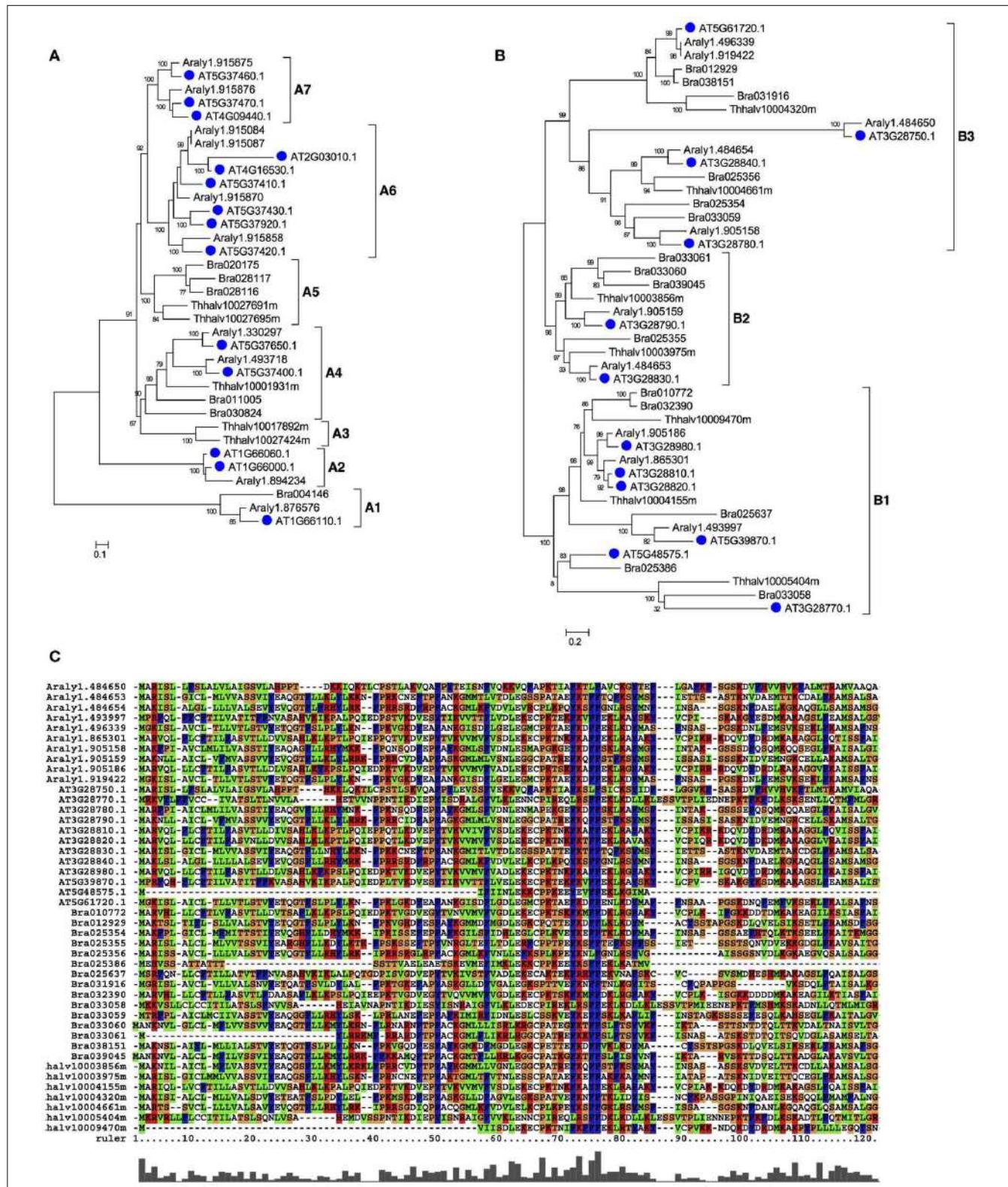
For meiocyte-specific genes, 424 genes were found with ~8-fold changes and showed enrichment for genes in an insertion of mitochondrial origin on chromosome II, as supported by similar preferential expression in meiocytes reported previously



**Table 4 | The specifically expressed genes in one sample compared with others.**

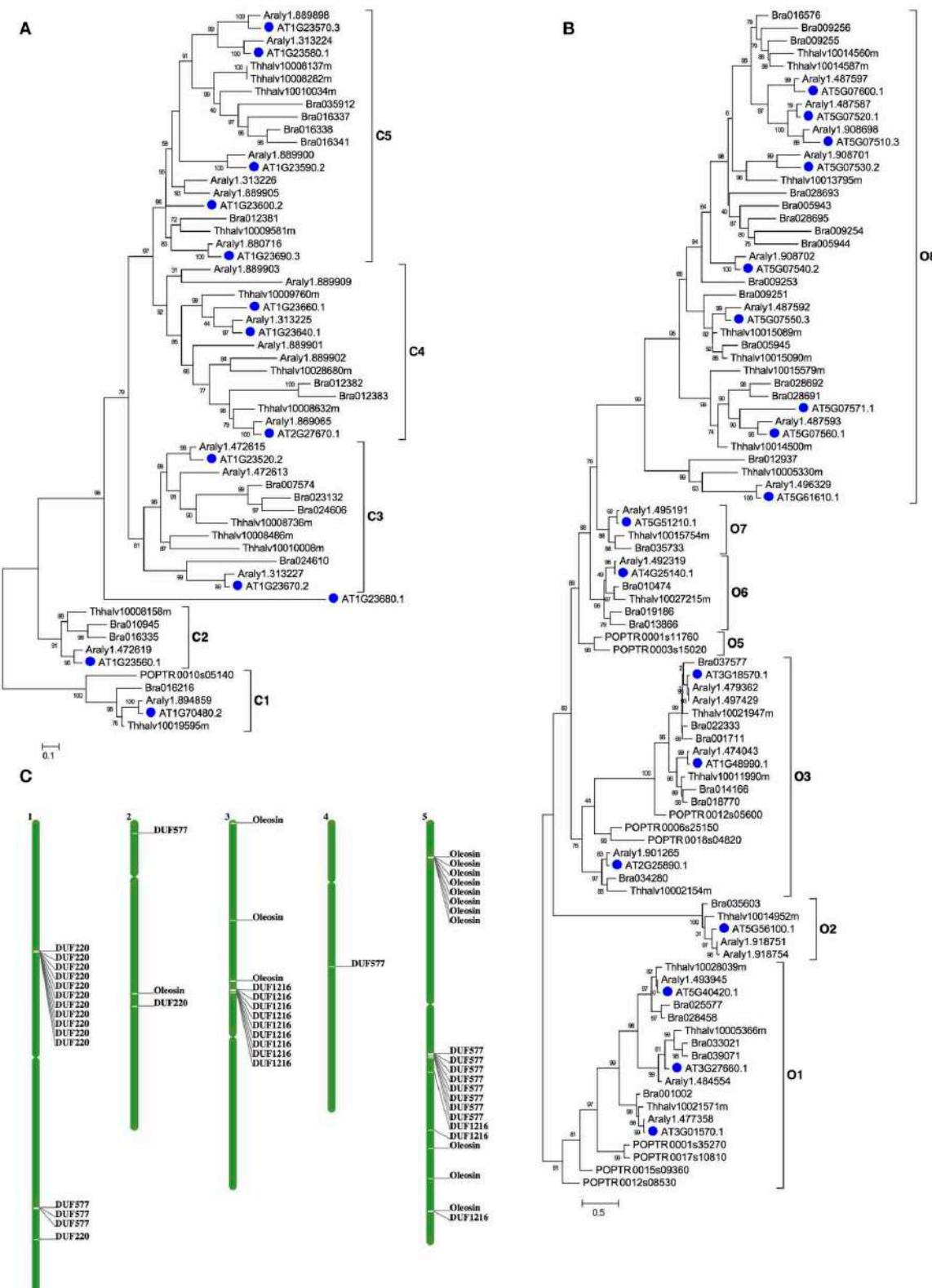
| Fold change | Seedling | IM   | F4  | F1-9 | F12  | Meiocyte |
|-------------|----------|------|-----|------|------|----------|
| 1           | 2656     | 1280 | 686 | 1740 | 1632 | 1636     |
| 2           | 1871     | 157  | 118 | 110  | 817  | 1200     |
| 4           | 1083     | 27   | 33  | 26   | 552  | 652      |
| 8           | 695      | 7    | 16  | 13   | 418  | 424      |

(Chen et al., 2010). The enriched genes also included 45 mitochondrial and 28 chloroplast genes, respectively. Moreover, in addition to previously reported gene families (Yang et al., 2011), we also detected several other enriched gene families, such as *Oxidored\_q1*, *Oxidored\_q2*, *Oxidored\_q3*, *Oxidored\_q4*, and *NADHdh*. In particular, most members of the *DUF577* family showed specific expression in meiocytes (Table S5). To further investigate this gene family, we performed phylogenetic analyses



**FIGURE 4 | The phylogenetic tree of *DUF577* and *DUF1216* gene families in plant.** The *DUF577* and *DUF1216* gene families were found only in plants, but not in other eukaryotic groups. **(A)** A Maximum likelihood (ML) tree of the *DUF577* gene family using representative species in eudicots and it can be divided into 8 clades, A1~A7. **(B)** An ML

tree of *DUF1216* gene family using representative species in eudicots and it can be divided into 3 clades, B1~B3. **(C)** An alignment of the N-terminal regions of *DUF1216* proteins. Species names are abbreviated as below: At-*Arabidopsis thaliana*, Araly-*Arabidopsis lyrata*, Bra-*Brassica rapa*, Thhalv-*Eutrema salsuginea*.



**FIGURE 5 | The phylogenetic tree of DUF220 and Oleosin gene families in plant.** (A) An ML tree of DUF220 gene family can be divided into 5 clades, C1~C5. (B) An ML tree of Oleosin gene family can be divided into 8 clades, O1~O8. Species names are abbreviated as below: AT-*Arabidopsis thaliana*,

Araly-Arabidopsis *lyrata*, Bra-*Brassica rapa*, Thhalv-Eutrema *salsugineum*; POPTR-*Populus trichocarpa*. **(C)** The chromosomal positions of the Arabidopsis DUF577, DUF1216, DUF220, and Oleosin genes. The names of genes refer to locus ID as listed in **Table S5**.

of this gene family with members from several representative plant species, including *Arabidopsis lyrata*, *Eutrema salsugineum*, *Brassica rapa*. As shown in **Figure 4A**, this family can be divided into seven subfamilies, designated as A1-A7. The tree supported that this gene family have experienced expansion and origin in Brassicaceae (**Figure 4A**). The A6 and A7 subfamilies only included *Arabidopsis lyrata* and *Arabidopsis thaliana*, suggesting an expansion that occurred since the divergence of *Arabidopsis* and other Brassicaceae species. Besides, functions of the DUF577 and other enriched family genes in meiosis need to be tested.

Similarly for F12, the enriched families included *DUF1216*, *Oleosin* and *DUF220*. Most members of the three gene families had specific expression in F12; these gene families contain lineages that originated and expanded within Brassicaceae (**Table S5** and **Figures 4, 5**). Phylogenetic analyses of the *DUF1216* family suggested that this gene family is specific to Brassicaceae, without homologs in other plants, and experienced gene duplication during Brassicaceae history (**Figure 4B**). Interestingly, the N-terminal region of *DUF1216* proteins had putative signal peptides with similar sequences, according to the SignalP prediction (<http://www.cbs.dtu.dk/services/SignalP/>). The predicted signal peptide contains a large number of hydrophobic amino acids, a conserved basic amino acid and a conserved cysteine at the ninth position (**Figure 4C**). *At5g07750* of the *Oleosin* family was reported to have experienced positive selection (Schein et al., 2004). However, expression of each of three tandem duplicated genes in the *Oleosin* family (*AT5G07510*: 10081.76, *AT5G07550*: 29536.45, *AT5G07560*: 10942.38) had extraordinarily high levels of more than RPKM of 10,000, suggesting that such high expression levels are important for F12 for later functions. Analysis of the *Arabidopsis* genome indicates that tandem duplication contributed to the expansion of *DUF1216* in Brassicaceae, as well as the expansion of the *Oleosin*, *DUF220* and *DUF577* families (**Figure 5C**). This pattern is different to those of *SET*, *JmjC*, and *Rhomboid* gene families, which are more likely to be retained after whole genome duplication events (Zhou and Ma, 2008; Zhang and Ma, 2012; Li et al., 2014).

## CONCLUSIONS

The analysis of *Arabidopsis* floral transcriptome datasets presented here provides a valuable resource of candidate genes for further studies to understand the flower development program. We provided evidence for at least 23,961 genes that are expressed in the *Arabidopsis* flower. Compared with seedling, over 10,000 DEGs were identified, revealing novel and different molecular characteristics in the developing flower such as regulatory genes, genes for high-energy production, and transposable elements. These results showed that flower development at different stages requires common and distinct transcription factor families. The gene expression in F12 was dramatically different from that for early flower development (F1–9, F4, and IM).

In addition to identifying floral developmental gene candidates, we found many genes or gene families specifically expressed at one stage. Many transposable element genes, at least 45 mitochondrial and 28 chloroplast genes showed specific expression in meiocytes. The *SCRL*, *SLR1-BP*, *DUF1216*, *Oleosin*, and *DUF220* gene families showed specific expression in F12 and *DUF577*

genes were detected to have specific expression meiosis. These specifically expressed genes have functions that are closely related to reproductive development, showed that mature flowers require many more specifically or differentially expressed genes than early flowers. These gene families expanded dramatically within the Brassicaceae lineage, suggesting novel functions that are possibly important for the origin and evolution of Brassicaceae. This dataset can be useful for discovering functional genes at different stages of the flower development and provide clues for the molecular and regulatory relationships between different stages.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00802/abstract>

**Figure S1 | Biological replicates were highly reproducible.**

**Figure S2 | Comparison between transcriptomes from RNA-Seq and microarray.**

**Table S1 | The expression of all genes in six samples.**

**Table S2 | The 126 known *Arabidopsis* flower development genes.**

**Table S3 | The expression of *SCRL* and *SLR1-BP* genes in different samples.**

**Table S4 | The expression of the 26 specific genes in F1–9.**

**Table S5 | The expression of *DUF577*, *DUF1216*, *DUF220* and *Oleosin* genes in different samples.**

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# Comparative transcriptomic analysis of male and female flowers of monoecious *Quercus suber*

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Monoecious species provide a comprehensive system to study the developmental programs underlying the establishment of female and male organs in unisexual flowers. However, molecular resources for most monoecious non-model species are limited, hampering our ability to study the molecular mechanisms involved in flower development of these species. The objective of this study was to identify differentially expressed genes during the development of male and female flowers of the monoecious species *Quercus suber*, an economically important Mediterranean tree. Total RNA was extracted from different developmental stages of *Q. suber* flowers. Non-normalized cDNA libraries of male and female flowers were generated using 454 pyrosequencing technology producing a total of 962,172 high-quality reads with an average length of 264 nucleotides. The assembly of the reads resulted in 14,488 contigs for female libraries and 10,438 contigs for male libraries. Comparative analysis of the transcriptomes revealed genes differentially expressed in early and late stages of development of female and male flowers, some of which have been shown to be involved in pollen development, in ovule formation and in flower development of other species with a monoecious, dioecious, or hermaphroditic sexual system. Moreover, we found differentially expressed genes that have not yet been characterized and others that have not been previously shown to be implicated in flower development. This transcriptomic analysis constitutes a major step toward the characterization of the molecular mechanisms involved in flower development in a monoecious tree with a potential contribution toward the knowledge of conserved developmental mechanisms in other species.

**Keywords:** flower development, monoecious, pyrosequencing, *Quercus suber*, RNA-seq, transcriptomics, cork oak, EST

## INTRODUCTION

*Quercus suber* (L.) is one of the most important forest species in Portugal, being the dominant tree of the oak woodlands (Aronson et al., 2009). Due to its ecological and socio-economic significance, the cork oak forest is a unique resource. There is a growing interest in the management of woods for the production of acorns destined either for nursery production or for animal feed stocks. Therefore, the knowledge of the molecular mechanisms that control flower induction and fertilization is crucial to fully understand the reproductive success of this species.

*Quercus suber* is a monoecious tree species with a protandrous system and a long progamic phase (period between pollination and fertilization). Male flowers are organized in catkins that emerge in reproductive buds of the previous growth season or at the base of the branches of the current season. Each individual catkin contains 15–25 staminate flowers that are radially set around the catkin's axis (Natividade, 1950). The staminate flowers present a perianth with four to six tepals with an equal or double number of anthers that do not burst simultaneously

(Boavida et al., 1999). Female inflorescences arise in spikes, with three to five flowers, on the axil of the new leaves. Female flowers are included in a cupule and contain three carpels, with two ovules each (Boavida et al., 1999). Male flowering buds occur in early spring and sometimes also in autumn, whereas female flowers appear in spring and only get fully developed a few months later, if pollinated. During spike elongation, three to five styles emerge from the cupule and the stigma becomes receptive (Ducousoo et al., 1993). At the time of pollination the ovary is still undifferentiated and the transmitting tissue extends only to the base of the styles. The wind driven pollen lays on the receptive stigmatic surface, germinates and the pollen tube grows throughout the transmitting tissue, until it reaches the base of the style. Usually, the pollen tube growth is arrested for 6 weeks, overlapping with ovule differentiation (Boavida et al., 1999; Kanazashi and Kanazashi, 2003). After fertilization, only one of the six ovules develops into a monospermic seed, which matures during autumn (Ducousoo et al., 1993; Boavida et al., 1999).

Flower development is a complex and dynamic process that requires the tight coordination of gene expression and environmental cues (Fornara et al., 2010). During the past several years, a significant progress has been made in elucidating the genetic networks involved in flower organ specification in hermaphroditic model (reviewed in Wellmer et al., 2014) and non-model species (Wu et al., 2010; Yoo et al., 2010; Zahn et al., 2010; Logacheva et al., 2011; Varkonyi-Gasic et al., 2011; Zhang et al., 2012). Unisexual flower specification requires developmentally regulated processes that initiate male and female organ primordia in separate parts of the plant (Dellaporta and Calderon-Urrea, 1993). Studies focusing on mutant isolation revealed that several genes affect the key steps of sex determination in a variety of species. For example, in maize, unisexuality is controlled by *TASSELSEED2* that is expressed in the male structure (tassel) and is involved in pistil primordia abortion (DeLong et al., 1993). Also, in melon, a single nucleotide change in the *1-AMINOCYCLOPROPANE-1-CARBOXYLIC ACID SYNTHASE* gene is responsible for the specific inhibition of the male reproductive organs (Boualem et al., 2008). With the advent of next generation sequencing (NGS) technology, the previous limitation of mutant isolation in important model and non-model species was surpassed (Rowan et al., 2011). In another Cucurbitaceae, prior knowledge established a link between the de-regulation of the homeotic ABC model genes and sex determinacy (Kater et al., 2001). Using NGS technology, Guo et al. (2010) further helped to understand the molecular mechanisms underlying sex determinacy in cucumber by comparing the transcriptomes of the two types of flowers (gynoecious and hermaphroditic). In *Quercus* spp., many studies have been conducted focusing on the morphology of reproductive organs (Kaul, 1985), life cycle (Ducousoo et al., 1993; Elena-Rossello et al., 1993), flowering process (Varela and Valdivieso, 1996), and embryogenesis (Stairs, 1964). However, molecular information regarding these mechanisms is still scarce. Ueno et al. (2010) described the first large-scale study of bud transcriptomes of the two main European white oak species (*Q. petraea* and *Q. robur*). Ueno et al. (2013) used the same pyrosequencing technology to characterize the bud transcriptomes of endo- and ecodormant sessile oak (*Q. petraea*). Recently, the transcriptome of *Q. suber* has been reported using 21 normalized cDNA libraries derived from multiple *Q. suber* tissues and organs, developmental stages and physiological conditions (Pereira-Leal et al., 2014). This work included two normalized libraries of *Q. suber* (male and female) flowers that could serve as a tool to mine genes in each flower type. However, data concerning differentially expressed genes during different developmental stages of each type of flower were still missing.

In the present work, with the aim of capturing the diversity of transcripts differentially expressed in male and female *Q. suber* flowers, inflorescences in different developmental stages were separately collected and non-normalized cDNA libraries were generated and sequenced using the 454 GS-FLX Titanium technology. This study provides a unique set of databases, invaluable for gene discovery, which might reveal the regulatory networks of sex-specific flower development of a non-model monoecious tree species.

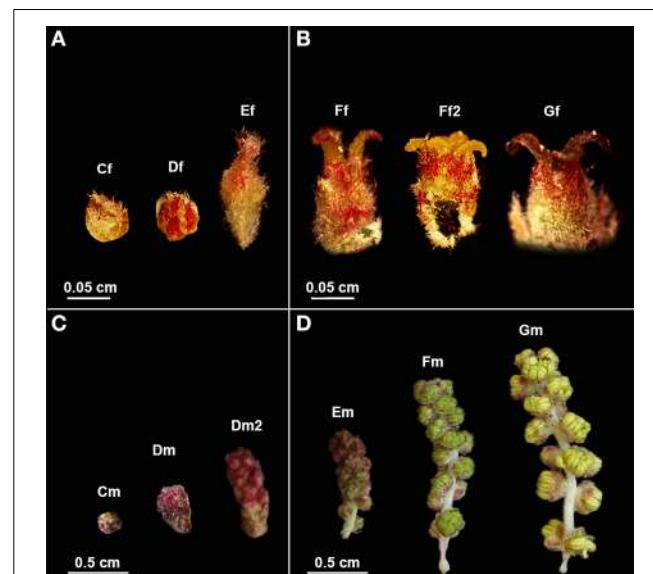
## MATERIALS AND METHODS

### PLANT MATERIAL

Six developmental stages of male and female cork oak flowers were collected from different trees in three different locations in Portugal (Lisbon, Porto, and Braga). The classification of the different phenological phases was based on visual observation, according to Varela and Valdivieso (1996) (Figure 1). Samples were harvested between the end of March and the beginning of June and were frozen in liquid nitrogen immediately after collection.

### RNA EXTRACTION AND cDNA PREPARATION

RNA was extracted from each sample using the RNAqueous® Kit (Ambion), following the manufacturer's instructions. The same amount of RNA was combined to create four specific RNA pools, two for female flowers (1F and 2F) and two for male flowers (1M and 2M), covering either early (1F or 1M) or late (2F or 2M) developmental stages. Pool 1F (Figure 1A) contained RNA from female buds enclosed by protective scales (Cf), female reddish buds with open scales (Df) and buds showing the elongation of the spike axe and the emergency of the first pair of flowers (Ef). The 2F pool (Figure 1B) included RNA



**FIGURE 1 |** *Quercus suber* female and male flowers in different developmental stages used in RNA-seq. (A) Early and (B) late stages of female flower development used in pools 1F and 2F, respectively. (C) Early and (D) late stages of male flower development used in pools 1M and 2M, respectively. (Cf) female bud enclosed by protective scales; (Df) female reddish bud with open scales; (Ef) elongation of the spike axe and the emergency of the first pair of flowers; (Ff) female flower showing distinct, erect, yellow stigmas with curved pinkish/brownish tips; (Ff2) flower with shining yellow and viscous pattern stigmas in clear divergent position; (Gf) female flower with closed stigmas that lost the receptivity, exhibiting a dark brown color. (Cm) catkin with red round shape due to the tight clustering of the flowers; (Dm) elongated cluster of male flowers; (Dm2) pendent catkin with some individualized flowers; (Em) male flowers with the anthers individualized; (Fm) flowers with individualized green/yellow anthers where pollen shedding begins; (Gm) catkin with male flowers in full anthesis.

from female flowers showing distinct, erect, yellow stigmas with curved pinkish/brownish tips (Ff), flowers with shining yellow and viscous pattern stigmas in clear divergent position (Ff2) and flowers with closed stigmas that lost the receptivity, exhibiting a dark brown color (Gf). The 1M pool (**Figure 1C**) comprised RNA from catkins with red round shape (Cm), elongated clustered male flowers (Dm), and pendent catkins with some individualized flowers (Dm2). The 2M pool (**Figure 1D**) included male flowers in which the anthers were becoming individualized (Em), flowers with individualized green/yellow anthers, prior to pollen shedding (Fm) and catkins with male flowers in full anthesis (pollen shedding occurring in half of the flowers with some anthers eventually empty) (Gm). RNA integrity was verified on an Agilent 2100 Bioanalyzer with the RNA 6000 Pico kit (Agilent Technologies) and the quantity assessed by fluorometry with the Quant-iT RiboGreen RNA kit (Invitrogen). A fraction of 2.0 µg of each pool of total RNA was used as starting material for cDNA synthesis using the MINT cDNA synthesis kit (Evrogen), where a strategy based on SMART double-stranded cDNA synthesis (Zhu et al., 2001) was applied. During the amplification of the poly RNA molecules, a known adapter sequence was introduced to both ends of the first strand of cDNA. The synthesis was also performed using a modified oligo-dT, containing a restriction site for *BsgI* that is needed to eliminate the tails, to minimize the interference of homopolymers during the 454-sequencing run. cDNA was quantified by fluorescence and sequenced in a full plate of 454 GS FLX Titanium system, according to the standard manufacturer's instructions (Roche-454 Life Sciences) at Biocant (Cantanhede, Portugal). Sequence reads were deposited in the NCBI Sequence Read Archive (SRA) under the accession number SRP044882.

#### SEQUENCE PROCESSING ASSEMBLY AND ANNOTATION

Prior to the assembly of sequences, the raw reads were processed in order to remove sequences with less than 100 nucleotides and low-quality regions. The ribosomal, mitochondrial and chloroplast reads were also identified and removed from the data set. The reads were then assembled into contigs using 454 Newbler 2.6 (Roche) with the default parameters (40 bp overlap and 90% identity).

The translation frame of contigs was assessed through BLASTx searches against Swissprot (*e*-value = 1e-6), and the corresponding amino acid sequences translated using an in-house script. Next, the contigs without translation were submitted to FrameDP software (Gouzy et al., 2009) and the remaining contigs were analyzed with ESTScan (Lottaz et al., 2003). Transcripts resulting from these two last sequence identification steps (FrameDP and ESTScan) were searched using BLASTp against the non-redundant NBCI (National Center for Biotechnology Information) database in order to translate the putative proteins.

The deduced aminoacid sequences were annotated using InterProScan (Hunter et al., 2009) and each was given the Gene ontology terms (GOs) (Ashburner et al., 2000).

To identify the differential gene expression between samples, the contigs were clustered using the CD-Hit 454 (Niu et al., 2010) application (90% similarity) in order to eliminate redundant sequences and generate reference contigs. After this step,

the contigs that codify non-redundant proteins were used as reference to map the reads. The mapping process was made using 454 Newbler Mapping 2.6 (Roche). The mapping results were quantified to obtain the number of reads from different samples and a contingency table with contig names was created using the number of reads per reference contig per sample. The contingency table was normalized at a 95 percentile using the MyRNA (Langmead et al., 2010) statistical analysis package and the differential gene expression was evaluated using a linear regression model based on a Gaussian distribution and taking into account only contigs with a minimum of eight mapped reads and FDR < 0.05. Differentially expressed were clustered using the Self-organizing Trees algorithm (SOTA), euclidean distance (Dopazo and Carazo, 1997; Herrero et al., 2001) and the default settings of the MeV: MultiExperiment Viewer program (<http://www.tm4.org/mev.html>).

#### qRT-PCR ANALYSIS

cDNA was synthesized from the same RNA samples used for the 454 sequencing, according to the manufacturer's instructions. cDNA was amplified using SsoFast™ EvaGreen® Supermix (Bio-Rad), 250 nM of each gene-specific primer (listed in **Supplementary Table S1**) and 1 µL of cDNA (1:100 dilution). Quantitative real-time PCR (qRT-PCR) reactions were performed in triplicates on the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). After an initial period of 3 min at 95°C, each of the 40 PCR cycles consisted of a denaturation step of 10 s at 95°C and an annealing/extension step of 10 s at the gene specific primer temperature. With each PCR reaction, a melting curve was obtained to check for amplification specificity and reaction contaminations, by heating the amplification products from 60°C to 95°C in 5 s intervals. Primer efficiency was analyzed with CFX Manager™ Software v3.1 (Bio-Rad), using the Livak calculation method for normalized expression (Livak and Schmittgen, 2001). Gene expression analysis was established based on three technical and biological replicates, and normalized with the reference gene *QsPP2AA3* (Marum et al., 2012).

#### RESULTS AND DISCUSSION

Due to the large number of reads attainable, the 454 DNA sequencing technology has great potential for discovering transcripts in non-model organisms. A prior study by Pereira-Leal et al. (2014) provided the first step toward the assembly of the monoecious tree *Q. suber* transcriptome using normalized libraries. In order to capture the diversity of transcripts differentially expressed during the development of female (F) and male (M) flowers, different developmental stages of flowers were collected covering either early (1F and 1M) or late (2F and 2M) developmental stages (**Figure 1**).

#### SEQUENCING AND ASSEMBLY OF *Q. SUBER* FLOWER TRANSCRIPTOME

Pyrosequencing resulted in 332,607 (1F), 312,282 (2F), 255,962 (1M), 270,871 (2M) raw reads for each library. After trimming, a total of 280,092 (1F), 252,024 (2F), 205,781 (1M), 224,275 (2M) high-quality reads were available with an average length of 264, 253, 269, and 270 bp, respectively (**Table 1**). Reads were

**Table 1 | Sequencing and annotation statistics of *Quercus suber* flower libraries.**

|   | <b>1F</b>   | <b>2F</b>   | <b>1M</b>   | <b>2M</b>   | <b>1F_1M_2F_2M</b> |
|---|-------------|-------------|-------------|-------------|--------------------|
| Number of raw reads                         | 332,607     | 312,282     | 255,962     | 270,871     |                    |
| Number of reads after trimming              | 280,092     | 252,024     | 205,781     | 224,275     | 837,163            |
| Average read length after trimming          | 264         | 253         | 269         | 270         | 263                |
| Number of contigs                           | 7565        | 6923        | 5267        | 5171        | 16,832             |
| Average contig length                       | 773         | 714         | 779         | 812         | 914                |
| Range of contig length                      | [60.. 3489] | [52.. 3394] | [36.. 3384] | [15.. 3392] | [16.. 3848]        |
| Number of translated contigs                | 7289        | 6600        | 5090        | 4981        | 16,152             |
| Peptide sequences with BLASTx matches       | 5723        | 5154        | 4083        | 3997        | 11,956             |
| Peptide sequences translated by FrameDP     | 2211        | 1907        | 1386        | 1398        | 6621               |
| Peptide sequences translated by ESTScan     | 108         | 117         | 116         | 114         | 297                |
| Total of amino acid sequences               | 8042        | 7178        | 5585        | 5509        | 18,874             |
| Peptide sequences with BLASTP matches       | 1294        | 1086        | 809         | 825         | 3372               |
| Amino acid sequence assigned InterPro terms | 5940        | 5312        | 4251        | 4217        | 12,698             |
| Amino acid sequence assigned to GO terms    | 4536        | 4056        | 3272        | 3269        | 9459               |

Individual libraries were generated from four specific RNA pools, two for female flowers (1F and 2F) and two for male flowers (1M and 2M), covering either early (1F and 1M) or late (2F and 2M) stages of flower development. The four individually EST projects were assembled into the 1F\_1M\_2F\_2M library. The libraries were assembled using 454 Newbler 2.6 (Roche) and annotated in a three-step process using BLASTx search, FrameDP and ESTScan using default parameters.

assembled into 7565, 6923, 5267, and 5171 contigs in 1F, 2F, 1M, and 2M, respectively, with an average contig length of 714 to 812 bp (**Table 1**).

High-quality reads from the four individual EST libraries were assembled together into a single library (1F\_2F\_1M\_2M), generating 837,163 high-quality reads with an average length of 263 bp that were assembled into 16,832 contigs with an average contig length of 914 bp (**Table 1**).

To annotate the *Q. suber* flower transcriptome, a three-step process (BLASTx search, FrameDP and ESTScan) was performed resulting in 16,152 (95.96%) translated contigs for the 1F\_2F\_1M\_2M library (**Table 1**). GO terms were then assigned, indicating a total of 9459 aminoacid sequences (50%) with at least one GO term (**Table 1**). Based on the GO annotations, cell, metabolic process and binding were the most abundant GO slims within the cellular component, molecular function, and biological process categories, respectively (**Figure 2**). Metabolic process (41.34%) and cellular process (30.40%) were the most highly represented groups within the biological process category, indicating that the floral tissues were undergoing extensive physiological activity in accordance with what was observed in *Arabidopsis thaliana* reproductive tissues (Hennig et al., 2004).

#### VALIDATION OF THE *Q. SUBER* FLOWER TRANSCRIPTOMES

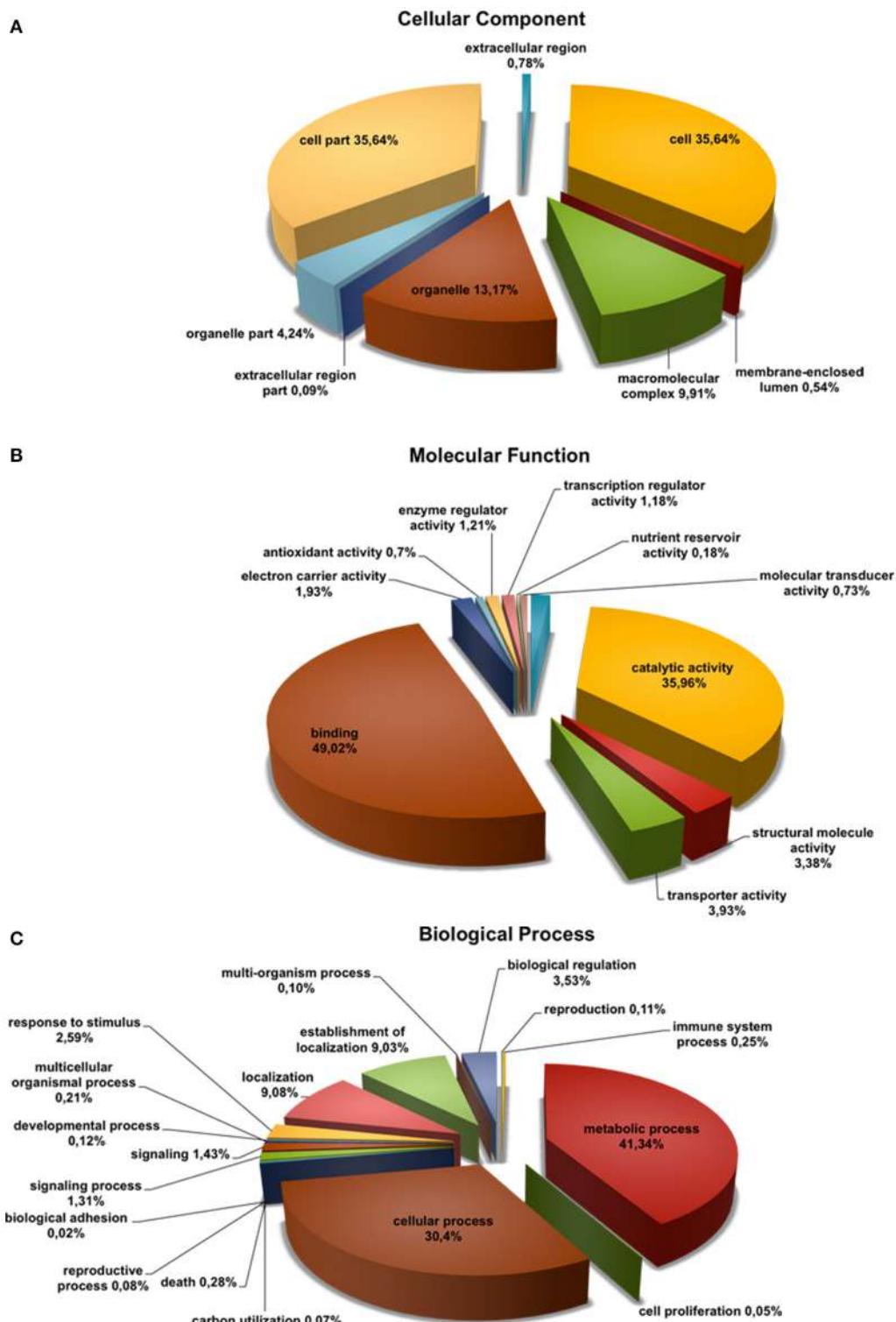
The *in silico* analysis of the transcriptomes allowed the identification of differences between the distinct developmental stages of male and female flowers. In order to validate the differences observed between male and female flower libraries, several contigs were identified by homology with functional important genes known to be involved in carpel or stamen development in model organisms. Homologs for *ABORTED MICROSPORES* (AMS, Xu et al., 2010), *LESS ADHERENT POLLEN3* (LAP3, Dobritsa et al., 2009), *LESS ADHESIVE POLLEN5* (LAP5, Dobritsa et al., 2010), and *LESS ADHESIVE POLLEN6* (LAP6, Dobritsa et al., 2010) were chosen as the male candidate genes

due to their involvement in pollen development. Homologs for the female candidate genes, *At4g27290* (Pagnussat et al., 2005), *CYTOCHROME P450 78A9* (CYP78A9, Ito and Meyerowitz, 2000), *POLYGALACTURONASE-1* (PG1, Tacken et al., 2010), and *STIGMA SPECIFIC1* (STIG1, Verhoeven et al., 2005) were selected based on their relevance in pollen recognition, stigma and transmitting tract development.

As expected, the *Q. suber* homologous genes presented differential expression ratios between male and female libraries (**Table 2**), and thus were considerate good candidates for qRT-PCR analysis. The qRT-PCR results confirmed that genes involved in pollen exine formation (LAP3, LAP5, and LAP6) and in the tapetum cell development (AMS) were more expressed in the early stages of male flower development (**Figure 3**), whereas genes involved in stigma-specific recognition (STIG1), in the recognition of pollen (*At4g27290*) and in fruit growth and development (CYP78A9, PG1) were more expressed in the female flowers. These results were in close agreement with the RNAseq data (**Table 2**) suggesting the reliability of the transcriptomic profiling data.

#### DIFFERENTIAL GENE EXPRESSION BETWEEN *Q. SUBER* FLOWER-TYPE LIBRARIES

In order to identify exclusive transcripts of early and late developmental stages of female and male flower development, the assembly of the four non-normalized libraries was analyzed. The analysis showed that there were 230 unique contigs for the early (1F) and 214 contigs unique for the late (2F) stages of female flower development (**Figure 4A**). The 1F unique contigs might correspond to genes controlling early flower development, whereas the 2F unique contigs might be associated with stigma maturation, ovule development and fertilization. Accordingly, there were 198 contigs unique in the early stages of male flower development (1M), most probably involved in early stages of anther development and 327 contigs specific for the late stages



**FIGURE 2 | Functional classification of *Quercus suber* unigenes.** Four EST projects were generated from four-specific RNA pools, two for female flowers (1F and 2F) and two for male flowers (1M and 2M), covering either early (1F and 1M) or the late (2F and 2M) developmental stages. The four individually EST projects were assembled into the 1F\_1M\_2F\_2M library and

the deduced aminoacid sequences of this library were annotated using InterProScan. The Gene Ontology terms (GOs) for each translated amino acid sequence were used to classify the transcript products within the category of (A) cellular component, (B) molecular function, and (C) biological process sub-ontologies.

**Table 2 | Candidate genes that were selected to validate the transcriptional levels determined by RNAseq results.**

| Candidate genes                   | Gene accession | 1F | 2F  | 1M  | 2M |
|-----------------------------------|----------------|----|-----|-----|----|
| • At4g27290                       | QSP078589.0    | 29 | 14  | 0   | 1  |
| • CYTOCHROME P450 78A9            | QSP091316.0    | 70 | 70  | 0   | 0  |
| • POLYGALACTURO-NASE1             | QS094531.0     | 19 | 337 | 0   | 0  |
| • STIGMA SPECIFIC1                | QS121989.0     | 5  | 34  | 0   | 0  |
| <hr/>                             |                |    |     |     |    |
| * ABORTED MICROSPORES             | QS049646.0     | 0  | 0   | 38  | 4  |
| * LESS ADHERENT POLLEN3           | QS049611.0     | 1  | 2   | 170 | 46 |
| * LESS ADHESIVE POLLEN5           | QS003695.0     | 0  | 0   | 566 | 6  |
| * LESS ADHERENT POLLEN6           | QS039918.0     | 0  | 0   | 924 | 49 |
| <hr/>                             |                |    |     |     |    |
| PROTEIN PHOSPHATASE 2A SUBUNIT A3 | QS092015.0     | 55 | 71  | 57  | 65 |

The number of ESTs represents their distribution in the female and male *Quercus suber* combined flower library, covering either early (1F and 1M) or late (2F and 2M) stages of flower development. The selected genes were identified by homology with functional important genes known to be involved in carpel or stamen development.

• Female candidate genes.

\* Male candidate genes.

PROTEIN PHOSPHATASE 2A SUBUNIT A3 was chosen as the reference gene. Gene accessions according to CorkOak database ([www.corkoakdb.org](http://www.corkoakdb.org)).

(2M) that could be indicative of genes controlling pollen development and maturation (Figure 4A). A normalization cut-off of eight reads at the 95th percentile was applied, resulting in 3760 differentially expressed genes (19.9%) for the 1F\_2F\_1M\_2M transcriptome. Differentially expressed genes were then clustered into different groups according to their expression profile similarity (Figure 4). Groups of genes that were either unique (Figure 4B) or significantly more expressed in the male samples (Figures 4C,D) were identified. At least 430 differentially expressed genes were predominantly expressed in the last stages of male flower development (Figures 4E,F), whereas 239 genes were absent from this stage and present in all the other libraries (Figure 4G). We also found genes that were more expressed in the early stages of both male and female flower development (Figure 4H). A group of genes (115) was more expressed in both female libraries (Figure 4I), whereas 217 genes appear to be preferentially expressed in late stages of female flower development (Figure 4J).

Out of the 3760 differentially expressed genes, a GO term was assigned to 1797 female and to 745 male transcripts. No significant differences were found between male and female GO categories apart from the molecular and cellular functions. In the former, 16% of the female GO terms were assigned to protein binding in contrast with the male GO terms (7%), whereas in

the latter, 8% of the female GO terms were assigned to the nucleus and just 1% of the male were appointed to the nucleus (Figure S1).

#### POTENTIAL ALLERGEN GENES PRESENT IN *Q. SUBER* LIBRARIES

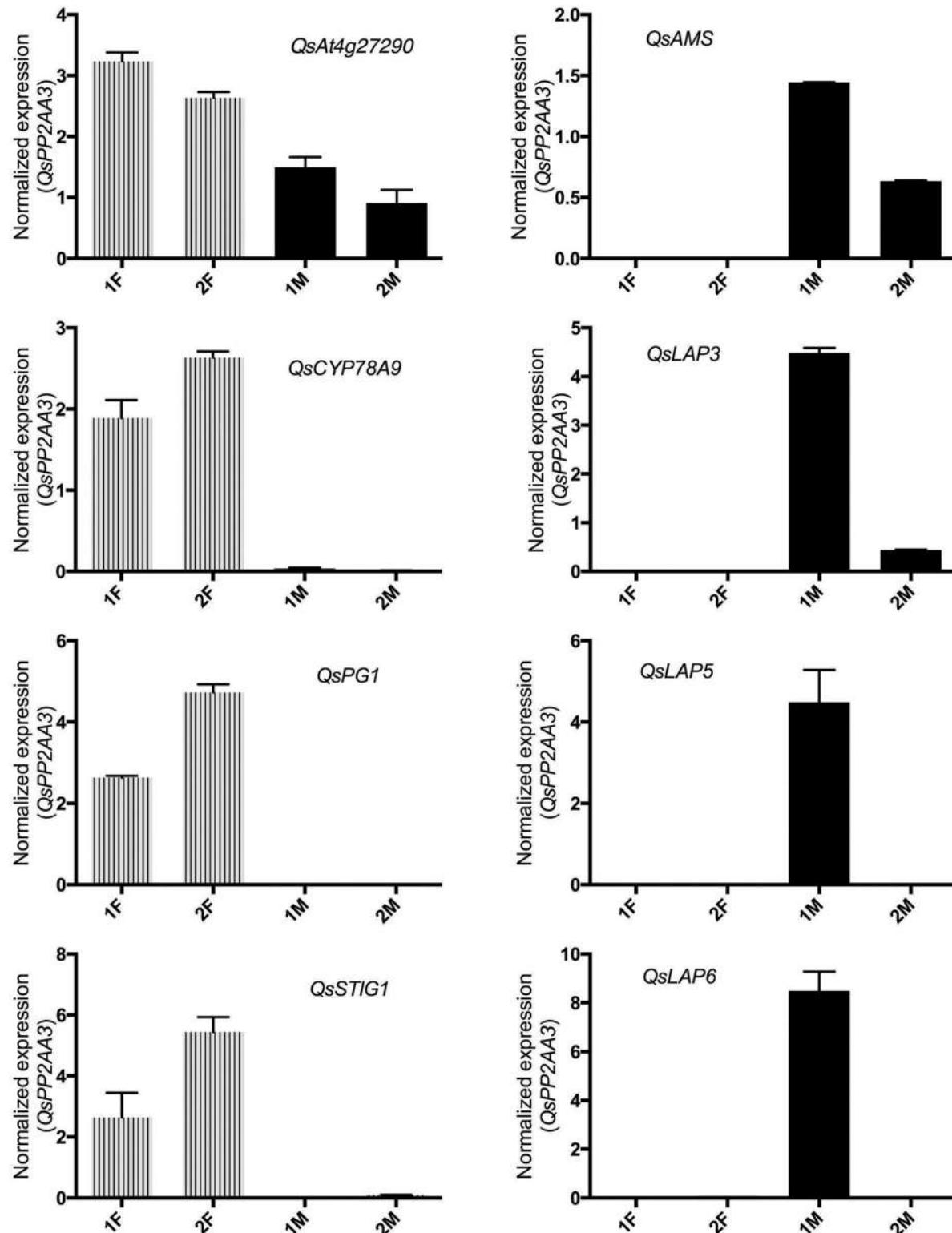
During spring, Fagales tree species produce and release large amounts of pollen. In Southern Europe, pollen from these plants and other anemophilous trees, like *Platanus acerifolia* and *Olea europaea*, has been proved to elicit allergic diseases, such as pollinosis rhinitis/rhino conjunctivitis (D'Amato et al., 2007; Esteve et al., 2012). The official site for the systematic allergen nomenclature (<http://www.allergen.org>), that was approved by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Sub-committee, lists 263 allergenic proteins to the taxonomic group Plantae Magnoliopsida. Among these 263 allergens, 34 were associated with the Fagales order and only one (Quea1) was related to the genus *Quercus*.

In order to identify transcripts encoding potential allergens in cork oak, blast searches were carried on the 1F\_1M\_2F\_2M transcriptome against the proteins reported as allergenic and included in the WHO-IUIS list. This analysis revealed several potential orthologs for genes coding for potential allergens in *Q. alba*, *Betula pendula*, *Corylus avellana*, *O. europaea*, *Hevea brasiliensis*, and *P. acerifolia* (Table 3). Of major interest was the identification of a potential ortholog of Quea1, which is the major allergen of *Quercus alba* (Wallner et al., 2009). As expected, almost all the potential orthologs of allergen genes were highly expressed in the male libraries (Table 3).

#### *Q. SUBER* MOST DIFFERENTIALLY EXPRESSED GENES BETWEEN FEMALE AND MALE TISSUES

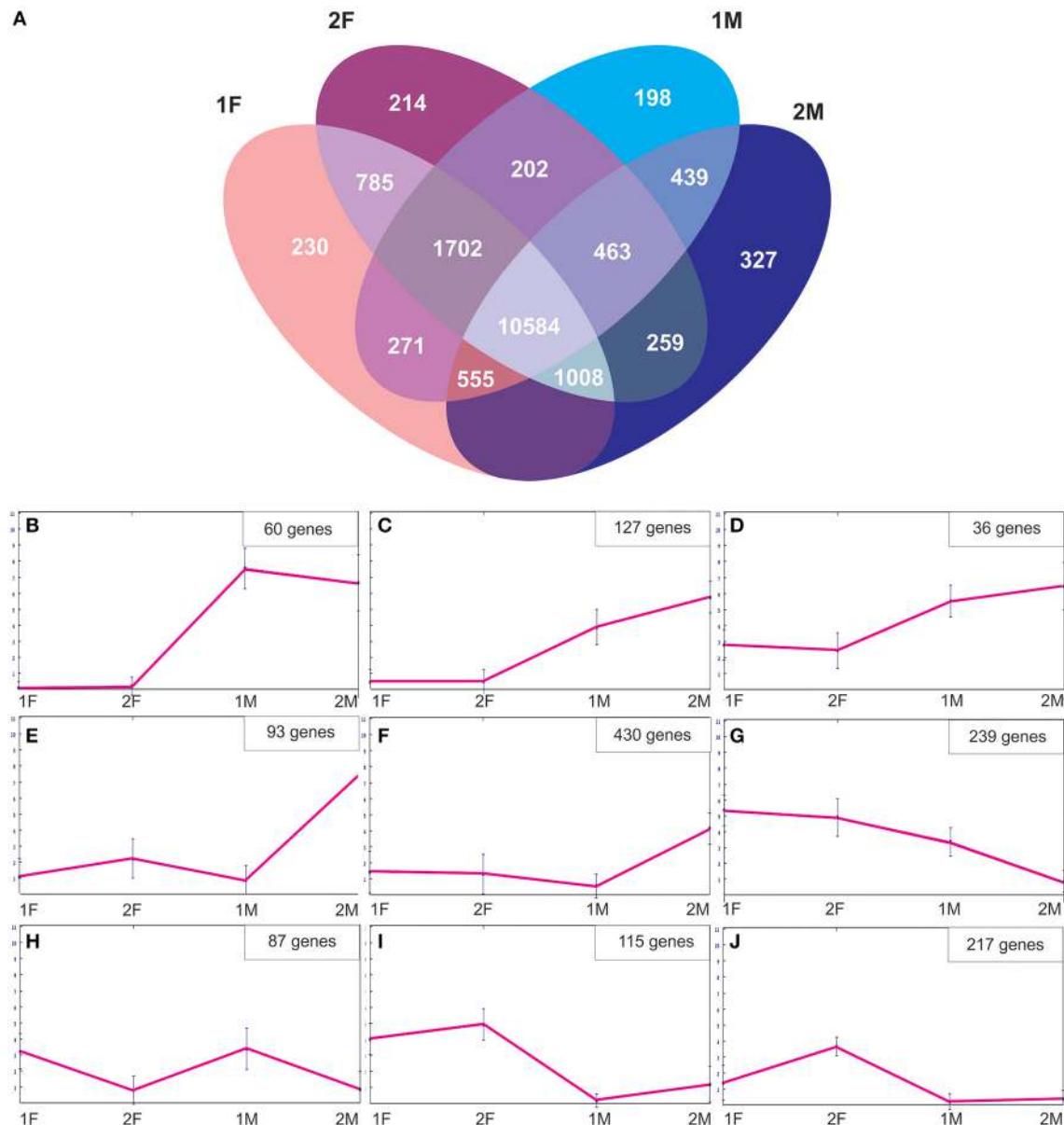
The ten genes most differentially expressed in both male and female tissues were identified by establishing a ratio between male and female EST counts (Table 4). Concerning the differentially expressed genes more represented in female flowers, we identified a homolog for POLYGALACTURONASE-1 that is comparatively 356 times more expressed in female tissues. Interestingly, several studies report the involvement of polygalacturonases associated genes to both carpel (Ogawa et al., 2009) and pollen development (Allen and Lonsdale, 1993; Tebbutt et al., 1994; Rhee et al., 2003). QsENDO-BETA-1,3-1,4 GLUCANASE, a member of the glycoside hydrolase family, is 199 times more expressed in female samples. In *Populus trichocarpa*, a member of this family, *PtrCel9A6*, is tightly involved in sexual determinism (Yu et al., 2013). Overexpression of *PtrCel9A6* in *A. thaliana* resulted in male sterility due to defects in anther dehiscence (Yu et al., 2013). It is possible that the QsENDO-BETA-1,3-1,4 GLUCANASE might have a similar function by inhibiting the development of male structures in female flowers.

A CYTOCHROME P450 transcript (QsCYTOCHROME P450 78A3) was also highly represented in the female samples with a possible role in carpel gametophyte and sporophyte development as it was shown for homologous genes in other species (Ito and Meyerowitz, 2000; Chakrabarti et al., 2013). Ito and Meyerowitz (2000) identified AtCYP450 78A9, a gene that when overexpressed in *A. thaliana* results in altered fruit and seed.



**FIGURE 3 | Relative expression of differentially expressed male and female genes chosen to validate RNAseq results.** *QsAMS*, *QsLAP3*, *QsLAP5*, *QsLAP6* were selected as male candidate genes, whereas *QsAt4g27290*, *QsCYP78A9*, *QsPG1*, and *QsSTIG1* as female candidate genes. Transcript abundance was determined using qPCR, and

normalized to *QsPP2AA3* using cDNA synthesized from distinct pools of RNA covering either early (1F and 1M) or late (2F and 2M) stages of male or female flower development. Reactions were performed in three biological and technical replicates. Error bars indicate standard deviation (SD).



**FIGURE 4 | Description of the *Quercus suber* unique and differentially expressed genes. (A)** Venn diagram indicating the number of exclusive and shared transcripts of early and late developmental stages of *Quercus suber* flower. Four EST projects were generated from four-specific RNA pools, two for female flowers (1F and 2F) and two for male flowers (1M and 2M), covering either early (1F and 1M) or the late (2F and 2M) developmental

stages. The four individually EST projects were assembled into the 1F\_1M\_2F\_2M library and the exclusive transcripts were identified using the Venny application (Oliveros, 2007). **(B–J)** Differentially expressed genes were clustered using the Self-organizing Trees algorithm (SOTA), euclidean distance (Dopazo and Carazo, 1997; Herrero et al., 2001) and the default settings of the MeV, MultiExperiment Viewer program (<http://www.tm4.org/mev.html>).

Another member of this family is *SIKLUH*, which controls not only plant architecture but also fruit mass and ripening in tomato (Chakrabarti et al., 2013). We also identified a homolog for *1-AMINOCYCLOPROPANE-1-CARBOXYLATE OXIDASE 66*, that belongs to a family of genes that has been associated to ethylene biosynthesis (Barry and Giovannoni, 2007). Several *RECEPTOR-LIKE PROTEIN KINASES (RLPK)* have been linked to key aspects of plant development: the brassinosteroids signaling pathway (Schumacher and Chory, 2000), meristem maintenance

(Clark, 2001), or pollen-pistil interaction (McCubbin and Kao, 2000). Thus, a 65 times more expressed *QsRLPK* transcript in female flowers might have an important role in carpel development.

Within the group of genes with the highest differential expression in male flowers, there were three unknown genes without a significant BLAST hit. These three genes may be specific to *Q. suber* and pivotal to anther differentiation and development in this species. Two highly represented *QsCHALCONE SYNTHASE*

**Table 3 | Quercus suber potential allergens in flower transcriptome.**

| Gene designation   | Gene accession | ESTs 1F+2F | ESTs 1M+2M | Allergen | Closest species homolog    | InterProScan description                  |
|--------------------|----------------|------------|------------|----------|----------------------------|---|
| <i>QsQUEA1</i>     | QS091157.0     | 11         | 367        | Quea1    | <i>Quercus alba</i>        | Pollen allergen Bet v 1                   |
| <i>QsBETV2</i>     | QS034447.0     | 2          | 22         | Betv2    | <i>Betula pendula</i>      | Profilin                                  |
| <i>QsBETV3</i>     | QSP068142.0    | 4          | 26         | Betv3    | <i>Betula pendula</i>      | Calcium-binding allergen Bet v 3          |
| <i>QsBETV6</i>     | QSP015348.0    | 40         | 100        | Betv6    | <i>Betula pendula</i>      | Isoflavone reductase homolog              |
| <i>QsCyP</i>       | QS017405.0     | 274        | 183        | Betv7    | <i>Betula pendula</i>      | Cyclophilin                               |
| <i>QsBETV4</i>     | QS115544.0     | 0          | 65         | Betv4    | <i>Betula pendula</i>      | Polyclacin Bet v 4                        |
| <i>QsBIP</i>       | QS126407.0     | 380        | 703        | Cora10   | <i>Corylus avellana</i>    | Luminal binding protein                   |
| <i>QsOLE1</i>      | QS069793.0     | 1          | 89         | Olee1    | <i>Olea europaea</i>       | Ole e1-like protein                       |
| <i>QsOLE9</i>      | QS095617.0     | 9          | 248        | Olee9    | <i>Olea europaea</i>       | $\beta$ -1.3 Glucanase                    |
| <i>QsPME</i>       | QS101292.0     | 1          | 72         | Olee11   | <i>Olea europaea</i>       | Pectin methyl esterase                    |
| <i>QsSOD_CU_ZN</i> | QS057690.0     | 115        | 96         | Olee5    | <i>Olea europaea</i>       | Superoxide dismutase. copper/zinc binding |
| <i>QsHEV1</i>      | QS150670.0     | 12         | 11         | Hevb6    | <i>Hevea brasiliensis</i>  | Pro-hevein                                |
| <i>QsPLAA2</i>     | QS060812.0     | 5          | 311        | Plaa2    | <i>Platanus acerifolia</i> | Exopolygalacturonase                      |
| <i>QsnsLTP</i>     | QS106891.0     | 1097       | 3077       | Plaa3    | <i>Platanus acerifolia</i> | Non-specific lipid-transfer protein       |

The number of ESTs represents their distribution in the female (1F+2F) and male (1M+2M) *Q. suber* combined flower library. Selected genes were identified by homology with functional important allergen genes included in the WHO-IUIS allergen list. The deduced aminoacid sequences of these genes were annotated using InterProScan. Gene accessions according to CorkOak database ([www.corkoakdb.org](http://www.corkoakdb.org)).

A genes were identified as male unique transcripts. A suppressor mutant of the *CHALCONE SYNTHASE A* homolog gene in *Petunia* generates viable pollen, however, pollen germination and tube growth are severely affected (Taylor and Jorgensen, 1992), which indicates that this gene is essential for proper anther development. *CHALCONE SYNTHASE A* associated genes are also known to be involved in the metabolic pathway that leads to the production of flavonoids and anthocyanin pigments in several species (Winkel-Shirley, 2002). Thus, it is possible that the coloration on the anthers might be due to the action of the highly expressed *QsCHALCONE SYNTHASE A* genes. A *Qs4-COURAMATE-CoA LIGASE-LIKE 1* gene that is related to the ACOS genes was also identified. These genes have been associated with proper pollen development (De Azevedo Souza et al., 2009). The *DEFECTIVE IN ANTER DEHISCENCE1 (DAD1)* is a lipase-like gene involved in pollen development (Ishiguro et al., 2001). In *A. thaliana dad1* shows defects in anther dehiscence, pollen maturation, and flower opening (Ishiguro et al., 2001). *QsLIPASE-LIKE* might also have a similar function.

To investigate whether these genes were flower specific, available root, leaf, bud and fruit libraries (Pereira-Leal et al., 2014) were used. Interestingly, all the male-associated genes are exclusive to the male flower except for *QsLIPASE-like*, which is expressed in almost all the other tissues with the exception of the female flower and leaves (Table 4). The majority of the female-associated genes analyzed are not flower specific. Interestingly, there is one gene that is present only in the buds, flowers and fruits (*QsNON-SPECIFIC LIPID-TRANSFER PROTEIN*), suggesting a putative role in the female reproductive determinism. At least two genes are expressed in all the organs (*QsPOLYGALACTURONASE-1* and *QsRECEPTOR-LIKE PROTEIN KINASE*) except for the male flowers. Considering that these genes are expressed in all *Q. suber* tissues analyzed

and absent from the male flowers might be indicative that these flowers go through a very distinctive developmental programme, or that the mentioned genes expression could be detrimental to proper male flower development. It will be very interesting to perform functional studies to analyse the involvement of the aforementioned genes in plant reproduction or flower development in *Q. suber* and other flowering species.

#### TRANSCRIPTION FACTORS DIFFERENTIALLY EXPRESSED IN FEMALE AND MALE FLOWERS

Differential expression of transcription factors (TF) has a pivotal role in the control of mechanisms that direct organ development (Latchman, 1997). Based on the analysis of the different TF groups, a group of biologically interesting genes that are sex-specific or differentially expressed in each library was identified (Table 5).

#### Zinc-finger TF family

The zinc-finger family of genes is an example of diversification in the Plant Kingdom and consists of a large number of proteins that are further classified into distinct subfamilies (Takatsuji, 1999). Among these, the C2H2-type and B-box zinc finger proteins constitute one of the largest families of transcriptional regulators in plants (Ciftci-Yilmaz and Mittler, 2008). Proteins containing zinc finger domains which play important roles in eukaryotic cells, regulating different signal transduction pathways and controlling processes such as development (Colasanti et al., 1998), homeostasis (Devaiah et al., 2007) and abiotic stress responses (Rizhsky et al., 2004; Sakamoto et al., 2004; Davletova et al., 2005). Some floral regulators contain a zinc-finger domain such as *CONSTANS (CO)*, which has been linked to floral induction in several species by integrating the circadian clock and light signals (Putterill et al., 1995;

**Table 4 | Quercus suber most differentially expressed genes in the flower transcriptome.**

|   | <b>Gene designation</b>                                   | <b>Gene accession</b> | <b>Organ</b>   | <b>ESTs 1F+2F</b> | <b>ESTs 1M+2M</b> | <b>InterProScan description</b>                               |
|---|---|-----------------------|----------------|-------------------|-------------------|---|
| <b>More expressed in Female Flowers</b> | <i>QsPOLYGALACTURONASE-1</i>                              | QS094531.0            | R, L, B, F, Fr | 356               | 1                 | BURP  |
|   | <i>QsENDO-BETA-1,3-1,4 GLUCANASE</i>                      | QS112778.0            | R, L, F, Fr    | 397               | 2                 | Glycoside hydrolase, family 17                                |
|   | <i>QsNON-SPECIFIC LIPID-TRANSFER PROTEIN</i>              | QS158755.0            | B, F, Fr       | 162               | 0                 | Plant lipid transfer protein/Par allergen                     |
|   | <i>QsCYTOCHROME P450 78A3</i>                             | QS073368.0            | R, F, Fr       | 140               | 0                 | Cytochrome P450   |
|   | <i>QsLEUCINE-RICH REPEAT EXTENSIN-LIKE PROTEIN3</i>       | QS109035.0            | R, F, Fr       | 273               | 2                 | Unintegrated  |
|   | <i>QsENDOCHITINASE PR4</i>                                | QS078296.0            | R, B, F, Fr    | 2553              | 22                | Glycoside hydrolase, family 19, catalytic                     |
|   | <i>QsISOFLAVONE REDUCTASE HOMOLOG P3</i>                  | QS124429.0            | R, L, F, Fr    | 86                | 1                 | NmrA-like   |
|   | <i>Qs1-AMINOCYCLOPROPANE-1-CARBOXYLATE OX. HOMOLOG 66</i> | QS023535.0            | L, F, Fr       | 78                | 1                 | Oxoglutarate/iron-dependent oxygenase                         |
|   | <i>QsACID PHOSPHATASE 1</i>                               | QS083994.0            | L, B, F, Fr    | 203               | 3                 | Acid phosphatase (Class B)                                    |
|   | <i>QsRECEPTOR-LIKE PROTEIN KINASE</i>                     | QS006331.0            | R, L, B, F, Fr | 65                | 1                 | Protein kinase, core  |
| <b>More expressed in Male Flowers</b>   | <i>QsUNKNOWN1</i>   | QS082953.0            | F              | 0                 | 1376              | PLT protein/seed storage/trypsin- $\alpha$ -amylase inhibitor |
|   | <i>QsKDEL-TAILED CYSTEINE ENDOPEPTIDASE</i>               | QS035274.0            | F              | 1                 | 1122              | Peptidase, cysteine peptidase active site                     |
|   | <i>QsCHALCONE SYNTHASE A1</i>                             | QS039918.0            | F              | 0                 | 973               | Chalcone/stilbene synthase, N-terminal                        |
|   | <i>QsUNKNOWN2</i>   | QS047555.0            | F              | 0                 | 759               | Bifunctional inhibitor/PLT protein/seed storage               |
|   | <i>Qs4-COURAMATE-CoA LIGASE-LIKE 1</i>                    | QS024768.0            | F              | 0                 | 712               | AMP-dependent synthetase/ligase                               |
|   | <i>QsBETA-1,3-GALACTOSYLTRANSFERASE 8</i>                 | QS087345.0            | F              | 0                 | 580               | Glycosyl transferase, family 31                               |
|   | <i>QsCHALCONE SYNTHASE A2</i>                             | QS003695.0            | F              | 0                 | 572               | Chalcone/stilbene synthase, N-terminal                        |
|   | <i>QsANTHRANILATE N-BENZOYLTRANSFERASE PROTEIN 2</i>      | QS009481.0            | F              | 0                 | 533               | Transferase   |
|   | <i>QsUNKNOWN3</i>   | (?)                   | F              | 0                 | 522               | Unintegrated  |
|   | <i>QsLIPASE-LIKE</i>                                      | QS011358.0            | R, B, F, Fr    | 0                 | 441               | Lipase, GDXG, active site                                     |

The 10 genes most differentially expressed in male or female tissues were identified by establishing a ratio between male and female EST count in female (1F+2F) and male (1M+2M) *Q. suber* combined flower library. The deduced aminoacid sequences of these genes were annotated using InterProScan. Gene accessions according to the CorkOak database [www.corkoakdb.org; (?) not present]. Transcript distribution of the most differentially expressed genes in the *Q. suber* organs was obtained by blasting the genes sequences against the organ libraries made available by Pereira-Leal et al. (2014). R, root; L, leaf; B, bud; F, flower; Fr, Fruit.

Böhnenius et al., 2006). A clear CO homolog was not identified, as expected for the type of biological sample (flowers) used in the RNAseq. However, four CO-like transcripts differentially expressed in female flowers as compared to male flowers were identified. Of these, the *QsCONSTANS-LIKE 9* (*QsCOL9*) homolog was five times more expressed in female flowers. In *A. thaliana*, *COL9* delays flowering by reducing expression of CO and *FLOWERING LOCUS T* in leaves (Cheng and Wang, 2005). Its high level of expression in female tissues, particularly in early stages of the reproductive program, could suggest a novel function yet undisclosed. A homolog for the *A. thaliana* zinc-finger protein *TRANSPARENT TESTA 1* (*TT1*) was also seven times more expressed in female than in male *Q. suber* flowers. In melon, *CmWIP1* (a homolog of *TT1*) has a masculinizing effect by indirect repression of the ethylene driven *CmACS-7* gene (Boualem et al., 2008). *CmWIP1* needs to be epigenetically silenced to generate a fully functional female flower (Martin et al., 2009). The expression of *QsTT1* in the female flowers might point out the differences between developmental programs that give rise to sexual dimorphism in monoecious and dioecious/hermaphrodite species.

#### Basic helix-loop-helix (bHLH) TF family

The bHLH family encloses one of the largest groups of plants TF (Heim et al., 2003). These TF are involved in, among others, wound and stress responses (De Pater et al., 1997; Smolen et al., 2002; Chinnusamy et al., 2003; Kiribuchi et al., 2004), hormonal regulation (Abe et al., 1997; Friedrichsen et al., 2002) stigma and anther development, and fruit development and differentiation (Rajani and Sundaresan, 2001; Liljegren et al., 2004; Szécsi et al., 2006; Gremski et al., 2007). From the differentially expressed bHLH genes in *Q. suber* floral libraries, three were up-regulated in male flowers. One was the homolog of *ABORTED MICROSPORES*, a gene essential to the development of pollen (Sorensen et al., 2003; Xu et al., 2010). The other two *QsbHLH* are homologs to genes associated with iron deficiency (Wang et al., 2013). In female flowers, nine *Q. suber* transcripts were significantly more expressed, with one transcript being exclusive to the female samples (*QsBR ENHANCED EXPRESSION 1*). In *A. thaliana*, *BEE1* is involved in the brassinosteroids signaling and associated with the development of the reproductive tract (Crawford and Yanofsky, 2011). Homologs for *GLABRA3*, *MYC2*, *INDUCER OF CBF*

**Table 5 | Transcription factors differentially expressed in male and female flowers of *Quercus suber*.**

| Gene designation                              | Gene accession | Organ          | ESTs<br>1F+2F | ESTs<br>1M+2M | InterProScan description         |
|---|----------------|----------------|---------------|---------------|----------------------------------|
| <i>QsCONSTANS LIKE 9</i>                      | QS124225.0     | F,L            | 34            | 7             | Zinc finger, B-box               |
| <i>QsCONSTANS LIKE 4</i>                      | QS050989.0     | R, L, B, F, Fr | 270           | 68            | Zinc finger, B-box               |
| <i>QsCONSTANS LIKE 5</i>                      | QS116713.0     | R, L, B, F, Fr | 82            | 35            | Zinc finger, B-box               |
| <i>QsTRANSPARENT TESTA 1</i>                  | QS064811.0     | R, L, B, F, Fr | 38            | 4             | Zinc finger, C2H2-type           |
| <i>QsABORTED MICROSPORES</i>                  | QS049646.0     | F              | 0             | 42            | bHLH dimerization region         |
| <i>QsBRASSINOSTEROID ENHANCED EXPRESSION1</i> | QS048556.0     | F              | 39            | 0             | bHLH dimerization region         |
| <i>QsINDUCER OF CBF EXPRESSION1</i>           | QS129356.0     | R, L, B, F, Fr | 38            | 4             | bHLH dimerization region         |
| <i>QsGLABRA3</i>                              | QS150704.0     | B, F Fr        | 31            | 5             | bHLH dimerization region         |
| <i>QsMYC2</i>                                 | QS073124.0     | R, L, B, F, Fr | 114           | 26            | bHLH dimerization region         |
| <i>QsIAA-LEUCINE RESISTANT3</i>               | QS154009.0     | R, L, B, F, Fr | 83            | 39            | bHLH dimerization region         |
| <i>QsPERIANTHIA</i>                           | QS095157.0     | F              | 39            | 15            | Basic-leucine zipper (bZIP)      |
| <i>QsCUC1</i>                                 | QS061789.0     | R, F, Fr       | 33            | 6             | No apical meristem (NAM) protein |
| <i>QsCUC2</i>                                 | QS009784.0     | R, F, Fr       | 33            | 7             | No apical meristem (NAM) protein |
| <i>QsIAA27</i>                                | QS075617.0     | R, L, B, F, Fr | 110           | 17            | AUX/IAA protein                  |
| <i>QsIAA9</i>                                 | QS117343.0     | R, L, B, F, Fr | 160           | 97            | AUX/IAA protein                  |
| <i>QsPIN1</i>                                 | QS117199.0     | R, B, F, Fr    | 20            | 1             | Auxin efflux carrier             |
| <i>QsEIN3</i>                                 | QS119163.0     | R, F           | 18            | 1             | Ethylene insensitive 3           |
| <i>QsMYB33</i>                                | QS020061.0     | F              | 0             | 13            | Homeodomain-like                 |
| <i>QsSUPPRESSOR OF CONSTANS1</i>              | QS149164.0     | R, L, B, F Fr  | 25            | 2             | Transcription factor, MADS-box   |
| <i>QsAP1</i>                                  | QS003005.0     | F Fr           | 68            | 8             | Transcription factor, MADS-box   |
| <i>QsFRUITFULL</i>                            | QS029922.0     | F Fr           | 53            | 7             | Transcription factor, MADS-box   |
| <i>QsSHORT VEGETATIVE PHASE 1</i>             | QS116365.0     | B, F Fr        | 36            | 6             | Transcription factor, MADS-box   |
| <i>QsSHORT VEGETATIVE PHASE 2</i>             | QS055926.0     | B, F Fr        | 9             | 23            | Transcription factor, MADS-box   |

Based on the analysis of the different transcription factor groups, a number of biological interesting genes were identified and their expression level was determined according to the number of ESTs in female (1F+2F) and male (1M+2M) *Q. suber* combined flower library Gene accessions according to CorkOak database ([www.corkoakdb.org](http://www.corkoakdb.org)). Transcript distribution of the most differentially expressed genes in the *Q. suber* organs was obtained by blasting the genes sequences against the organ libraries made available by Pereira-Leal et al. (2014). R, root; L, leaf; B, bud; F, flower; Fr, fruit.

*EXPRESSION1*, and *IAA LEUCINE RESISTANT3* were also up-regulated in female flowers. These genes are involved in hormonal regulation, cold acclimation, cell fate and double fertilization (Bernhardt et al., 2003; Chinnusamy et al., 2003; Yadav et al., 2005; Rampey et al., 2006; Dombrecht et al., 2007). However, there were three bHLH transcripts differentially expressed whose function is yet to be characterized in other species, making them good candidates at least to be involved in carpel development.

#### Basic Leucine Zipper (bZIP) TF Family

The bZIP TFs regulate diverse biological processes in plants including flower development (Jakoby et al., 2002). Eight *Q. suber* bZIP associated genes were differentially expressed in female samples. Among them is the homolog of *VIP1*, an *A. thaliana* bZIP TF that regulates pathogen responses and rehydration responses (Tzfira et al., 2001; Tsugama et al., 2012). Another bZIP TF up-regulated in female samples is the homolog of *PERIANTHIA* (*PAN*), a gene involved in flower development in *A. thaliana* by altering floral organ number and initiation pattern (Running and Meyerowitz, 1996; Wynn et al., 2014). *PAN* is also involved in the activation of the C-class MADS box protein *AGAMOUS* (*AG*), a gene essential for carpel development (Das et al., 2009; Maier et al., 2009).

#### CUC/NAM TF Family

Data also showed several *CUP-SHAPED COTYLEDON/NO APICAL MERISTEM* (*CUC/NAM*) genes highly represented in female flowers and the majority of them is differentially expressed. The *CUC/NAM* family encloses genes that control boundary formation and lateral organ separation, which are critical for proper leaf and floral patterning (Aida et al., 1997; Vroemen et al., 2003). A *CUC/NAM* gene in *Medicago trunculata* is needed for proper regulation of floral organ identity (Cheng et al., 2012). Also, in *A. thaliana*, *Petunia* and rice, mutants for *CUC/NAM* genes lead to the fusion of the cotyledons and some floral organs, as well as severe defects of the primary apical meristem (Souer et al., 1996; Aida et al., 1997; Mao et al., 2007). Out of *QsCUC/NAM* genes that are differentially expressed, homologs for *CUC1* and *CUC2* genes were up-regulated in female flowers.

#### MADS-Box TF Family

The MADS family of TF include a group of genes that play prominent roles in plant development (Smacznak et al., 2012). Particularly, MADS TFs were found to be crucial for proper flower development in several species across the angiosperm lineage (reviewed in Theissen and Melzer, 2007). According to the canonical ABC model, which explains how homeotic genes control flower identity, stamens are formed by the activity of the

B-Class and a C-class gene, whereas the same C-class is responsible for carpel development (Coen and Meyerowitz, 1991). As expected, B-class genes were differentially expressed in the male flowers (*QsAPETALA3* and *QsPISTILLATA*), and a similar level of expression of *QsAGAMOUS* (C-class gene) in both male and female libraries. The E-function genes (*SEPALLATA1-4*) that act as cadastral genes for proper organ development and identity (Pelaz et al., 2000) were also identified in both libraries but there was no sex differential expression. Several other homologs for MADS genes (*QsAPETALA1*, *QsFRUITFUL*, *QsFLOWERING LOCUS*, or *QsSUPPRESSOR OF CONSTANS1*) that influence flowering in model and non-model species were also identified both in female and male libraries. Two transcripts similar to the *SHORT VEGETATIVE PHASE* gene were identified. It was interesting to detect a *QsSVP* gene differentially expressed in the female libraries and another in the male libraries. Genes of the *SVP* lineage in peach (*dormancy-associated genes, DAM*) are involved in growth cessation, bud set and break (Li et al., 2009).

#### HORMONE RELATED GENES DIFFERENTIALLY EXPRESSED IN FEMALE AND MALE FLOWERS

Flower development is strongly affected by hormonal regulation (reviewed in Chandler, 2011). Auxin is tightly linked to the initiation of floral organ primordia and the disruption of auxin biosynthesis, polar auxin transport or auxin signaling leads to failure of flower formation (reviewed in Aloni et al., 2006). In agreement, the floral meristem identity gene *LEAFY* was recently shown to act through the regulation of the auxin response pathway (Li et al., 2013). Aux/IAA proteins, Auxin Efflux Carriers, and AUXIN RESPONSIVE FACTORS (ARF) are core components of the auxin-signaling cascade (Guilfoyle and Hagen, 2007). Several genes associated with the auxin regulatory network are highly represented and several are differentially expressed in female flowers. Particularly, homologs for the *A. thaliana* *IAA27* and *IAA9* genes were differentially expressed. The latter is a gene involved in fruit development and leaf embryogenesis in tomato (Wang et al., 2005) while mutants for *IAA27* showed altered fruit and flower development. A homolog of the *ARF4* gene that in *A. thaliana*, together with *ARF3*, control perianth organ number and spacing, as well as organ borders (Sessions et al., 1997) was identified in female flowers. Mutants for these genes showed defects in the stamens and gynoecium, as well as in the perianth organs, indicating an involvement in regional identity determination (Sessions et al., 1997). An auxin efflux carrier *QsPIN1* was also found to be up-regulated 20 fold in female flowers. Loss of *PIN1* function severely affects organ initiation; *pin1* mutants are characterized by an inflorescence meristem that does not initiate any flowers, resulting in the formation of a naked inflorescence stem (Okada et al., 1991). The abundance of auxin related machinery in female tissues leads to the possibility that female tissue determination might be under strong control of this hormone. Another hormone strongly correlated with sex determination is ethylene (Byers et al., 1972; Rudich et al., 1972). In cucumber, ethylene signaling is important in the inhibition of stamen development (Yamasaki et al., 2001). Interestingly, all the differentially expressed genes containing an ETHYLENE RESPONSIVE

FACTOR (ERF) domain were detected in the female flowers, in agreement with the aforementioned role of ethylene in the feminization of the flower meristem. Another gene linked to ethylene signaling pathway is *EIN3*, a nuclear TF that initiates downstream transcriptional cascades for ethylene responses (Potuschak et al., 2003; Yanagisawa et al., 2003). *QsEIN3* was unique to the female samples. Interestingly in *Arabidopsis*, activated ethylene signaling reduces bioactive GA levels, thus enhancing the accumulation of DELLA (repressors of the gibberellins pathway) and this most likely happens downstream of the transcriptional regulator *EIN3* (Achard et al., 2007). This is very interesting because the gibberellin hormone is thought to be essential for the developmental of a fully functional stamen in several species like *A. thaliana*, *Oryza sativa* or *Cucurbita maxima* (Pimenta Lange and Lange, 2006). This goes in agreement with our RNAseq results, in which several gibberellin related genes are found exclusively in the male flowers equally expressed in early and late stages of the developmental program, indicating a role in floral primordia and anther differentiation. Also, a *GAMYB* firmly involved in anther development, *QsMYB33*, was only expressed in male samples (Millar and Gubler, 2005). Interestingly, all the GRAS associated transcripts (known to be important regulators in GA signaling) that includes the gibberellin repressors DELLA, were only present in the female database.

#### CONCLUSION

Monoeious and dioecious species have been long considered unique tools to study the developmental programs involved in the formation of separate male and female flowers. However, for the majority of these species, insufficient or nonexistent genomic and transcriptomic data availability has hampered functional studies. Advances in NGS technologies have made possible to perform a rapid and cost-effective compilation of large RNA sequence data sets in non-model organisms with no or little prior genomic data available. Here, a broad flowering transcriptome composed of four independent libraries was obtained for early and late developmental stages of male and female flowers of *Q. suber*, a monoecious tree. In the future, to further enhance our knowledge on sex-specific genetic networks, individual EST libraries could be obtained for each phenological stage to fine map male and female flower specific regulators. Comparative studies revealed a subset of transcripts that were differentially expressed in the different libraries, many of which have a known role in flower and/or plant development. Transcriptome analysis also revealed a group of genes expressed exclusively in each type of flower gender that may have a functional role in male and female flower organ development or in sex specification. Some of the genes that showed differential expression have not been previously characterized in other species and others have not, to our knowledge, been implicated in flower development. Thus, it would be very interesting to perform functional studies using the above mentioned genes to identify its roles in plant reproduction or flower development in *Q. suber* and other flowering species. The analysis of *Q. suber* flower transcriptome may therefore contribute to uncover sex-specific regulatory networks hidden by hermaphroditism and serve as a platform to future studies in model and non-model species independently of their sexual habit.

## AUTHOR CONTRIBUTIONS

Leonor Morais-Cecílio, Maria M. R. Costa—Conceived and designed the experiments. Margarida Rocheta, Rómulo Sobral, Maria I. Amorim, Teresa Ribeiro, Leonor Morais-Cecílio, Maria M. R. Costa—Preparation of plant material and RNA. Margarida Rocheta, Rómulo Sobral, Joana Magalhães, Maria I. Amorim, Leonor Morais-Cecílio, Maria M. R. Costa—Performed the experiments. Miguel Pinheiro, Conceição Egas—Transcriptome sequencing and Bioinformatics. Margarida Rocheta, Rómulo Sobral, Joana Magalhães, Maria I. Amorim, Miguel Pinheiro, Leonor Morais-Cecílio, Maria M. R. Costa—Data analysis. Margarida Rocheta, Rómulo Sobral, Joana Magalhães, Maria I. Amorim, Miguel Pinheiro, Leonor Morais-Cecílio, Maria M. R. Costa—Paper writing and discussion. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2014.00599/abstract>

**Figure S1 | Functional classification of *Quercus suber* differentially expressed unigenes.**

**Table S1 | List of PCR primers used to amplify candidate genes.**

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# Transcriptomic insights into antagonistic effects of gibberellin and abscisic acid on petal growth in *Gerbera hybrida*

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Petal growth is central to floral morphogenesis, but the underlying genetic basis of petal growth regulation is yet to be elucidated. In this study, we found that the basal region of the ray floret petals of *Gerbera hybrida* was the most sensitive to treatment with the phytohormones gibberellin (GA) and abscisic acid (ABA), which regulate cell expansion during petal growth in an antagonistic manner. To screen for differentially expressed genes (DEGs) and key regulators with potentially important roles in petal growth regulation by GA or/and ABA, the RNA-seq technique was employed. Differences in global transcription in petals were observed in response to GA and ABA and target genes antagonistically regulated by the two hormones were identified. Moreover, we also identified the pathways associated with the regulation of petal growth after application of either GA or ABA. Genes relating to the antagonistic GA and ABA regulation of petal growth showed distinct patterns, with genes encoding transcription factors (TFs) being active during the early stage (2 h) of treatment, while genes from the “apoptosis” and “cell wall organization” categories were expressed at later stages (12 h). In summary, we present the first study of global expression patterns of hormone-regulated transcripts in *G. hybrida* petals; this dataset will be instrumental in revealing the genetic networks that govern petal morphogenesis and provides a new theoretical basis and novel gene resources for ornamental plant breeding.

**Keywords:** abscisic acid, antagonistic regulation, *Gerbera hybrida*, gibberellin, petal growth, RNA-seq

## Introduction

Petals are a particularly important component of the reproductive system of higher plants. As well as protecting the stamen and pistil, petals are instrumental in attracting the correct pollinator(s) to ensure successful pollination, which depends on their specific size, shape, color and arrangement (Glover and Martin, 1998). These unique characteristics are established during petal morphogenesis and are fundamentally connected with the identity, growth and development of petal primordium (Krizek and Fletcher, 2005; Alvarez-Buylla et al., 2010).

A number of gene regulatory networks (GRNs) that govern petal development, together with the associated transcription factors (TFs), have been identified recently (Alvarez-Buylla et al., 2010; O’Maoileidigh et al., 2014). The ABCDE model indicates that petal identity is determined

by the combined actions of class A (*AP1*), B (*AP3* and *PI*), and E (*SEP*) genes, whereas petal growth is negatively regulated by class C (*AGAMOUS*, *AG*) genes (Krizek and Fletcher, 2005; Alvarez-Buylla et al., 2010). In early flower primordium formation, *AINTEGUMENTA* (*ANT*), *JAGGED* (*JAG*), and *ARGOS* (an Auxin-Regulated Gene involved in Organ Size) function as positive regulators of cell proliferation (Krizek, 1999; Dinneny et al., 2004; Ohno et al., 2004). By contrast, Big Brother (*BB*), *KLU* and *DA1* are repressors of cell division in the flower (Disch et al., 2006; Anastasiou et al., 2007; Li et al., 2008). The *A1NAP* gene was shown to function at the transition point between cell division and cell expansion in *Arabidopsis thaliana* petals and stamens, acting downstream of class B genes (*AP3/PI*) (Sablowski and Meyerowitz, 1998). In the later stages of petal growth, *BIG-PETAL* (*BPEp*), a basic helix-loop-helix (bHLH) TF, is known to regulate petal size in *A. thaliana* by restricting cell expansion (Szecsi et al., 2006).

Phytohormones are well-known mediators of flower organ morphogenesis. In *A. thaliana*, auxin regulates many aspects of floral growth (Aloni et al., 2006) and the auxin response factor 8 (ARF8) interacts with *BPEp* to modulate cell expansion in petals (Varaud et al., 2011). Moreover, mutation that affect jasmonic acid (JA) biosynthesis leads to reduced petal growth (Brioudes et al., 2009) and JA regulates the expression of *BPEp*, suggesting that *BPEp* may also have a role in JA-mediated petal growth (Brioudes et al., 2009; Varaud et al., 2011). Furthermore, ARF6 and ARF8 induce the production of JA to promote the growth of petals and stamen by triggering the expression of *MYB21* and *MYB24* (Reeves et al., 2012). Therefore, auxin and JA function coordinately in the regulation of petal growth in *A. thaliana*, representing a very close association within the GRN involved (Varaud et al., 2011).

It is known that gibberellin (GA) regulates many critical biological events in plants, including seed germination, stem elongation and flowering (Sun, 2008; Heden and Thomas, 2012). Recent evidence has revealed that GA signaling is crucial to petal growth (Sun, 2008). As a versatile regulator, abscisic acid (ABA) has been shown to act antagonistically to the function of GA in a variety of developmental processes, including floral transition and fruit development (Razem et al., 2006). However, it is unknown whether such an antagonistic relationship exists in the regulation of petal growth. In addition, in contrast to the significant progress made in elucidating the GRN involving auxin and JA that governs petal growth (Brioudes et al., 2009; Varaud et al., 2011), the GRN associated with GA and ABA remains poorly understood.

*Gerbera hybrida*, a member of the sunflower family, is emerging as a model for the investigation of the genetic regulation of organ growth and development (Kotilainen et al., 1999; Laitinen et al., 2007; Zhang et al., 2012). The ray petals in *G. hybrida* only exhibit substantial cell expansion after stage 3 when the proliferation-to-expansion phase transition occurs (Meng and Wang, 2004; Laitinen et al., 2007), and can serve as a useful system for investigation of the regulatory network governing cell expansion. Previously, we presented a morphological description and the cellular basis of the ray petal growth in *G. hybrida*, thereby establishing the necessary groundwork for the molecular

characterization of petal growth (Meng and Wang, 2004; Zhang et al., 2012). In the current study, we used powerful second-generation sequencing technology to determine the transcriptome of the ray petals of *G. hybrida* at stage 3. This allowed us to produce high-resolution digital profiles of global gene expression relating to petal growth, thereby revealing the GRN that underpins the antagonistic control of petal growth by GA/ABA signaling. Since samples were collected from well-characterized stages and tissues, the transcriptome data are highly conducive to cross-lab or cross-species comparisons. In addition, the initial analysis of the wealth of molecular information has generated unprecedented molecular insights into petal growth.

## Materials and Methods

### Plant Material and Growth Conditions

*G. hybrida* “Shenzhen No. 5” seedlings were grown in a greenhouse at Zengcheng Ornamental Center (Guangzhou, China) as described by Zhang et al. (2012) at a temperature of 26/18°C (day/night) and relative humidity of 65–80%. The development stages of the inflorescence were defined according to Meng and Wang (2004). Inflorescences at stage 1.5 (between stages 1 and 2), which are approximately 1.5 cm in diameter with a ray petal (petal) length of 6 mm, were used for the *in vivo* experiment. For the *in vitro* experiment, petals at stage 3 were used.

### Hormone and Inhibitor Treatments

For the evaluation of petal length as described below, GA and/or ABA treatments were employed in *in vivo* or *in vitro* experiments, depending on the purpose of the analysis. Five to six inflorescences of similar size were included for each treatment. *In vivo* treatments were performed by spraying the stage 1.5 inflorescences with 3–5 ml 10 μM GA<sub>3</sub> or 50 μM ABA once a day; inflorescences were sampled after 9 days. As a control, inflorescences sprayed with 0.1% ethanol in deionized water were sampled in parallel. *In vitro* treatments were in accordance with the previously described procedures (Huang et al., 2008; Zhang et al., 2012) using stage 3 inflorescences. Briefly, about 10 petals of the outermost whorl of ray flowers were detached from the inflorescences, placed on two layers of Whatman filter paper soaked in 3% sucrose solution, with or without hormones (10 μM GA<sub>3</sub> or 50 μM ABA) as supplementary elements, and treated for 9 days. Ten or more petals were used in the *in vitro* experiments; the duration of treatment varied depending on the purpose of the assay performed, as indicated below.

To evaluate the interaction between the effects of GA and ABA, we performed *in vitro* experiments using a combination of hormones, in which, for example, after preculturing the petals with GA for 2, 12, or 24 h, ABA was added to the medium, with the final measurements being made after 72 h. Conversely, where ABA was the initial hormone used, GA was added during the experiment.

The widely used inhibitors of GA and ABA biosynthesis (White et al., 2000; Kusumoto et al., 2006; Martinez-Andujar et al., 2011; Heden and Thomas, 2012), paclobutrazol (PAC) and fluridone (FLU), were also used in this study. In the *in vitro*

experiments described above, the phytohormones were replaced by PAC (10  $\mu$ M) or FLU (0.1  $\mu$ M).

Hormones and inhibitors were acquired from Sigma-Aldrich Chemical Co. (Shanghai, China). Both *in vivo* and *in vitro* experiments were each replicated at least three times.

### Measurement of Petal and Cell Length

To measure petal elongation, whole petals from each *in vivo* experiment were harvested and images of the petals were scanned using an EPSON-G850A scanner (EPSON, China) and photographed. Measurement of petal length was performed using Image J software (<http://rsb.info.nih.gov/ij/>, NIH, MD, USA). In total, more than six inflorescences were collected for each treatment and the lengths of 10 petals from each inflorescence were measured. Data from at least 60 petals were thus averaged to estimate the petal length under each treatment condition.

Elongation of three petal regions, namely top, middle or basal, was also recorded for *in vitro* treated petals. The lengths of three regions of the same petals before and after treatment were measured. For each individual measurement, a total of 10 petals were used and three independent measurements were made. The elongation rates were calculated according to the equation: Elongation rate =  $(Lt - Li)/Li \times 100\%$ , where  $Lt$  is the petal length after treatment, while  $Li$  is the initial length of each petal before treatment. Data from individual measurements were averaged.

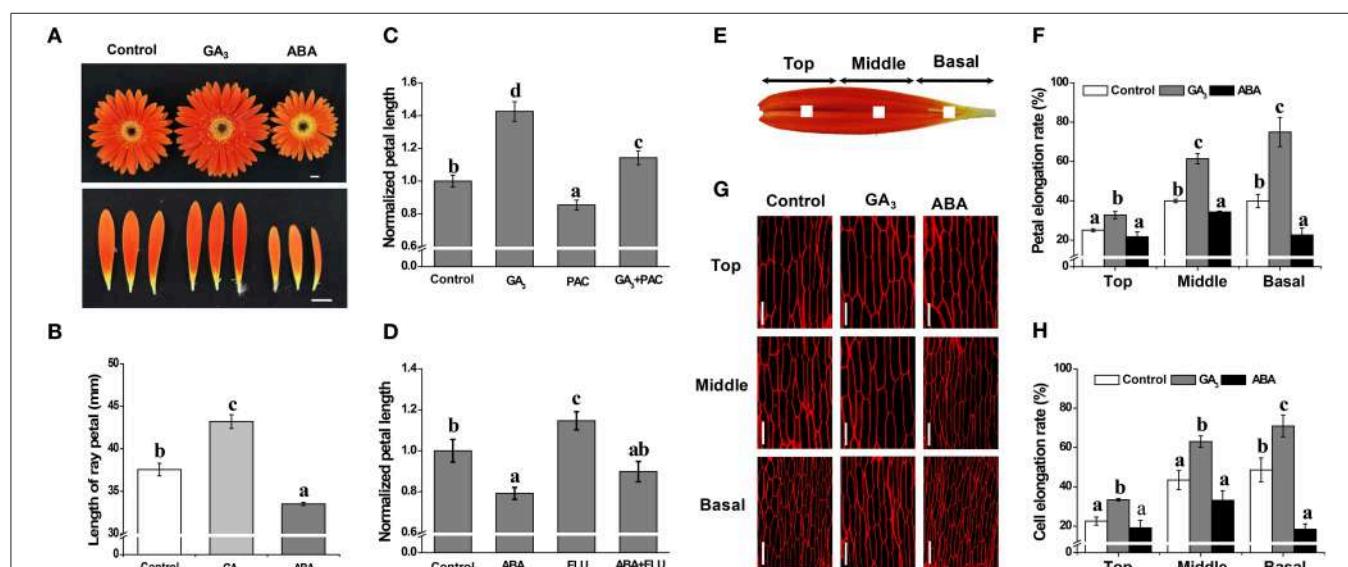
For measurement of cell length, petals were sampled after *in vitro* treatments. A 1 mm<sup>2</sup> petal block was dissected from the center of each of the top, middle and basal regions, and

was stained by immersion in 0.1 mg mL<sup>-1</sup> propidium iodide for 5 min at 25°C, followed by rinsing thoroughly with deionized water to remove excess stain solution, before flattening samples on a glass slide. Abaxial epidermal cell images were obtained with a laser confocal scanning microscope (LSM710/ConfoCor2, Carl-Zeiss, Jena, Germany), after which the length of individual cells was measured using Image J software. From at least 10 petals detached from different inflorescences, more than 100 cells were randomly selected for length measurement, which was performed before and after treatment. Untreated samples gave the initial length,  $Li$ , while samples after treatment provided  $Lt$  values. The elongation rate was estimated using the equation described above and data from three independent measurements of biological replicate samples were averaged.

After measurement, One-Way ANOVA was conducted to test for statistical significance using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Duncan's test was applied to assess the differences between treatments.

### RNA-seq

Before treatment with GA or ABA, petals were precultured on 3% sucrose medium (pH 5.8) for 2 days (Huang et al., 2008). Petals were treated for either 2 or 12 h with GA or ABA. At least 200 petals from 20 inflorescences at stage 3 were used for each combination of phytohormone and treatment duration. Petals cultured on 3% sucrose solution without addition of phytohormone were used as the control. The entire basal regions of petals (**Figure 1E**) were collected and pooled for each



**FIGURE 1 |** Antagonistic effects of GA and ABA on growth of petals in *G. hybrida*. *G. hybrida* was grown in a greenhouse under the conditions described in Materials and Methods. Plants with inflorescences at stage 1.5 were sprayed with deionized water (with 0.1% ethanol) (Control), 10  $\mu$ M GA<sub>3</sub> or 50  $\mu$ M ABA and were subjected to morphological characterization (**A**) and petal length measurement (**B**) after 9 days of treatment. Sixty or more ray petals were measured for each treatment and the value are given as average lengths  $\pm$  SE. Representative examples of inhibition of GA- or ABA-derived effects on petal growth by PAC (10  $\mu$ M) or FLU (0.1  $\mu$ M) are shown in (**C,D**).

for which the control petal length was set as 1. One mm<sup>2</sup> blocks at the center of the top, middle or basal regions of the petal are indicated in (**E**). Detached petals treated with Control, 10  $\mu$ M GA<sub>3</sub> or 50  $\mu$ M ABA for 9 days were used for morphological characterization of abaxial epidermal cell using a confocal microscope (**G**) and measurement of elongation rate of each petal region (**F**,  $n = 10$ ) or cell (**H**,  $n > 100$ ). Three biological replicates were performed for each measurement. Values are given as mean  $\pm$  SE. Letters above the bars indicate significant differences between the respective values ( $p < 0.05$ ). Scale bar represents 1 cm (**A**) or 50  $\mu$ m (**G**).

treatment combination. Total RNA was extracted from each basal pool, resulting in six samples, corresponding to the six treatment combinations of each hormone and treatment duration; these were denoted Control2h, Control12h, GA2h, GA12h, ABA2h, and ABA12h. TRIzol® reagent (Invitrogen, USA) was used for total RNA extraction according to the manufacturer's instructions. DNase I (Takara, Japan) was used to remove genomic DNA. The quality of total RNA was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Those samples with an RNA integrity number (RIN) > 8 were used to prepare cDNA libraries, as previously described (Kuang et al., 2013). The libraries were used for paired-end 45 × 2 sequencing using Illumina HiSeq™ 2000 at the Beijing Genomics Institute (BGI) (Shenzhen, China). In total, six sets of raw reads were obtained, corresponding to Control2h, Control12h, GA2h, GA12h, ABA2h, and ABA12h. All sequence data were deposited at the NCBI in the Short Read Archive (SRA) database under the accession numbers SRX850776, SRX850779, SRX850784, SRX850787, SRX850789, and SRX850790 for Control2h, GA2h, ABA2h, Control12h, GA12h, and ABA12h, respectively.

## Data Processing and Analysis

Raw read processing and primary bioinformatics analysis of the transcript datasets were conducted at Genedenovo Biotechnology Co., Ltd (Guangzhou, China). In brief, raw reads were filtered to remove "dirty" data, including adaptor sequences, the reads in which unknown bases are greater than 10% and low-quality reads containing more than 50% bases with  $Q \leq 5$ . The clean reads thus generated were mapped to the previously assembled *G. hybrida* transcriptome (Kuang et al., 2013) using SOA-Paligner/soap2 (Li et al., 2009). Mismatches of no more than two bases were allowed, with separate alignments being performed for each sample independently. Unigenes mapped by at least one read, in at least one sample, were identified for further analysis. Estimation of gene expression and identification of differentially expressed genes (DEGs) were conducted using a modified method of that described previously (Audic and Claverie, 1997). Transcript abundance was expressed as RPKM (reads mapped per 1000 bp per million sequenced reads) (Mortazavi et al., 2008). RPKM values presenting as "0" were artificially set to "0.001" for subsequent analysis. Comparisons of RPKM between treatments (treatment2h vs. Control2h, treatment12h vs. Control12h, treatment12h vs. treatment2h) were performed for each Unigene. Those with a fold-change of  $\geq 2$  and a false discovery rate (FDR) < 0.001, in at least one comparison, were considered as significant DEGs.

DEGs were subjected to enrichment analysis for both KEGG pathway and GO annotation terms. Before KEGG pathway analysis, KEGG Orthology terms for DEGs were retrieved from the KEGG pathway database (<http://www.genome.jp/kegg/>). The enrichment analysis was performed by comparing the observed DEG count to the expected count of the genes involved in a given pathway with a random distribution of the previously reported transcriptome (Kuang et al., 2013). A hypergeometric test was performed for statistical analysis and the  $p$ -value cut off was 0.05. For GO enrichment analysis, Gene Ontology (GO) annotations for each DEG were retrieved by mapping

to GO terms in the database at <http://www.geneontology.org>. For DEGs with opposite regulation patterns, GO terms were also retrieved according to the annotations of *A. thaliana* homologs at <http://www.arabidopsis.org>, followed by performing GO enrichment analysis using the BinGO App in Cytoscape 3.2.0 (<http://cytoscape.org/>) against the whole *A. thaliana* genome. GO terms for Biological Processes (GO-BP) with a FDR  $\geq 0.05$  were considered significant. Hierarchical clustering analysis was performed using MeV 4.9.0 (<http://www.tm4.org/mev.html>) by considering the RPKM value as the normalized transcript level for a given gene.

## Quantitative Real Time PCR (qRT-PCR) Validation

Total RNA was extracted from the basal region of petals (**Figure 1E**) using TRIzol® Reagent (Invitrogen, USA) according to the manufacturer's instructions and quantified with a NanoDrop 1000 Spectrophotometer (Fisher Rochester, NY, USA). Two  $\mu$ g RNA was treated with DNase I (Takara, Japan) according to the manufacturer's instructions, followed by cDNA synthesis using the SuperScript® III First-Strand Synthesis System (Invitrogen, USA) in a 40  $\mu$ l total reaction volume with Random Primer 6 (Takara, Japan). For qRT-PCR, transcripts of target genes were amplified in a 20  $\mu$ l reaction containing 2  $\mu$ l cDNA (corresponding to 20 ng RNA), 1  $\mu$ l primers and 5  $\mu$ l SsoFast™ EvaGreen® Supermix (Bio-Rad, USA). Quantitation of each transcript was repeated using total RNA from three independent samples as starting materials and each qPCR was performed in triplicate. The primers are listed in Supplemental Table S1. Expression levels of the tested genes were normalized to that of the ACTIN (AJ763915) gene as previously described (Kuang et al., 2013).

## Results

### Effect of GA and ABA on Petal Growth

The *in vivo* experiments performed with intact inflorescences (stage 1.5) revealed that, compared with the average petal length of 37.6 mm in the control, GA promotes elongation of the petal to an average length of 43.2 mm, whereas ABA treatment results in petals that are shorter than controls, with an average length of 33.3 mm. Thus, GA treatment produces a significant increase in petal length, while ABA treatment produces a significant decrease (**Figures 1A,B**;  $p < 0.01$ ), i.e., the two phytohormones have opposite effects on inflorescence size. We also found that treatment with PAC or FLU can suppress or enhance petal elongation (**Figures 1C,D**;  $p < 0.05$ ), respectively. Moreover, PAC-mediated suppression and FLU-mediated enhancement of petal length can be reversed by the application of exogenous GA and ABA (**Figures 1C,D**), respectively. We interpret these data as illustrating that GA and ABA have contrasting effects on petal elongation.

The *in vitro* experiments showed that 9 days of GA treatment significantly increased the elongation rate of petal tissue in the top, middle and basal regions by 33, 61, and 75%, respectively, compared to increases of 25, 40, and 40% in the control for the same regions (**Figure 1F**;  $p < 0.05$ ). Elongation rates following ABA treatment, however, were 22, 34, and 23%, respectively,

indicating a significant inhibition in the middle and basal regions (**Figure 1F**;  $p < 0.05$ ). We further showed that cell elongation rates were greatly increased in the presence of GA, by 33, 63, and 71% in the top, middle and basal regions, respectively, but only in the basal region was cell elongation rate significantly suppressed by ABA treatment (an increase of only 18% vs. a 49% increase in the control) (**Figures 1G,H**;  $p < 0.05$ ). These results indicate that petal elongation is associated with cell elongation, and that the antagonistic effects of GA and ABA are predominantly limited to the basal region.

The combined effects of GA and ABA on petal elongation were further tested *in vitro*. The growth dynamics of the petal indicated that GA-mediated promotion and ABA-mediated repression of petal elongation could both be attenuated by the co-application of ABA and GA, respectively, suggesting that the effects of GA and ABA are antagonistic (**Figure 2A**). Interestingly, the promotion of petal elongation by GA was only significantly attenuated when ABA was applied within 2 h of the initial GA treatment: no significant attenuation in petal length was seen if ABA was added at 12 or 24 h (**Figure 2B**). Moreover, ABA-mediated repression of petal growth was overcome by supplementation with GA 2 or 12 h after the initial application of ABA (**Figure 2C**).

### Effect of GA and ABA on the Petal Transcriptome

An investigation of the GA/ABA-associated GRN that modulates petal growth was performed using RNA-seq data. After removing contaminated and low-quality sequences, all reads were mapped onto the published transcriptome, which contains 47,104 Unigenes (Kuang et al., 2013). Unigenes represented by at least one mapped read were accepted for subsequent analyses. In total, we generated 42,773 Unigenes and the coverage for individual RNA samples ranged from 76 to 87% (**Table 1**). The global distribution of the relative expression level, which is determined by a

log<sub>2</sub>-transformed fold-change relative to the control, is shown in **Figure 3A**. ABA treatment for 2 h resulted in greater variation of the relative expression level, with the distribution ranging from -4.34 to 5.11 and a higher mean value of 0.46, whereas GA treatment for 2 h gave a distribution range from -4.41 to 3.98 and a mean of -0.08. At the 12 h time point, the range of transcription levels was spread more broadly, from -6.24 to 4.49 and -4.20 to 7.38 for GA and ABA treatment, respectively. The mean transcription level after 12 h, however, decreased to 0.30 with ABA treatment, but increased to 0.18 with GA treatment.

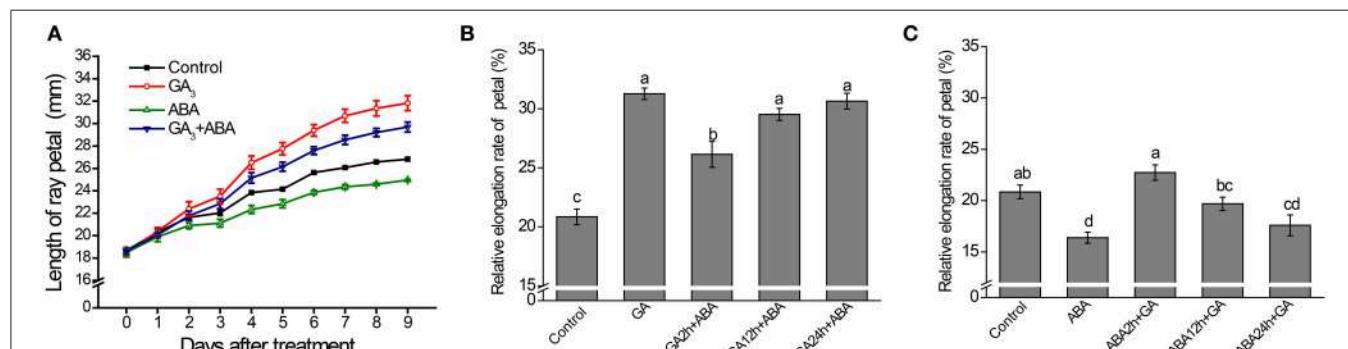
### Identification of DEGs Compatible with Antagonistic Effects of GA and ABA

We then screened the DEGs from the collection of 42,773 Unigenes (**Table 1**). Using the criteria of fold-change  $\geq 2$  and FDR  $< 0.001$ , we identified 222 and 938 DEGs after GA treatment for 2

**TABLE 1 | Summary of the mapping reads and Unigenes identified by RNA-seq.**

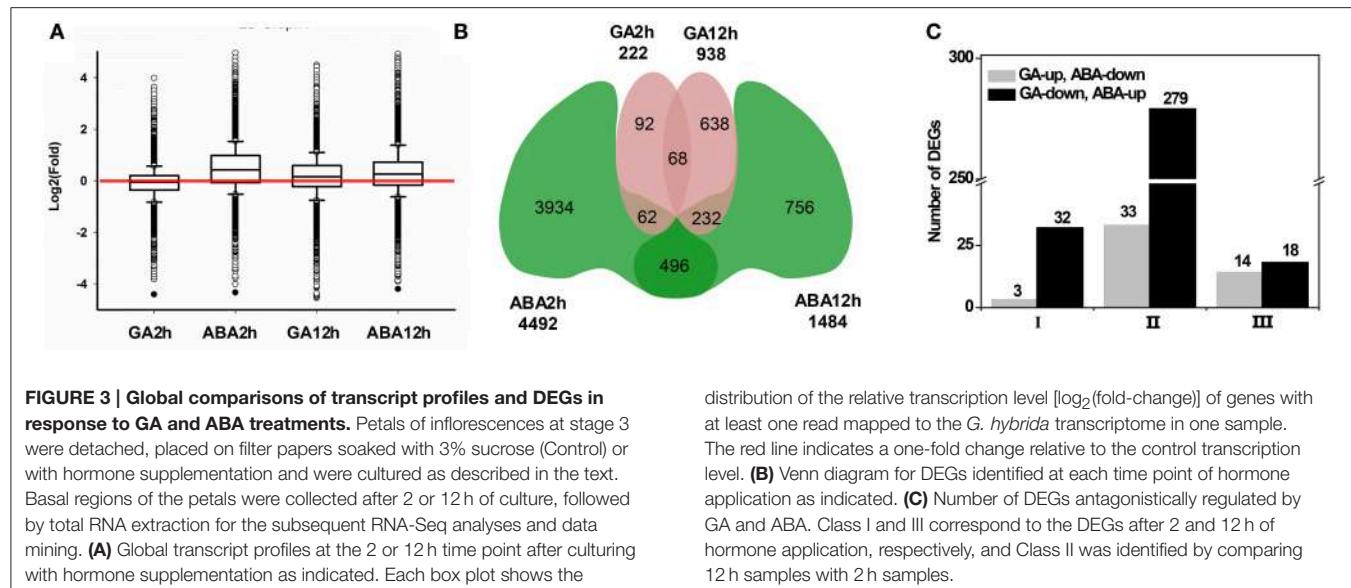
|                                     | Number of Unigenes |
|-------------------------------------|--------------------|
| Total Unigenes (Kuang et al., 2013) | 47,104             |
| Total mapped Unigenes               | 42,773             |
| <b>MAPPED IN SAMPLE</b>             |                    |
| Control2h                           | 39,503 (84%*)      |
| GA2h                                | 38,824 (82%*)      |
| ABA2h                               | 40,767 (87%*)      |
| Control12h                          | 35,788 (76%*)      |
| GA12h                               | 36,438 (77%*)      |
| ABA12h                              | 35,928 (76%*)      |

\*Percent of Unigenes with at least one mapped read.



**FIGURE 2 | Effects of GA or ABA on petal growth are attenuated by co-application of the hormones.** Petals of inflorescences at stage 3 were detached and placed on filter paper soaked with 3% sucrose (Control) or with hormone supplementation and were cultured as described in the text. **(A)** Time-course dynamics of petal length under control conditions or after treatment with 10  $\mu$ M GA<sub>3</sub>, 50  $\mu$ M ABA or a mixture of 10  $\mu$ M GA<sub>3</sub> and 50  $\mu$ M ABA. A total of 10 petals for each treatment were cultured for 9 days. **(B)** Effect on petal elongation rate of addition of ABA after pre-culture with GA alone for 2, 12, and 24 h. **(C)** Effect on petal elongation rate of addition of GA after pre-culture with ABA alone for 2, 12, and 24 h. Petals were cultured for a total of

72 h. Control: petals were continuously cultured with 3% sucrose; GA: petals were continuously cultured with 3% sucrose plus GA alone; ABA: petals were continuously cultured with 3% sucrose plus ABA alone; GA+ABA: petals were continuously cultured with a mixture of GA and ABA; GA2h+ABA/ABA2h+GA: petals were pre-treated with GA or ABA for 2 h followed by addition of ABA or GA. In other cases, the duration of GA or ABA pre-culture before ABA or GA supplementation was as indicated. Each value is the mean  $\pm$  SE ( $n = 6$  petals). The experiment was repeated at least three times with similar results. Representative data are presented. Letters above the bars in **(B,C)** indicate significant differences between the respective values ( $p < 0.05$ ).



and 12 h, respectively, of which 68 Unigenes were common in both datasets (Figure 3B). There were 4492 DEGs in response to ABA treatment for 2 h (Figure 3B), which is 20-fold higher than the response to GA treatment for the same time. After treatment with ABA for 12 h, 1484 DEGs were identified, of which 496 DEGs also occurred in the group treated with ABA for 2 h (Figure 3B; Supplemental Tables S2–S5). Notably, with increasing duration of treatment, the number of DEGs in response to GA treatment increased, but decreased in response to ABA. In total, we obtained a set of 6278 DEGs for the subsequent identification of genes involved in the antagonistic regulation of petal growth by GA and ABA.

We further analyzed those DEGs antagonistically regulated by GA and ABA. Firstly, we defined three classes of DEGs. Class I and Class III DEGs refer to those antagonistically regulated by GA and ABA after 2 and 12 h treatment, respectively. We found that, relative to control (Control2h), three DEGs up-regulated by GA (GU) were shown to be down-regulated by ABA (AD) after 2 h treatment, in contrast to 32 DEGs down-regulated by GA (GD) but up-regulated by ABA (AU) (Figure 3C; I). Thus, a total of 35 DEGs (57.38% of the DEGs that co-regulated by GA and ABA at 2 h) were antagonistically regulated by GA and ABA at 2 h (Figure 3C; Supplemental Tables S6, S7). When the hormone treatments were extended to 12 h, the number of antagonistically regulated Class III DEGs was nearly the same (Figure 3C; III). Although the number of Class III DEGs (32) was similar to that of Class I DEGs, the ratio was decreased to 13.8% (Figure 3C; Supplemental Tables S6, S7). Class II DEGs, which were identified by comparing the datasets representing treatment for 12 h and treatment for 2 h, had an opposite pattern of change between GA and ABA treatment during the test period. For Class II DEGs, we identified 312 DEGs showing such an opposite dynamic change from 2 to 12 h, among which 33 DEGs showed a GU/AD pattern, whereas 279 DEGs showed a GD/AU pattern (Figure 3C; Supplemental Tables S8, S9).

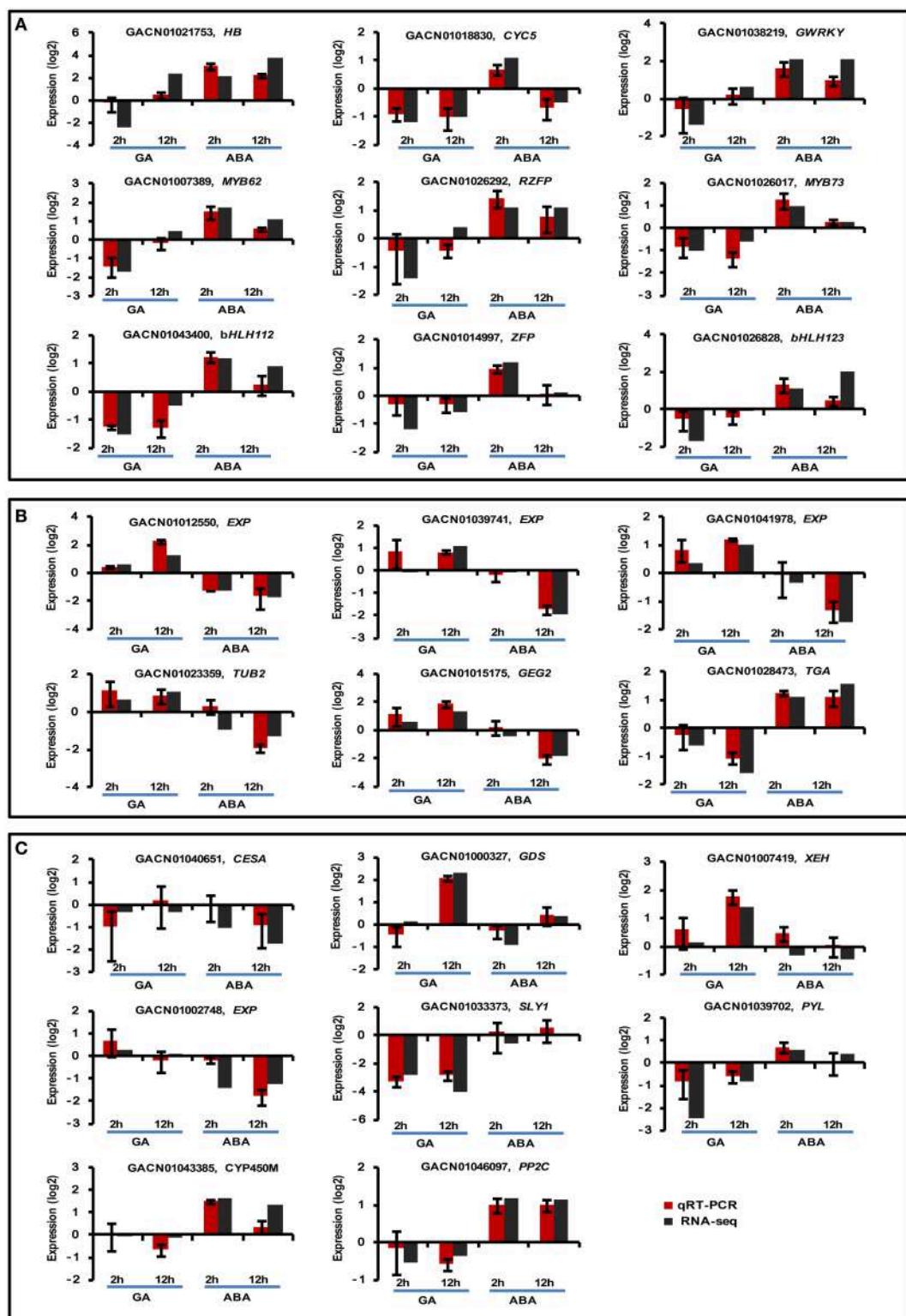
distribution of the relative transcription level [ $\log_2(\text{fold change})$ ] of genes with at least one read mapped to the *G. hybrida* transcriptome in one sample. The red line indicates a one-fold change relative to the control transcription level. **(B)** Venn diagram for DEGs identified at each time point of hormone application as indicated. **(C)** Number of DEGs antagonistically regulated by GA and ABA. Class I and III correspond to the DEGs after 2 and 12 h of hormone application, respectively, and Class II was identified by comparing 12 h samples with 2 h samples.

Twenty-three DEGs were selected for qRT-PCR. Specifically, eight DEGs were randomly selected (Figure 4C), nine DEGs were selected from Class I (Figure 4A) and six were selected from Class III (Figure 4B). Overall, the qRT-PCR data showed patterns similar to those obtained from RNA-Seq for these DEGs, although the particular values of fold-change were different.

### Enrichment Analysis of DEGs Antagonistically Regulated by GA and ABA

To retrieve GO annotations for DEGs, we searched the TAIR 10 protein database using BlastX; GO annotations were assigned to each DEG according to the best hit. Enrichment analysis of DEGs was performed using BinGO with the default setting of FDR < 0.05, and compared with the *A. thaliana* whole genome GO annotation. By separately analyzing the three DEG classes, we observed that the GO-Biological Process (GO-BP) termed “cell wall organization” was overrepresented at 12 h within the set of Class III DEGs having a GU/AD pattern (Table 2). The sub-category “cell wall loosening” indicates the specific processes involved. For Class II DEGs with a GU/AD pattern, the GO-BP termed “apoptosis” was overrepresented (Table 2). Overrepresented categories were not found for Class I DEGs with a GU/AD pattern, but genes with the opposite pattern, GD/AU, involved in “regulation of transcription”, were mostly enriched in the 2 h dataset of Class I DEGs (Table 2).

KEGG pathway enrichment analysis was also carried out to elucidate the interaction of GA/ABA mediated pathways in petal growth. Of the ~47,000 Unigenes in *G. hybrida* (Kuang et al., 2013), 20,483 can be annotated and mapped to different pathways (data not shown). Not surprisingly, transcripts encoding proteins involved in plant hormone signal transduction were significantly enriched in all samples (Table 3), showing that 24 of the 103 annotated DEGs in the GA2h treatment were associated with these pathways. In addition, GA and ABA also regulated the expression of genes involved in multiple hormone signaling



**FIGURE 4 | Real-time quantitative PCR validation of transcript profiles for a subset of DEGs.** Twenty-three DEGs were selected for real-time qPCR validation, including nine Class I (**A**), six Class III (**B**), and eight randomly selected DEGs (**C**). *G. hybrida* ACTIN (AJ763915) was used as the normalization control. Sample collection was conducted

as described in the text. Three biological repeats were included for each condition. The y-axis indicates the expression of each DEG relative to control by log<sub>2</sub>-transformed RQ value for qPCR or fold-change value for RNA-seq. Error bars indicate the interval between the log<sub>2</sub>-transformed values of maximum or minimum RQ.

**TABLE 2 | DEGs with enriched GO terms.**

| Unigene ID   | Description   | GA2h         | GA12h        | ABA2h        | ABA12h       |
|--|---|--------------|--------------|--------------|--------------|
| <b>CELL WALL ORGANIZATION/CELL WALL LOOSENING (III)*</b> |   |              |              |              |              |
| GACN01006243   | Expansin  | -0.91        | <b>-2.42</b> | 0.61         | <b>1.05</b>  |
| GACN01012550   | Expansin  | 0.59         | <b>1.27</b>  | <b>-1.28</b> | <b>-1.76</b> |
| GACN01038094   | Expansin  | 0.77         | <b>1.32</b>  | <b>-1.55</b> | <b>-1.99</b> |
| GACN01039741   | Expansin  | 0.10         | <b>1.09</b>  | -0.07        | <b>-1.94</b> |
| GACN01041978   | Expansin  | 0.34         | <b>1.02</b>  | -0.33        | <b>-1.74</b> |
| GACN01023151   | Expansin  | 0.81         | <b>2.16</b>  | <b>-1.07</b> | <b>-1.90</b> |
| <b>APOPTOSIS (II)*</b>                                   |   |              |              |              |              |
| GACN01018625   | BCL-2-associated athanogene 5                         | 0.30         | <b>1.68</b>  | <b>1.69</b>  | -0.62        |
| GACN01031726   | Probable disease resistance protein                   | 0.20         | <b>3.26</b>  | <b>1.44</b>  | <b>-7.36</b> |
| GACN01015754   | TIR-NBS-LRR class disease resistance protein          | -0.83        | 0.32         | <b>3.21</b>  | <b>1.12</b>  |
| GACN01011634   | TIR-NBS-LRR class disease resistance protein          | -0.11        | <b>1.25</b>  | <b>2.32</b>  | <b>1.21</b>  |
| GACN01026830   | RPM1 interacting protein 13                           | -0.15        | <b>1.09</b>  | <b>3.46</b>  | 0.38         |
| GACN01022318   | NB-ARC domain-containing disease resistance protein   | <b>-1.00</b> | <b>1.68</b>  | <b>2.09</b>  | 0.97         |
| GACN01023277   | Putative TIR-NBS-LRR class disease resistance protein | <b>-2.64</b> | <b>1.09</b>  | <b>1.85</b>  | <b>-1.20</b> |
| GACN01009587   | NB-ARC domain-containing disease resistance           | -0.70        | 0.42         | <b>1.33</b>  | 0.19         |
| GACN01043665   | Putative TIR-NBS-LRR class disease resistance protein | 0.17         | <b>1.19</b>  | <b>2.74</b>  | 0.16         |
| GACN01017010   | Disease resistance protein RGC2                       | <b>-1.70</b> | -0.35        | <b>2.05</b>  | -0.53        |
| <b>REGULATION OF TRANSCRIPTION (I)*</b>                  |   |              |              |              |              |
| GACN01007389   | MYB domain protein 62 like                            | <b>-1.73</b> | 0.48         | <b>1.70</b>  | <b>1.05</b>  |
| GACN01014997   | Transcription factor zinc finger protein              | <b>-1.16</b> | -0.62        | <b>1.18</b>  | 0.14         |
| GACN01026828   | Transcription factor bHLH123                          | <b>-1.66</b> | -0.08        | <b>1.13</b>  | <b>2.02</b>  |
| GACN01018830   | Cycloidea-like 5                                      | <b>-1.18</b> | <b>-1.02</b> | <b>1.13</b>  | -0.49        |
| GACN01038219   | WRKY DNA-binding protein 30                           | <b>-1.35</b> | 0.59         | <b>2.11</b>  | <b>2.06</b>  |
| GACN01021753   | Homeobox-leucine zipper protein AtHB-7                | <b>-2.41</b> | <b>2.42</b>  | <b>2.18</b>  | <b>3.76</b>  |
| GACN01026017   | MYB domain protein 73                                 | <b>-1.02</b> | -0.64        | <b>1.04</b>  | 0.33         |
| GACN01043400   | Transcription factor bHLH112-like                     | <b>-1.47</b> | -0.47        | <b>1.24</b>  | 0.87         |
| GACN01026292   | Ring zinc finger protein                              | <b>-1.39</b> | 0.40         | <b>1.11</b>  | <b>1.05</b>  |

\*The bracketed Roman numeral indicates the DEG class under consideration. Fold changes greater than 2 relative to the corresponding control are in bold.

pathways (Supplemental Table S10). Moreover, we also identified crosstalk between the biosynthesis and metabolism pathways of multiple hormones. For example, genes involved in diterpenoid biosynthesis, which is associated with the gibberellin biosynthesis and metabolism pathway (Sun, 2008), were significantly enriched by both GA and ABA treatment. Similarly, the carotenoid biosynthesis pathway, which contributes to ABA biosynthesis and metabolism, was overrepresented after 12 h GA treatment. Interestingly, genes involved in the biosynthesis of zeatin, a class of cytokinin (CK), were identified after 2 h GA or ABA treatment, suggesting crosstalk between the metabolic pathways of the three hormones.

### Transcript Profiling of DEGs Antagonistically Regulated by GA and ABA

The three sets of DEGs annotated “regulation of transcription,” “cell wall organization” or “apoptosis” were loaded separately into MeV for hierarchical clustering analysis. It was apparent that “regulation of transcription” genes were repressed at the 2 h time point under GA treatment conditions; however, they were gradually activated from 2 to 12 h (Figure 5A; Table 2). ABA treatment promoted transcription of all these genes at 2 h,

but most of them were repressed from 2 to 12 h. Five of the six genes involved in “cell wall organization” were significantly activated from 2 to 12 h with GA treatment, indicating constitutive activation by GA. ABA led to a slight increase in transcript abundance at the 2 h point, but reduced transcript levels at 12 h (Figure 5C; Table 2). The DEGs grouped under “apoptosis” all presented GU/AD patterns from 2 to 12 h (Figure 5B; Table 2).

Genes involved in biosynthesis, metabolism and signaling pathways associated with GA and ABA were retrieved from the DEG set according to the KEGG annotation. Clustering analysis indicated that a large proportion of these DEGs exhibited opposite expression patterns at 2 h of GA vs. ABA treatment, but a somewhat similar pattern at 12 h (Figures 5D–G). For example, four putative members of the gibberellin 2-oxidase (GA2ox) gene family exhibited GD/AU patterns. Genes encoding ent-kaurene synthase (KS) and ent-kaurene oxidase (KO), which initially showed the GD/AU expression pattern at 2 h, were up-regulated by GA but repressed by ABA from 2 to 12 h (Figure 5D). Gibberellin 3-oxidase which contribute to the generation of active GAs, were regulated by GA and ABA in a similar manner (Figure 5D). However, two genes (GACN01003714, GACN01001277) for

**TABLE 3 | Pathways differentially regulated by GA and ABA.**

| Pathway                               | GA2h              | GA12h              | ABA2h                | ABA12h             |
|---------------------------------------|-------------------|--------------------|----------------------|--------------------|
| Plant hormone signal transduction     | <b>24 (6.8)**</b> | <b>46 (29.7)**</b> | <b>165 (146.2)*</b>  | <b>78 (54.2)**</b> |
| Plant-pathogen interaction            | 11 (6.6)          | 38 (29.0)          | <b>239 (143.0)**</b> | <b>91 (53.0)*</b>  |
| Phosphatidylinositol signaling system | –                 | 5 (3.8)            | <b>38 (20.7)**</b>   | 8 (7)              |
| Cyanoamino acid metabolism            | <b>5 (0.8)**</b>  | <b>7 (3.3)*</b>    | 14 (15.7)            | <b>17 (6.3)**</b>  |
| Starch and sucrose metabolism         | 1 (2.3)           | <b>20 (10.3)**</b> | 51 (50.8)            | <b>40 (18.8)**</b> |
| Diterpenoid biosynthesis              | 0 (0.3)           | <b>10 (1.1)**</b>  | <b>17 (5.6)**</b>    | 3 (2.1)            |
| Carotenoid biosynthesis               | 1 (0.6)           | <b>8 (2.8)**</b>   | 18 (14.0)            | 7 (5.2)            |
| Photosynthesis—antenna proteins       | 0 (0.2)           | 1 (0.8)            | 2 (3.8)              | <b>16 (1.4)**</b>  |
| Flavonoid biosynthesis                | 1 (0.9)           | <b>17 (3.8)**</b>  | 22 (18.9)            | 11 (7.0)           |
| Anthocyanin biosynthesis              | 1 (0.0)           | 0 (0.3)            | 1 (1.3)              | <b>4 (0.5)**</b>   |
| ABC transporters                      | 2 (0.8)           | 2 (3.6)            | <b>27 (17.6)*</b>    | <b>15 (6.5)**</b>  |
| Zeatin biosynthesis                   | <b>3 (0.8)*</b>   | 5 (3.4)            | <b>28 (16.7)**</b>   | 8 (6.2)            |

The number of DEGs in the corresponding pathway is shown. Expected counts from a random distribution based on the statistical analysis described by Nemhauser et al. (2006) are shown in parenthesis. The p-value was calculated using Fisher's Exact Test. Numbers significantly different to controls are shown in bold. \* and \*\* indicate  $p < 0.05$  and  $p < 0.01$ , respectively.

NCED (9-cis-epoxy-carotenoid dioxygenase) homologs, encoding the rate limiting enzyme in ABA biosynthesis (Thompson et al., 2000), displayed GD patterns (Figure 5G).

## Discussion

### GA and ABA Differentially Modulate Petal Growth

Petal development is related to cell division and cell expansion, in which many phytohormones and genes are involved (Alvarez-Buylla et al., 2010; Krizek and Anderson, 2013). Previous studies have shown that at stage 3 of petal development in *G. hybrida*, the petal size is mainly determined by cell expansion, and not by cell division (Meng and Wang, 2004; Laitinen et al., 2007; Zhang et al., 2012). Two findings in our current study extend these conclusions, showing that GA and ABA have antagonistic effects on petal growth by modulation of cell elongation at the basal region: (1) petal/cell elongation is enhanced by GA but repressed by ABA when each phytohormone is applied alone (Figure 1); (2) the increase in petal length by GA and the reduction in petal length by ABA are attenuated by the co-application of ABA and GA, respectively (Figure 2).

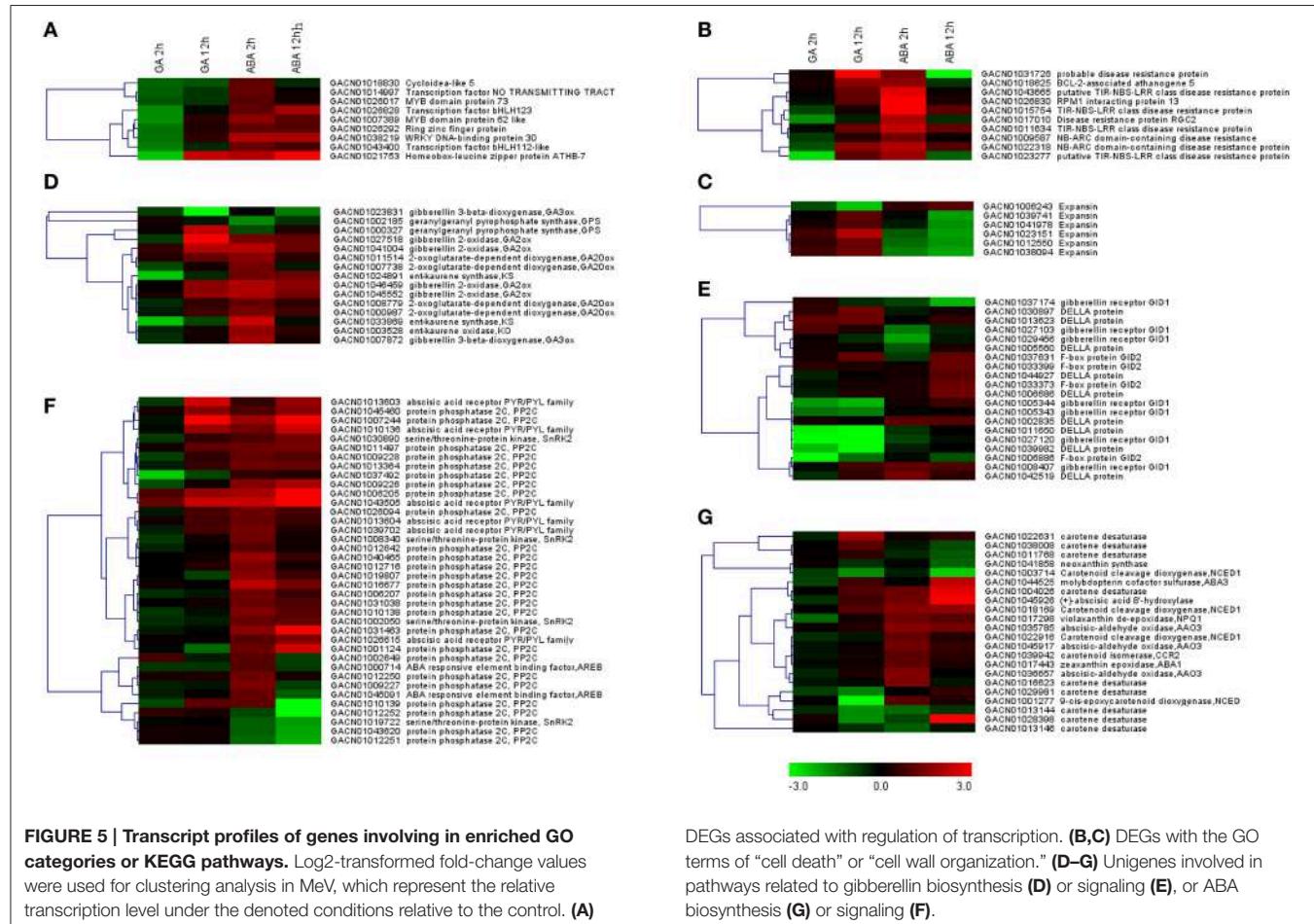
Further evidence at the transcriptional level demonstrates that GA and ABA have different and contrasting effects on global transcription profiles in petal, especially after 2 h treatment. Thus, the number of DEGs identified at 2 h following ABA treatment was higher than after GA treatment (Figure 3), similar to the findings obtained in *A. thaliana* (Nemhauser et al., 2006). Treatment for 12 h, however, did not result in dramatic differences, implying that time-course expression changes of many genes occurred from 2 to 12 h. These opposing effects on transcription were further backed by analyses of the DEGs and

the corresponding transcript profiles in response to GA or ABA treatment. It was found that the number of Class II DEGs (Supplemental Tables S8, S9) was greater than that of Class I or III (Figure 3C). Furthermore, while most Class II DEGs showed an elevation in transcript abundance between 2 and 12 h during GA treatment, they showed the opposite trend when treated with ABA. As expected, genes involved in pathways associated with the metabolism of diverse hormones were affected by both phytohormones. Taken together, these observations suggest that GA and ABA perturb various GRNs, resulting in antagonistic effects on petal growth.

### DEGs Associated with Hormone Pathways are Enriched by GA and ABA Treatment

Hormones have been reported to play a role in petal development in several plants. GA levels transiently increase in *Gaillardia* petals at the start of the corolla's fast growth stage, then decrease later on (Koning, 1985). In an *Arabidopsis* GA-deficient mutant, petal growth is arrested, but the defect is completely eliminated by application of GA (Goto and Pharis, 1999). A tomato GA-deficient mutant initiates flower buds, but floral development is not completed unless the mutant is treated with GA (Jacobsen and Olszewski, 1991). ABA, on the other hand, is usually associated with petal senescence, and accumulates to high levels in senescent rose petals (Kumar et al., 2008). Silencing a homeodomain-leucine zipper I transcription factor gene in rose delays ABA-induced petal senescence (Lü et al., 2014). In the current study, we demonstrated that GA treatment promotes, and ABA treatment suppresses, petal growth in *G. hybrida*. When the biosynthesis of endogenous GA and ABA are inhibited by PAC and FLU, respectively, the effects of GA and ABA treatment are reversed. These results suggest that the perturbation of endogenous GA and ABA biosynthesis in *G. hybrida* partly contributes to the antagonistic action of these two hormones on petal growth.

Our RNA-seq data also support the above hypothesis, showing that DEGs involved in hormone-associated pathways, including GA or ABA biosynthesis and signaling, are oppositely perturbed by GA or ABA treatment for 2 h. However, these differences are no longer apparent when the treatment is extended to 12 h (Figures 5D–G), indicating that transcriptional regulation of hormone-associated pathways is an early and transient event during petal growth. We interpret our results to further suggest that inhibition by GA and ABA of each other's effects on petal growth could result from at least two scenarios: (1) GA and ABA could target the same genes. For example, KS and KO, which contribute to GA biosynthesis (Sun, 2008), are regulated by both GA and ABA (Figure 5D). In addition, genes for TPR (tetra-tripeptide) repeat-containing protein (GACN01042178), CBL-interacting protein kinase (GACN01010590) and leucine-rich repeat transmembrane protein kinase (GACN01030993), which are annotated in both GA- and ABA-associated pathways (Van der Knaap et al., 1999; Rosado et al., 2006; Pandey et al., 2008), are also perturbed by both GA and ABA (Supplemental Tables S6, S8); (2) GA and ABA interfere with each other's biosynthetic or signaling pathways by an effect on the components of these pathways. For example, *GA2ox* proteins, which play roles in converting active GAs to inactive forms (Sun, 2008), are activated



**FIGURE 5 | Transcript profiles of genes involving in enriched GO categories or KEGG pathways.** Log<sub>2</sub>-transformed fold-change values were used for clustering analysis in MeV, which represent the relative transcription level under the denoted conditions relative to the control. (A)

by ABA, suggesting that ABA treatment could contribute to GA inactivation. That the *NCED* gene, which contributes to ABA biosynthesis (Finkelstein and Rock, 2002), is down-regulated by GA treatment (**Figure 5G**) also indicates that GA could affect ABA production. We suppose that this latter antagonistic effect of GA and ABA on each other's biosynthesis, in addition to their antagonistic effects on hormone signaling, contributes directly to the rapid responses in petal to the presence of both hormones.

Putative crosstalk nodes coupling diverse hormone-associated pathways were also identified in this study. *PAO5* (GACN01003808) and *MMS21* homologs (GACN01025802), involved in the cytokinin signaling pathway (Brenner et al., 2005; Huang et al., 2009), respond to GA and ABA antagonistically (Supplemental Table S8). The homolog of a member of the PP2C family (GACN01010139), which is predicted to be enriched during an ethylene- and auxin-induced response or jasmonic acid- and salicylic acid-mediated signaling (Heyndrickx and Vandepoele, 2012), is up-regulated by GA but down-regulated by ABA (Supplemental Table S9). Other hormone pathways, including CK, ET, brassinosteroi (BR), JA and salicylic acid (SA) signaling, are also altered by GA and/or ABA (Supplemental Table S5). These data, in accordance with the report by Nemhauser et al. (2006), support the hypothesis that

*G. hybrida* petal growth is regulated by a substantial network of interconnected hormonal pathways and feedback circuits.

## **DEGs Involved in Transcriptional Regulation, Apoptosis and Cell Wall Organization**

Our data show that cell expansion is critical for petal growth at later stages. GO-BP enrichment analyses for Class III DEGs at 12 h show that the category “cell wall organization” especially the sub-category “cell wall loosening” is overrepresented (**Table 2**), and the DEGs involved, encoding a group of expansins, are activated by GA, but repressed by ABA. Expansins are considered to be molecular markers of cell elongation (Bai et al., 2012; Ikeda et al., 2012). The enrichment of these genes after 12 h hormone treatment is consistent with the notion that GA-induced cell wall loosening contributes to cell elongation during petal growth.

Analysis of the Class II DEGs indicated that the GO category "apoptosis" changes rapidly during the 10 h interval between the two sampling periods, apparently in a GU/AD pattern (**Table 2**). As explored further below, this suggests an intriguing relationship between petal development and apoptosis during GA and ABA treatment. BlastX searches indicated that the genes involved code for a group of pathogen-associated disease resistance proteins. No clear association between these proteins and petal

development has yet been described, but a TIR-NB-LRR protein (Kim et al., 2012) provides one example where disease resistance proteins contribute to plant development, suggesting that these proteins are functional in diverse biological processes. On the other hand, it is well established that pathogen-induced plant immunity is regulated by GA and ABA. For example, ABA can induce plant immunity-associated callose deposits by which an efficient pathogen-resistant barrier is formed (Luna et al., 2011). Mutations in the genes involved in ABA biosynthesis or signaling can enhance resistance to some pathogens (Sánchez-Vallet et al., 2012). It is also evident that GA and DELLA proteins are linked to disease responses and the associated cell wall modification (De Bruyne et al., 2014). Apoptosis is a critical event during plant development, as well as in pathogen-induced plant immunity. For example, vascular development in the plant coordinates the different phases of xylem maturation, including secondary wall formation, cell death and other processes (Bollhoner et al., 2012). Cell death is included in the hypersensitive responses induced by pathogens in the plant (Lam et al., 2001). However, the contribution of apoptosis to GA/ABA-regulated petal development remains to be elucidated.

The third group of DEGs enriched in GA- and ABA-treated petals is involved in regulation of transcription. There were nine TFs that appeared after 2 h treatment and showed a GD/AU pattern (**Table 2**). The homologs of some of these proteins are reported to be regulated by GA and ABA in other plant species. For example, the *Arabidopsis* homolog of the putative *G. hybrida* MYB62 (GACN01007389) plays a role in GA biosynthesis and signaling pathways. Overexpression of the *AtMYB62* gene results in a GA-deficient phenotype that can be partially reversed by exogenous application of GA (Devaiah et al., 2009). The *G. hybrida* DEG, GACN01021753, encodes a putative member of the homeodomain leucine zipper (HD-Zip) family. Overexpression of some members of the HD-Zip family in *Arabidopsis* and *Oryza sativa* (rice) affects organ elongation and expansion by modulation of GA and/or ABA metabolism and signaling (Agalou et al., 2008; Son et al., 2010). The DEG GACN01038219 encodes a putative WRKY protein. Two WRKY homologs in rice, OsWRKY51 and OsWRKY71, interact in aleurone cells and establish a novel mechanism of crosstalk between ABA and GA signaling (Xie et al., 2006). In addition, we found that GACN01018830 codes for a homolog of the TCP-domain containing protein, CYCLOIDEA-like 5 (CYC5). CYC homologs are

reported to be involved in flower symmetry regulation in many plant species (Martin-Trillo and Cubas, 2010). In *G. hybrida*, genes for CYC homologs were also identified previously (Brolholm et al., 2008; Tahtiharju et al., 2012). Expression analysis during ray flower ligule development indicates that *GhCYC3* contributes to early petal growth and correlates with cell division, while *GhCYC5* is instead activated at late stages when elongation growth is ceasing (Kotilainen et al., 1999; Juntheikki-Palovaara et al., 2014). Integrating the previously reported findings with our data in the current study, we conclude that these various transcription regulators contribute to cell growth in the petal of *G. hybrida*. Under the influence of GA, genes involved in “cell wall loosening” and “apoptosis” are released from transcriptional repression by these TFs. ABA, on the contrary, activates these TF genes and result in repression of cell elongation.

In summary, we have identified a group of DEGs from the basal region of the petals of *G. hybrida* that show antagonistic transcription profiles during GA and ABA treatment. Annotation enrichment analyses further clarified the biological processes and pathways involved, as well as the co-targets for both hormones. Our data support the hypothesis that cell expansion in *G. hybrida* petals at inflorescence stage 3 is attributed to the regulation of transcription and apoptosis, which consequently lead to activation of cell wall loosening. GA and ABA work antagonistically to balance the responses to developmental signals and guarantee the smooth running of this network.

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## Supplementary Material

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# Molecular phenotypes associated with anomalous stamen development in *Alternanthera philoxeroides*

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*Alternanthera philoxeroides* is a perennial amphibious weed native to South America but has now spread to diverse parts of the world. *A. philoxeroides* reproduces both sexually and asexually in its native range, but propagates solely through vegetative means in its introduced range. Traits associated with sexual reproduction become degraded for sexual dysfunction, with flowers possessing either pistillate stamens or male-sterile anthers. Degradations of sexual characters for loss of sexuality commonly take place in clonal plants. The underlying molecular-genetic processes remain largely unknown. We compared the gene expression profiles of abnormal stamens with that of normal stamens by RNA-Seq analysis, and identified a large number of differentially expressed genes between abnormal and normal stamens. In accordance with flower morphology, the expression of B-class MADS-box genes (*ApAP3*, *ApTM6*, and *ApPI*) was markedly reduced in pistillate stamens. However, most of the genes involved in meiosis were expressed normally in stamens with male-sterile anthers. In addition to verifying the expression patterns of genes previously known to be related to stamen and pollen grain development, we also identified previously unknown molecular phenotypes associated with sexual dysfunction in *A. philoxeroides*, that is helpful for dissecting the molecular mechanisms underpinning various male-sterile phenotypes and the molecular processes underlying the transition from sexuality to asexuality in clonal plants.

**Keywords:** *Alternanthera philoxeroides*, sexual dysfunction, aberrant stamen development, male sterility, molecular phenotypes

## Introduction

*Alternanthera philoxeroides*, commonly known as alligator weed, is a perennial amphibious weed native to South America, but has now spread to diverse parts of the world, showing up in North and South America, France, Italy, Australia, New Zealand, China, and other parts of Asia. *A. philoxeroides* can grow in a variety of habitats, including open lands, waterway banks, ponds, and lakes. Individuals growing in aquatic and terrestrial habitats showed extensive variations in leaf size and shape, stem diameter and internode length, but exhibited little genetic differentiation within and among populations (Ye et al., 2003; Geng et al., 2007). It has thus been proposed that phenotypic plasticity, rather than locally adapted ecotypes, allows *A. philoxeroides* to colonize a wide range of habitats (Geng et al., 2006, 2007; Li and Ye, 2006).

*Alternanthera philoxerooides* reproduces both sexually and asexually in its native range, but propagates mainly through vegetative means via storage root and stem fragmentation in its introduced range and does not produce viable seeds (Julien and Stanley, 1999; Sosa et al., 2007). Extensive field survey of the introduced *A. philoxerooides* in China revealed various patterns of anomalous floral development (Chen, 1964). The most striking aberration is the homeotic transformation of stamens into pistils or pistil-like structures (**Figure 1**). The complete pistillate flowers do not have stamens but five pistil-like structures and one normal pistil. Unlike the normal pistil, the ‘pistils’ transformed from stamens often develop ovary-like structures but contain no ovule inside (Chen, 1964; Hu et al., 2011). Monoclinous flowers possessing both stamens and pistils are common in natural populations. However, the anthers of these flowers are often shriveled bearing no or few non-viable pollen grains (Hu et al., 2011; Wang et al., 2011). There also exist some incomplete pistillate flowers with intermediate phenotypes between monoclinous and complete pistillate plants.

The ABC(DE) model is now widely used as a framework for understanding the molecular mechanisms controlling floral organ identity (Coen and Meyerowitz, 1991; Theissen, 2001). According to the model, the differentiation of floral organs is controlled by the differential expression of several subsets of homeotic genes belonging to the MADS-box gene family, except for the A-class gene *APETALA2* (Riechmann and Meyerowitz, 1997; Vandebussche et al., 2003). The co-expression of B- and C-class MADS-box genes establishes the identity of the stamens (Coen and Meyerowitz, 1991; Jack et al., 1992). B-function mutants produce homeotic transformation of stamens into carpels (Jack et al., 1992; Goto and Meyerowitz, 1994). It is unclear whether pistillody in *A. philoxerooides* was caused by altering the expression pattern of the B-class MADS-box genes. Additionally, the cytotype of *A. philoxerooides* found in China is a hexaploid (Cai et al., 2009). Sosa et al. (2007) suggested a hybrid origin of the invasive hexaploid of *A. philoxerooides*, and that meiotic abnormalities due to the formation of univalents/multivalents led to microspore degeneration which resulted in anthers bearing no pollen grains. Hu et al. (2011) found, however, that the microspore tetrads were formed and separated normally in the anthers of the hexaploid *A. philoxerooides*, but

the protoplasm of most pollen grains disintegrated at the post-maturation stage and pollen grains became empty, with only a few non-viable pollen grains left in the anthers.

Male sterility in plants has received considerable attention because of its potential value in breeding and hybrid seed production. It is also of great importance in evolutionary studies on the origin of dioecy (Sawhney and Shukla, 1994; Singh et al., 2012). The phenotypic manifestations of male sterility are diverse in plants, including the complete absence of male organs, the failure to develop normal sporogenous tissues (no meiosis), the abortion of pollen at any step of its development, and the inability of mature pollen to germinate on compatible stigma (Budar and Pelletier, 2001; Singh et al., 2012). The conversion of stamens to different type of floral organs also represents a male-sterile condition (Sawhney and Shukla, 1994). Although multiple genes and proteins related to microspore and pollen abortion have been characterized (Yang et al., 2003a; Jung et al., 2006; de Azevedo Souza et al., 2009), the genetic and molecular mechanisms underpinning various male-sterile phenotypes are still poorly understood. Furthermore, loss of sexuality is common in invasive clonal plants (Eckert and Barrett, 1993; Eckert, 2002; Barrett et al., 2008). Degeneration of sex can be caused by environmental and/or genetic factors. It is possible that the sexual infertility in sterile polyploids is due to polyploidy *per se* (Eckert, 2002). Traits associated with sexual reproduction may become degraded for sexual dysfunction, especially in plants that are sexually infertile and reproduction is solely clonal. However, there seems to have been a general lack of interest in dissecting the molecular-genetic processes associated with sexual infertility and degradation of sexual characters in clonal plants, even though they have arisen repeatedly in many groups of plants (Eckert, 2002). Investigation of the genetic architecture and molecular mechanisms underlying the transition from sexuality to asexuality in clonal plants will not only extend our understanding of the genetic control of reproductive organ development, but may also provide insights into the mechanisms and evolutionary pathways of sexual sterility in clonal plants.

Molecular phenotypes are important links between genomic information and organismic functions, fitness, and evolution (Held et al., 2014). In this study, we compared the gene expression profiles of abnormal stamens with that of normal stamens by RNA-Seq analysis. A large number of differentially expressed genes between abnormal and normal stamens were captured. The pistillate stamens exhibited a molecular phenotype distinct from that of the stamens with male-sterile anthers. In addition to verifying the expression patterns of genes previously known to be related to stamen and pollen grain development, we identified molecular phenotypes previously unknown to be associated with sexual dysfunction in *A. philoxerooides* that will be helpful in future analyses.

## Materials and Methods

### Plant Materials

Plants producing normal fertile flowers were collected from Argentina and maintained in the botanical garden of Yunnan



**FIGURE 1 |** Images of representative *Alternanthera philoxerooides* flowers: normal flowers (left), pistillate flowers (middle), and male-sterile flowers (right).

University (E102°42', N25°03', Kunming, China). Pistillate flowers and male-sterile flowers were collected from plants growing in natural habitats close to the botanical garden. Flower heads containing flowers at different developmental stages were collected and preserved in RNAlater solution (Life Technologies, Gaithersburg, MD, USA). Five individuals were sampled from each type of flowers.

## RNA Extraction, cDNA Library Construction and Illumina Sequencing

RNAs of normal flowers, male-sterile flowers, and pistillate flowers were extracted using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) and purified with the on-column DNase I digestion (Qiagen) following the manufacturer's instructions. RNA quality was visually checked on a 1% agarose gel and by a Nanodrop 2000c Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). RNA integrity was further verified by an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

cDNA libraries were constructed following the High-Throughput Illumina Strand-Specific RNA Sequencing Library protocol (Zhong et al., 2011). Briefly, poly A containing mRNA was purified from total RNA and then fragmented into small pieces. Double-stranded cDNA was synthesized from the fragmented cDNA, and Illumina sequencing adapters were ligated to the ends of the fragments. Libraries were sequenced using the HiSeq 2000 System (Illumina, San Diego, CA, USA).

## *De novo* Transcriptome Assembly and Gene Annotation

Raw reads generated by the sequencing machine were filtered to obtain high-quality reads. Reads containing adaptor sequences were discarded. Reads with a PHRED quality score below 20 were also removed. *De novo* assembly was carried out using the Trinity software with default settings and a minimum contig length of 200 bp (Grabherr et al., 2011). Assembled contigs were used as input for a second assembly made with CAP3 (Huang and Madan, 1999). Redundancy was reduced using CD-HIT with a sequence similarity threshold of 95% (Li and Godzik, 2006). *De novo* assembled sequences were annotated using BLASTX against the *Arabidopsis thaliana* protein database<sup>1</sup> (TAIR10\_peptide), with an *e*-value cut-off of  $10^{-10}$ . BLAST searches against the Phytozome database<sup>2</sup> were then done for unannotated sequences.

Clean reads from each sample were mapped back to the *de novo* assembled reference transcriptome. Gene expression levels were calculated from the number of uniquely aligned clean reads and then normalized into units of Reads Per Kilobase per Million reads mapped (RPKM; Mortazavi et al., 2008). Differentially expressed transcripts were detected using an False Discovery Rate (FDR) value cut-off  $\leq 0.001$  and the absolute value of log<sub>2</sub> ratio  $\geq 1$ . GO enrichment analysis for biological processes was carried out utilizing Fisher's exact test with default parameters ( $p < 0.01$ ) by the R package topGO (Alexa and Rahnenfuhrer,

2010). The REViGO web server<sup>3</sup> was used to reduce the redundancy and visualize the overrepresented GO terms based on semantic similarity (Supek et al., 2011).

## Identification and Cloning of B-class MADS-Box Genes and Meiotic Genes in *A. philoxerooides*

Putative *A. philoxerooides* AP3, TM6, and PI sequences were used as queries to conduct BLAST searches against the NCBI databases<sup>4</sup> to find homologous sequences. Multiple alignments of the retrieved sequences were constructed using ClustalW 2.0 (Larkin et al., 2007). A neighbor-joining tree was reconstructed by MEGA 6.0 (Tamura et al., 2013) using the Jones-Taylor-Thornton (JTT) model. Support for each node was tested using bootstrap method with 1000 replicates. Gene-specific primers were designed for amplifying conserved motif of each gene. PCR products were cloned into pMD 19-T vector (TaKaRa, Dalian, China) and confirmed by Sanger sequencing. Based on conserved motif sequences, gene-specific primers (Supplementary Table S1) were designed for conducting RACE-PCR to amplify target 5' and 3' cDNA ends, using SMARTer RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). Amplification products of 5' and 3' RACE were then cloned and sequenced to get full-length cDNAs. Four meiotic genes identified from *A. philoxerooides*, *ApASY1*, *ApMLH3*, *ApMPK4*, and *ApMMD1*, were also cloned and sequenced. They are responsible for homologous chromosome synapsis (Armstrong et al., 2002), crossover formation (Jackson et al., 2006), male-specific meiotic cytokinesis (Zeng et al., 2011) and general meiotic cell cycle progression (Yang et al., 2003b), respectively.

## Quantitative Real-Time PCR (qRT-PCR) Analysis

The expression patterns of B-class MADS-box genes and genes involved in meiosis were analyzed by quantitative real-time PCR (qRT-PCR). For B-class gene analysis, total RNAs were isolated from leaves, sepals, stamens, and carpels of normal and pistillate flowers, respectively. For meiotic gene analysis, total RNAs were isolated from the stamens at early developmental stages of normal and sterile flowers. The first-strand cDNA was made from 2 µg of total RNA using PrimeScript™ RT Master Mix Perfect Real Time (TaKaRa, Dalian, China) following the manufacturer's recommendations. The gene-specific primers used for qRT-PCR (Supplementary Table S1) were designed using PRIMERS3<sup>5</sup>. Real-time PCR was performed on a Roche LightCycler® 2.0 machine (Roche diagnostics, Mannheim, Germany) using SYBR® Premix Ex TaqTM II (TliRNaseH Plus; TaKaRa, Dalian, China). The cycling parameters are as follows: initial denaturation (95°C for 30 s), 40 amplification cycles (95°C for 5 s and 60°C for 20 s), and followed by a melt cycle (60°C for 15 s). All reactions were run with three biological replicates and each with three technical replicates. *UBC10* was used as the reference gene to normalize the gene expression level. Quantification of the relative changes

<sup>1</sup><http://www.arabidopsis.org/>

<sup>2</sup><http://www.phytozome.net/>

<sup>3</sup><http://revigo.irb.hr/>

<sup>4</sup><http://www.ncbi.nlm.nih.gov/>

<sup>5</sup><http://bioinfo.ut.ee/primer3/>

in gene expression was performed using the  $2^{-\Delta\Delta CT}$  method (Livak and Schmittgen, 2001). Data represented three biological replicates with three technical replicates and were shown as average, with error bars representing standard deviations. Duncan's test was used to determine the statistical significance of differences.

## Results

### **De novo Transcriptome Assembly and Annotation**

cDNA libraries were constructed and sequenced for the normal flower, male-sterile flower, and pistillate flower, respectively. A total of 107,160,189 raw reads accounting for 21.6 Gb of raw data were generated for the three libraries. After filtering, 104,487,087 clean reads (20.1 Gb) were retained and used for *de novo* assembly (**Table 1**). Each library was assembled independently, and then merged to generate the final assembly. After redundancy removal, a final set of 208,082 transcripts ( $\geq 200$  bp) were obtained, with a mean length of 870 bp and N50 of 1,514 bp (**Table 2**). Of the transcripts retained, 169,183 (81.3%) transcripts were expressed in all three samples and 4.3% only in normal flowers.

A total of 83,878 (40.3%) transcripts were matched to 15,273 *A. thaliana* genes, covering 56.4% of *A. thaliana* genome. 3,980 (1.9%) transcripts were further identified by BLAST searches against the Phytozome database. Among the annotated transcripts, 56 were associated with A-, B-, C-, and E-class MADS-box genes (Supplementary Table S2), and 168 associated with 31 known meiotic genes responsible for homologous chromosome synapsis (Armstrong et al., 2002), male-specific meiotic cytokinesis (Zeng et al., 2011), general meiotic cell cycle progression (Yang et al., 2003b), and meiotic recombination (Osman et al., 2011), respectively (Supplementary Table S3).

### **Detection of Differentially Expressed Genes**

To identify molecular phenotypes associated with different patterns of anomalous stamen development in *A. philoxerooides*, expression patterns of annotated transcripts were compared between different types of flowers. Comparison between normal and pistillate flowers revealed 11,015 up-regulated and 8,591 down-regulated transcripts in the pistillate flower, using a FDR of 0.1% (**Figure 2**). 12,761 and 12,364 transcripts were up- and down-regulated, respectively, in the male-sterile flower compared to the normal flower. Transcripts associated with B-class MADS-box genes exhibited lower expression in the pistillate flower, while transcripts associated with A-, C-, and E-class genes showing no significant differences between normal and pistillate flowers

(Supplementary Table S2). Transcripts associated with meiotic genes did not show significant decreases in male-sterile flowers compared with normal flowers, with the exception of transcripts associated with *AtMSH5* that showed decreased expression in the male-sterile flower (Supplementary Table S3).

GO term enrichment analysis of differentially expressed genes revealed additional enriched functional categories. Genes involved in polyketide biosynthesis, oligopeptide transport, anther wall tapetum development, pectin catabolism, and negative regulation of endopeptidase activity, showed decreased expressions in the pistillate flower (**Figure 3**). GO terms associated with the response to red or far red light, negative regulation of circadian rhythm, ATP-dependent chromatin remodeling and protein acetylation were also enriched in the down-regulated genes of the pistillate flower. In addition, GO term annotation highlighted that genes involved in the jasmonic acid (JA) mediated signaling pathway were strongly overrepresented among the differentially expressed genes between normal and male-sterile flowers (**Figure 4**), and most of these genes were expressed increasingly in the male-sterile flower. Genes involved in the biosynthesis of constituents required for pollen wall development and pollen maturation, such as sporopollenin, xanthophyll, cellulose, pectin, lipid, sugar, and various pollen proteins, were included in the supercluster of JA mediated signaling pathway (**Figure 4**).

### **Validation the Expression of B-Function and Meiotic Genes in Anomalous Stamens**

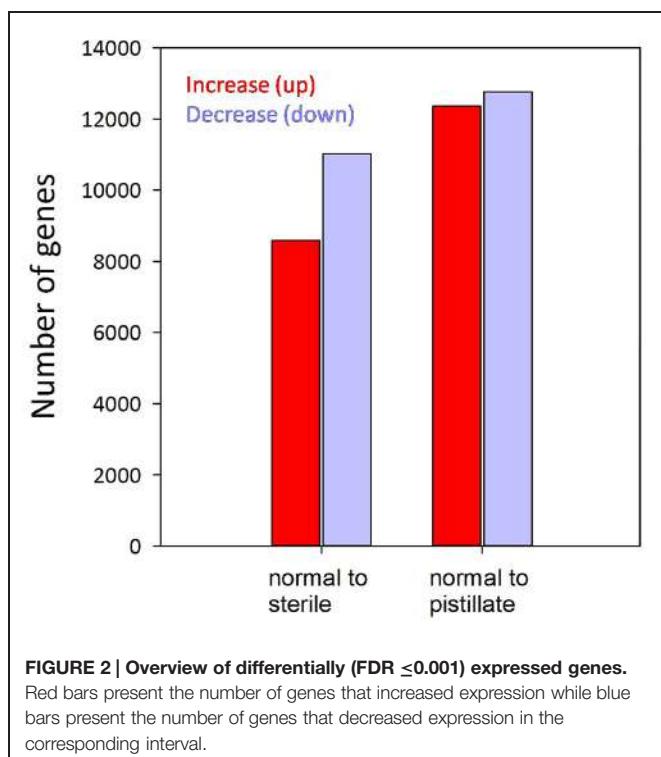
Full-length cDNAs of three B-class MADS-box genes were obtained from *A. philoxerooides*. They were clustered, respectively, with the *AP3*, *TM6*, and *PI* orthologs from other plant species in the phylogenetic tree (Supplementary Figure S1), and were thus designated, respectively, as *ApAP3*, *ApTM6*, and *ApPI*. *ApAP3* was 675 bp in length with an open reading frame corresponding to 224 deduced amino acid residues. *ApTM6* was 717 bp long, encoding a 238 amino acid protein, while *ApPI* containing a 654 bp open reading frame. The expression patterns of *ApAP3*, *ApTM6*, and *ApPI* in normal and pistillate flowers were validated by qRT-PCR. The results showed that three B-class genes were all expressed in the sepals of both flowers. However, the expression levels of *ApTM6* and *ApPI* were very low, and there were no significant differences in expression levels of three genes between two types of flowers (**Figure 5**). Three B-class genes were all highly expressed in the stamens of normal flowers, but the expression levels decreased by 73.2, 70.1, and 54.2%, respectively, in the stamens of pistillate flowers. Expressions of *ApAP3* and *ApTM6* were also detected in the carpels of both flowers but not for *ApPI*.

**TABLE 1 | Summary of sequencing statistics.**

|                                     | Normal flowers | Pistillate flowers | Male-sterile flowers | Total          |
|-------------------------------------|----------------|--------------------|----------------------|----------------|
| Total raw reads                     | 49,113,802     | 34,075,245         | 23,971,142           | 107,160,189    |
| Total raw bases (bp)                | 9,920,988,004  | 6,883,199,490      | 4,842,170,684        | 21,646,358,178 |
| Number of reads after trimming      | 48,046,265     | 33,347,238         | 23,093,584           | 104,487,087    |
| Number of bases after trimming (bp) | 9,286,674,933  | 6,463,064,464      | 4,432,813,071        | 20,182,552,468 |

**TABLE 2 | Summary of *de novo* assembly results.**

|  | No          |
|--|-------------|
| Total number of high quality assembled reads | 104,487,087 |
| Number of transcripts                        | 208,082     |
| Mean length (bp)                             | 870         |
| N50 (bp)                                     | 1,514       |
| Longest transcript (bp)                      | 18,515      |
| Number of transcripts > 5 Kb                 | 663         |
| Number of transcripts > 10 Kb                | 23          |

**FIGURE 2 | Overview of differentially (FDR ≤ 0.001) expressed genes.**

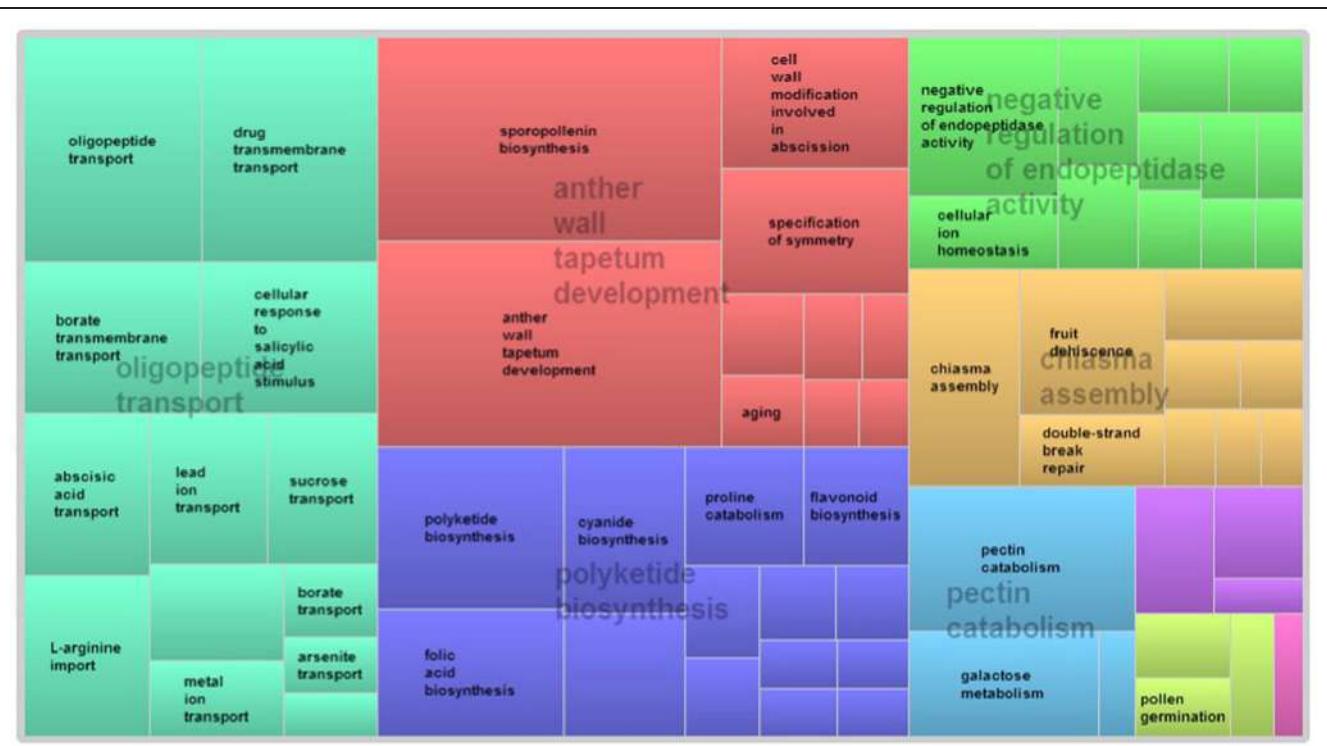
Red bars present the number of genes that increased expression while blue bars present the number of genes that decreased expression in the corresponding interval.

The expression patterns of four meiotic genes, *ApASY1*, *ApMLH3*, *ApMPK4*, and *ApMMD1* involved in different processes of meiosis, were also validated by qRT-PCR. They were all expressed in the stamens of normal and male-sterile flowers, and did not show significant differences in expression levels between normal and male-sterile flowers (Figure 6).

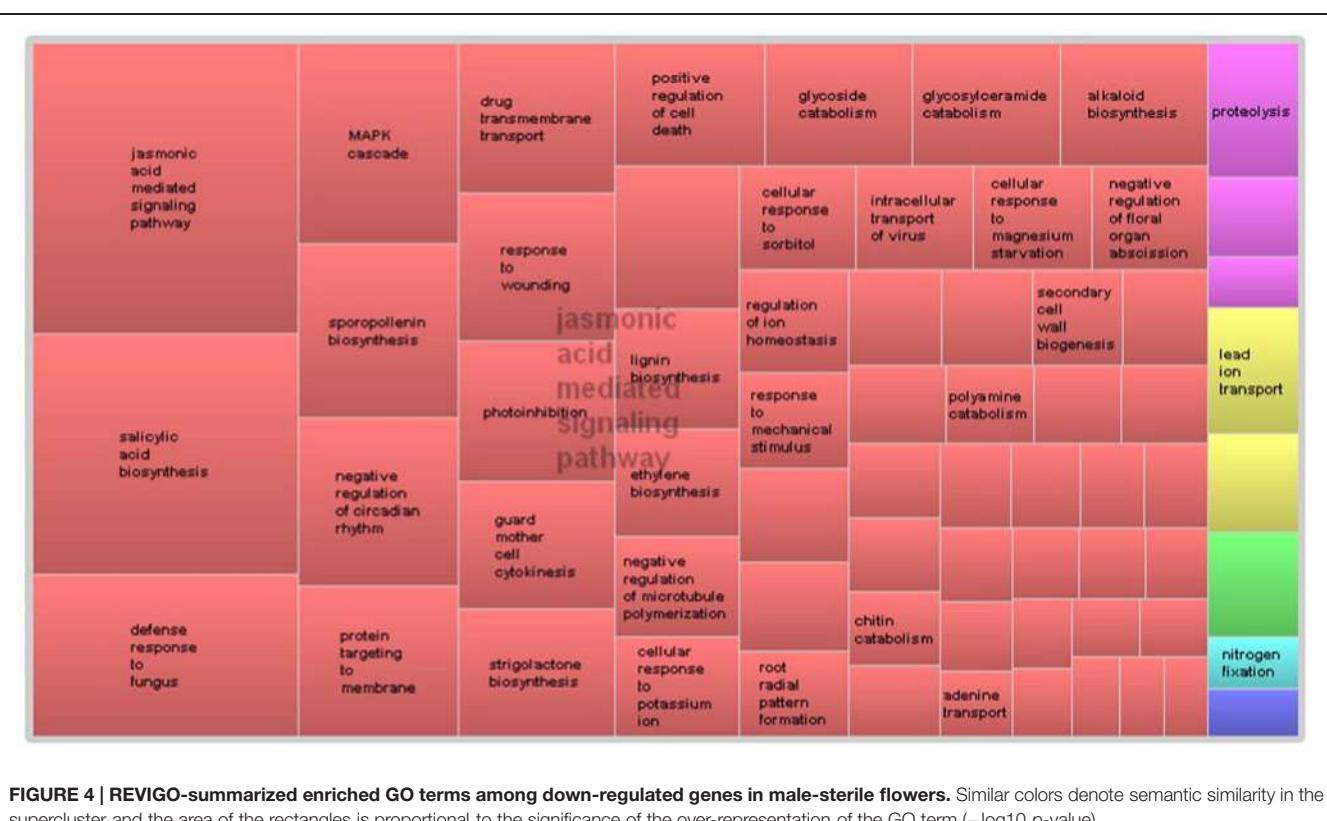
## Discussion

Comparative transcriptome analysis revealed overall differences in gene expression between normal and anomalous flowers. Altered expressions of genes associated with stamen development were confirmed with qRT-PCR analyses. As revealed in other plants (de Martino et al., 2006), the expression levels of B-function MADS-box genes were significantly decreased in the stamens of *A. philoxerooides* pistillate flowers. B-function genes physically interact with C- and E-function genes to form quaternary complexes to specify stamen development (Airoldi,

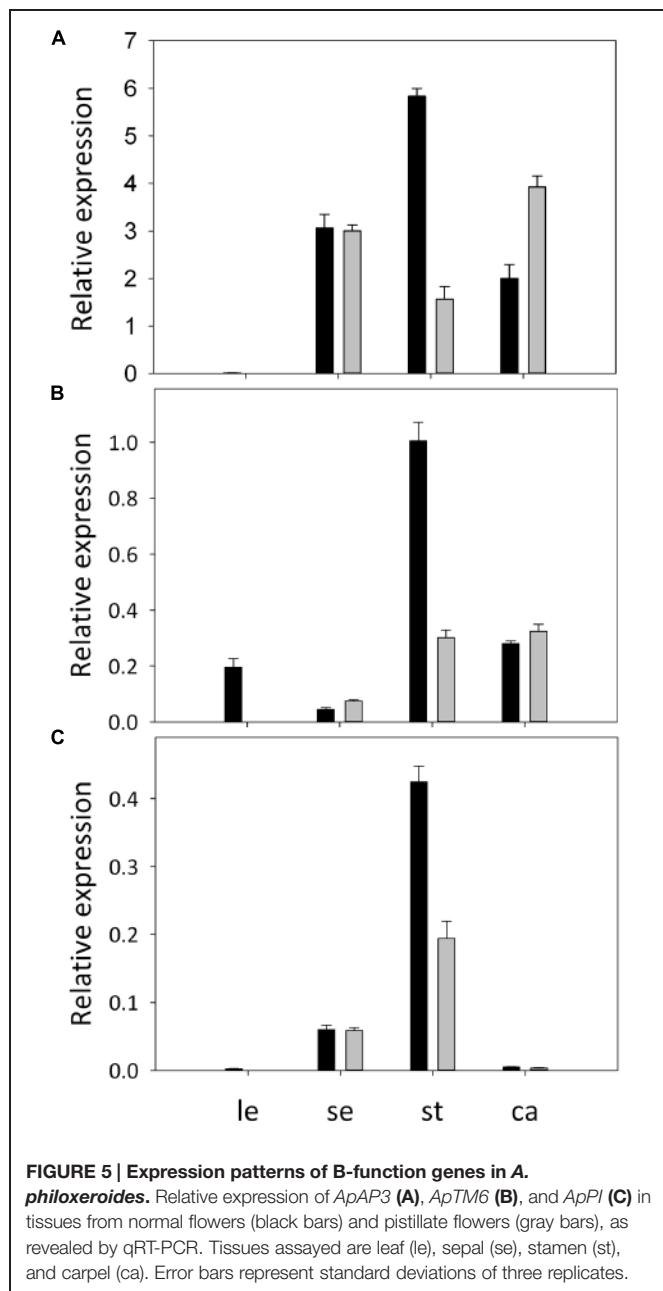
2010). Because the expression levels of C- and E-function genes remained in *A. philoxerooides* pistillate flowers, the decreased expression of B-class genes was evidently responsible for the homeotic transformation of stamens into carpels in *A. philoxerooides*. Our results are in agreement with previous studies on homeotic variation in flowers. de Martino et al. (2006) showed that decreased expression of only one B-class gene could result in a complete transformation of the stamens into carpel-like organs in tomato. The deficiency of B-class MADS-box genes also caused homeotic conversions of stamens into carpels in *Arabidopsis* (Jack et al., 1992; Goto and Meyerowitz, 1994), *Antirrhinum* (Schwarz-Sommer et al., 1992; Tröbner et al., 1992), tomato (Rasmussen and Green, 1993; Olimpieri and Mazzucato, 2008) and wheat (Hama et al., 2004; Yamada et al., 2009). The reduced expression of B-function genes in *A. philoxerooides* seems not to result from the loss-of-function mutation in B-class genes because, by cloning and sequencing *ApAP3*, *ApTM6*, and *ApPI* from different plants, we did not find significant sequence variation between normal and pistillate flowers. Deng et al. (2011) and Liu et al. (2011) showed that environmental variation, especially soil nutrient heterogeneity, can induce floral gender transformation in *A. philoxerooides*, with the stamens of monoclinous flowers being completely or partially transformed into carpels (Deng et al., 2011; Liu et al., 2011). It is unclear, however, by which mechanisms the change in environment is sensed, transduced, and finally elicits modifications to the selective expression of B-class genes in different habitats. In addition to B-class genes, transcriptome analysis also revealed other genes that were differentially expressed between normal and pistillate flowers, and were enriched for a wide range of molecular function categories. The differential expression of genes involved in GA signaling and epigenetic regulation is of special interest. It has been revealed that floral homeotic genes (*AP3* and *PI*) were targets of GA signaling in flower development (Yu et al., 2004). GA probably promoted stamen development by upregulating expression of the floral meristem identity gene *LEAFY* (*LFY*), which in turn upregulates expression of the B-class MADS-box gene *AP3* (Plackett et al., 2011). Reduction in GA synthesis might lead to a reduced expression of *AP3*, and thereby produces abnormal flowers with carpelloid stamens (Kamata et al., 2013). Studies on the *stamenless* mutant also showed evidences that stamen identity in tomato depended on gene–hormone interactions (Quinet et al., 2014). Additionally, it has been shown that epigenetically regulated ectopic expression of flower homeotic genes may alter floral organ identity (Kapoor et al., 2005; Pu et al., 2013). Histone modification and ATP-dependent chromatin remodeling are also involved in the regulation of spatiotemporal-specific expression of genes that lead to patterning, specification, and morphogenesis of flowers (Gan et al., 2013). Mutation in the chromatin-remodeling ATPases BRAHMA led to the occurrence of carpelloid structures in the third whorl of *Arabidopsis* flowers (Hurtado et al., 2006; Wu et al., 2012). It has been suggested that MADS-domain proteins may closely interact with chromatin remodeling factors to facilitate chromatin opening and transcription initiation (Smaczniak et al., 2012; Guo et al., 2015).



**FIGURE 3 |** REVIGO-summarized enriched GO terms among down-regulated genes in pistillated flowers. Similar colors denote semantic similarity in the supercluster and the area of the rectangles is proportional to the significance of the over-representation of the GO term ( $-\log_{10} p\text{-value}$ ).

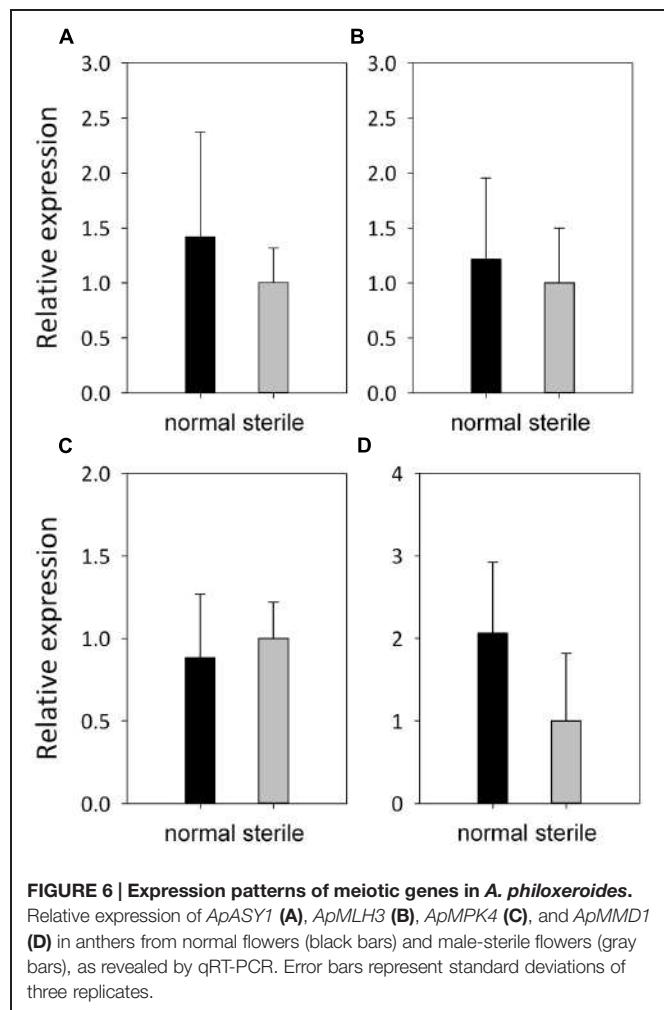


**FIGURE 4 |** REVIGO-summarized enriched GO terms among down-regulated genes in male-sterile flowers. Similar colors denote semantic similarity in the supercluster and the area of the rectangles is proportional to the significance of the over-representation of the GO term ( $-\log_{10} p\text{-value}$ ).



**FIGURE 5 | Expression patterns of B-function genes in *A. philoxerooides*.** Relative expression of *ApAP3* (A), *ApTM6* (B), and *ApPI* (C) in tissues from normal flowers (black bars) and pistillate flowers (gray bars), as revealed by qRT-PCR. Tissues assayed are leaf (le), sepal (se), stamen (st), and carpel (ca). Error bars represent standard deviations of three replicates.

Most of the meiotic genes investigated in this study were normally expressed in the male-sterile flower of *A. philoxerooides*. This result was inconsistent with our original hypothesis. The cytotype of *A. philoxerooides* found in China is a hexaploid, with approximately 100 chromosomes. Abnormal male meiosis is often used as a cytological explanation for pollen sterility in polyploid plants. The most common meiotic abnormalities were those related to irregular chromosome segregation due to polyploidy, leading to the formation of chromosomally imbalanced gametes and aneuploidy. To date, little cytogenetic work has been done on the meiotic process of the invasive *A. philoxerooides*, due to the small size and apparent similarity of the chromosomes. It is unclear whether meiosis proceeds normally in the



*A. philoxerooides* male-sterile flower. The normal expression of meiotic genes in the male-sterile flower seems to suggest that the meiotic abnormality is unlikely responsible for the pollen sterility observed in *A. philoxerooides*, or segregation defects sometimes occur during meiosis II after meiosis I has proceeded normally. Other anther developmental defects may also generate male-sterile phenotypes (Sanders et al., 1999; Sakata and Higashitani, 2008). In consistent with this prediction, we found that many genes involved in the JA mediated signaling pathway were strongly down-regulated in the male-sterile flower. JA is critical for late stages of stamen development, regulating filament elongation, anther opening, and pollen maturation. (Turner et al., 2002; Song et al., 2013; Wasternack and Hause, 2013). *Arabidopsis* mutants impaired in JA biosynthesis exhibited non-viable pollen and delayed anther dehiscence (Wasternack and Hause, 2013). JA signaling also played crucial roles in a variety of biosynthetic pathways for the components of pollen intine and exine, and various storage materials accumulated during pollen maturation (Mandaokar et al., 2003, 2006; Wasternack and Hause, 2013). As a result of defects in the JA signaling pathway,

a lot of genes involved in the biosynthesis of constituents required for pollen wall development and pollen maturation were also down-regulated in the male-sterile flower of *A. philoxerooides*. Thus, defects in JA synthesis and/or JA signaling, as well as subsequent physiological disorders, might be potential causes for male sterility in *A. philoxerooides*.

Overall, the invasive *A. philoxerooides* exhibited a high level of plasticity in stamen development. This high level of plasticity is clearly resulted from relaxed selective constraints on sexual reproduction. After being introduced into China, *A. philoxerooides* spreads mainly by vegetative (clonal) propagules, though it retains the principal ability to reproduce both sexually and asexually in its native range. Although genetic factors, such as changes in ploidy, may play a role in causing reduced sexual fertility, the shift toward asexual reproduction is more likely promoted by biotic and/or abiotic limiting factors of the environment in exotic *A. philoxerooides* populations. Clonal reproduction probably helps the plants of *A. philoxerooides* to overcome the negative effects associated with low population densities during colonization and enhances exploitation of ubiquitous environmental heterogeneity, facilitating range expansion. Asexual reproduction is particularly common among introduced species (Kronauer et al., 2012), and shifts from sexual to asexual reproduction in the exotic range have been observed in several clonal invaders (Sculthorpe, 1967; Ornduff, 1987; Hollingsworth and Bailey, 2000). Repeated cycles of colonization and low-density may favor uniparental reproduction because selfing and asexuality provide plants with reproductive assurance (Eckert et al., 2006; Barrett et al., 2008). Meanwhile, genetic sterility may be induced by environmental suppression of sexual recruitment because natural selection no longer strongly maintains the traits involved in sex (Eckert, 2002). As a result, 'neutral' sterility mutations and developmental abnormalities accumulate in highly clonal populations, as shown in exotic *A. philoxerooides* plants. Thus, the occurrence of various types of stamen abnormalities could be explained by the hypothesis that sex were degraded for they no longer increase fitness (Larkin et al., 2007; Tamura et al., 2013). Sexual sterility may be first

induced by ecological factors, the resulting genetic sterility may, in turn, further hamper sexual recruitment in clonal populations, facilitating the evolution of asexual reproduction in clonal plants.

## Author Contributions

ZZ collaborated in the design of the research, collected plant materials, prepared RNA samples for high-throughput sequencing, performed the bioinformatics analyses, interpreted the results and wrote the first draft of the manuscript. CZ helped in the design of the experiments as well as RNA samples preparation, performed the experiments with B-class MADS-box genes, including plant materials collection, cDNA full-length cloning, phylogenetic analysis and qRT-PCR. Moreover, she made graphs and wrote the draft about B-class MADS-box genes. JY made substantial contributions to the design of the research, analysis of next-generation sequencing data and manuscript revision. All authors have read the final version of the manuscript and agree with its content.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2015.00242/abstract>

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# TCP24 modulates secondary cell wall thickening and anther endothecium development

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miR319-targeted *TCP* genes are believed to regulate cell division in leaves and floral organs. However, it remains unknown whether these genes are involved in cell wall development. Here, we report that *TCP24* negatively regulates secondary wall thickening in floral organs and roots. The overexpression of the miR319a-resistant version of *TCP24* in *Arabidopsis* disrupted the thickening of secondary cell walls in the anther endothecium, leading to male sterility because of arrested anther dehiscence and pollen release. Several genes linked to secondary cell wall biogenesis and thickening were down-regulated in these transgenic plants. By contrast, the inhibition of *TCP24* using the ectopic expression of a *TCP24-SRDX* repressor fusion protein, or the silencing of *TCP* genes by miR319a overexpression, increased cell wall lignification and the enhanced secondary cell wall thickening. Our results suggest that *TCP24* acts as an important regulator of secondary cell wall thickening and modulates anther endothecium development.

**Keywords:** *Arabidopsis*, *TCP24*, male sterility, anther dehiscence, secondary wall thickening, SRDX

## Introduction

Anther dehiscence is a multistage process that involves coordinated programmed events in specific cells, including degeneration of the middle layer and the tapetum, thickening of the endothecium, degradation of septum cells, and breakage of stomium cells (Goldberg et al., 1993; Sanders et al., 1999; Wilson et al., 2011). Secondary wall thickening of the endothecium generates the tensile force necessary to rupture the stomium and in turn, release the pollen grains (Keijzer, 1987; Bonner and Dickinson, 1989). The importance of this process has been demonstrated by genetic analysis. A loss-of-function mutation of *MYB26* disrupts secondary thickening of the anther walls, resulting in non-dehiscent anthers (Dawson et al., 1999; Steiner-Lange et al., 2003; Yang et al., 2007). Two NAC domain transcription factors, *NST1* and *NST2*, function redundantly in regulating endothecium wall thickening and act downstream of *MYB26*. Overexpression of these two genes results in ectopic secondary thickening in various tissues (Mitsuda et al., 2005). Mutations in *IRREGULAR XYLEM* (*IRX*) and receptor-like protein kinase 2 (*RPK2*) genes also lead to the defective secondary wall thickening of the anther (Brown et al., 2005; Mizuno et al., 2007; Hao et al., 2014). Other genes, such as *CA2* (carbonic anhydrase 2), *AHP4* (*Arabidopsis* histidine-containing phosphotransfer factor 4), *SAF1* (secondary wall thickening-associated F-box 1) and *CBSX2* (cystathionine  $\beta$ -synthase domain-containing protein), negatively regulate this process, and the overexpression of these genes in *Arabidopsis* leads to anther non-dehiscent phenotypes (Jung et al., 2008, 2013; Villarreal et al., 2009; Kim et al., 2012).

The *TEOSINTE BRANCHED1*, *CYCLOIDEA*, and *PCF* (*TCP*) family encodes plant-specific transcription factors, which contain a conserved bHLH motif that allows DNA binding and protein-protein interactions (Cubas et al., 1999; Martin-Trillo and Cubas, 2010). The *TCP* members are grouped into two classes based on sequence homology: class I and class II *TCPs* (Cubas et al., 1999). It is believed that class I *TCP* genes promote cell division, while class II genes act antagonistically to inhibit cell division (Li et al., 2005). Duplication and diversification events over millions of years have generated a large family of 24 *TCP* genes in *Arabidopsis* of which 11 belong to the class II subfamily (Martin-Trillo and Cubas, 2010). A functional analysis shows that the class II *TCP* genes regulate several aspects of plant development. *Arabidopsis BRANCHED1* (*BRCA1*) and *BRCA2*, both closely related to the *TEOSINTE BRANCHED1* from maize (Doebley et al., 1997), are involved in suppressing axillary bud outgrowth (Aguilar-Martinez et al., 2007). *TCP2*, *TCP3*, *TCP4*, *TCP10*, and *TCP24* are the targets of miR319a/JAW. The down-regulation of these genes by overexpression of miR319a in *jaw-D* mutants generates larger leaves with crinkled surfaces owing to the extended cell proliferation along leaf margins (Palatnik et al., 2003). Conversely, hyper-activation of *TCP4* results in decreased cell proliferation, resulting in smaller leaves (Sarvepalli and Nath, 2011). miR319a-targeted genes function redundantly with *TCP5*, *TCP13*, and *TCP17* to coordinate the maintenance of undifferentiated fates in the shoot apical meristem and the promotion of the differentiated status in leaves (Koyama et al., 2007; Efroni et al., 2008). This coordination is achieved via the negative regulation of *CUP-SHAPED COTYLEDON* (*CUC*) genes, and *TCP3* can directly activate the expression of *miR164A*, *ASYMMETRIC LEAVES1*, *INDOLE-3-ACETIC ACID3/SHORT HYPOCOTYL2* (*IAA3/SHY2*), and *At1g29460* to suppress *CUC* expression (Koyama et al., 2010). miR319a-targeted *TCPs* can interact with *ASYMMETRIC LEAVES2* and repress the expression of *BREVIPEDICELLUS* and *KNAT2* genes by binding to their promoters causing normal leaf development (Li et al., 2012b). It is reported that a transcriptional repressor, TIE1, recruits co-repressors TOPLESS/TOPLESS-related proteins to repress the activities of class II *TCP* genes (Tao et al., 2013).

Previous studies showed that these *CINCINNATA* (*CIN*)-like *TCP* genes were expressed differentially in various organs, indicating that they might play important roles in many aspects of plant development (Koyama et al., 2007). In this study, we used a reverse genetic approach to investigate the function of *TCP24*. Overexpression of *TCP24* led to non-dehiscent anthers owing to the lack of secondary wall thickening in the endothecium, while fusing it with an EAR motif repressor domain (SRDX) caused enhanced lignin deposition in the anther endothecium, as well as other tissues, suggesting that *TCP24* functions as a negative regulator of secondary wall thickening.

## Materials and Methods

### Plant Materials and Growth Conditions

The wild type and transgenic plants of *Arabidopsis thaliana* used in this study were of Columbia ecotype (Col-0). Seeds were

surface sterilized in 70% ethanol for 1 min, followed by 0.1% HgCl<sub>2</sub> for 10 min, then washed five times in sterile distilled water, and plated on solid 1% sugar Murashige and Skoog medium. The plates were sealed with parafilm, incubated at 4°C in the dark for 2 days, and then moved to a growth room at 22°C with 16 h light. Two weeks later, the seedlings were transplanted carefully to peat soil in plastic pots, moved to a growth chamber in the phytotron of Institute of Plant Physiology and Ecology, and grown at 22°C with 16 h of light per day.

### Gene Cloning and Transformation

The full length CDS of *TCP24* was amplified from cDNA. *mTCP24* was generated by site-directed mutagenesis method using the QuikChange® Lightning Site-Directed Mutagenesis Kit (Stratagene Catalog #210518) with appropriate primers. To construct the plasmid *p35S:mTCP24*, vector PJP100 (*p35S:mTCP2*, obtained from Dr. Weigel's lab) was modified by replacing *mTCP2* with *mTCP24*. The 35S promoter of *p35S:mTCP24* was replaced with the *TCP24* promoter (2.7 kb fragment upstream from the translational start site) for the construction of *pTCP24:mTCP24*. *p35S:TCP24SRDX* was generated in our modified pCAMBIA3301 binary vector by fusing *TCP24* with the EAR motif repressor domain SRDX under the control of the 35S promoter. The plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101 for plant transformation using the floral dip method as described previously (Li et al., 2012a). The transgenic plants of *p35S:mTCP24* and *pTCP24:mTCP24* were selected on plates containing 1/2 Murashige and Skoog media supplemented with 50 mg/L kanamycin, while *p35S:TCP24SRDX* plants were selected using 40 mg/L phosphinothricin.

### In Situ Hybridization

The full-length coding sequence of *TCP24* was polymerase chain reaction (PCR) amplified and cloned into pBluescript SK. Digoxigenin-labeled sense and antisense probes were synthesized with T7 or T3 RNA polymerase (Roche). Inflorescences from wild type and transgenic plants were pretreated and hybridized as described previously (Liu et al., 2011). Locked nucleic acid (LNA)-modified probe of miR319a was synthesized and labeled with DIG at the 3' end and used for *in situ* hybridization.

### Real-Time PCR

The total RNA of inflorescences (with opened flowers removed) was extracted using TRIzol Reagent (Invitrogen) and treated with DNase I (TaKaRa) to remove DNA contamination. For cDNA synthesis, ~4 µg RNA was reverse-transcribed using PrimeScript® Reverse Transcriptase (TaKaRa) with oligo(dT) primers according to the manufacturer's protocol. A quantitative real-time PCR analysis was performed using the Rotor-Gene 3000 system (Corbett Research, Mortlake, NSW, Australia) using SYBR Premix Ex Taq (Takara). ACTIN mRNA was used as an internal control, and the comparative threshold cycle ( $2^{-\Delta\Delta Ct}$ ) method was used to determine relative transcript levels. Three biological replicates and three technical replicates were performed. The gene specific primers

for reverse-transcription PCR were shown in Supplementary Table S1.

## Histology

Inflorescences of 5- to 6-week-old wild type and transgenic plants were fixed in formalin/acetic acid/alcohol (FAA) and embedded in paraffin (Sigma). Then, 7  $\mu\text{m}$  sections were stained with 0.05% (w/v) toluidine blue (Sigma) at 37°C for 15 min and then washed with water. For the analysis of semi-thin sections, samples fixed in FAA were embedded in epoxy resin. Then, 2- $\mu\text{m}$ -thick sections were cut with glass knives, affixed to glass slides, and stained in 0.05% (w/v) toluidine blue. The sections were observed under a light microscope (Olympus model BX 51).

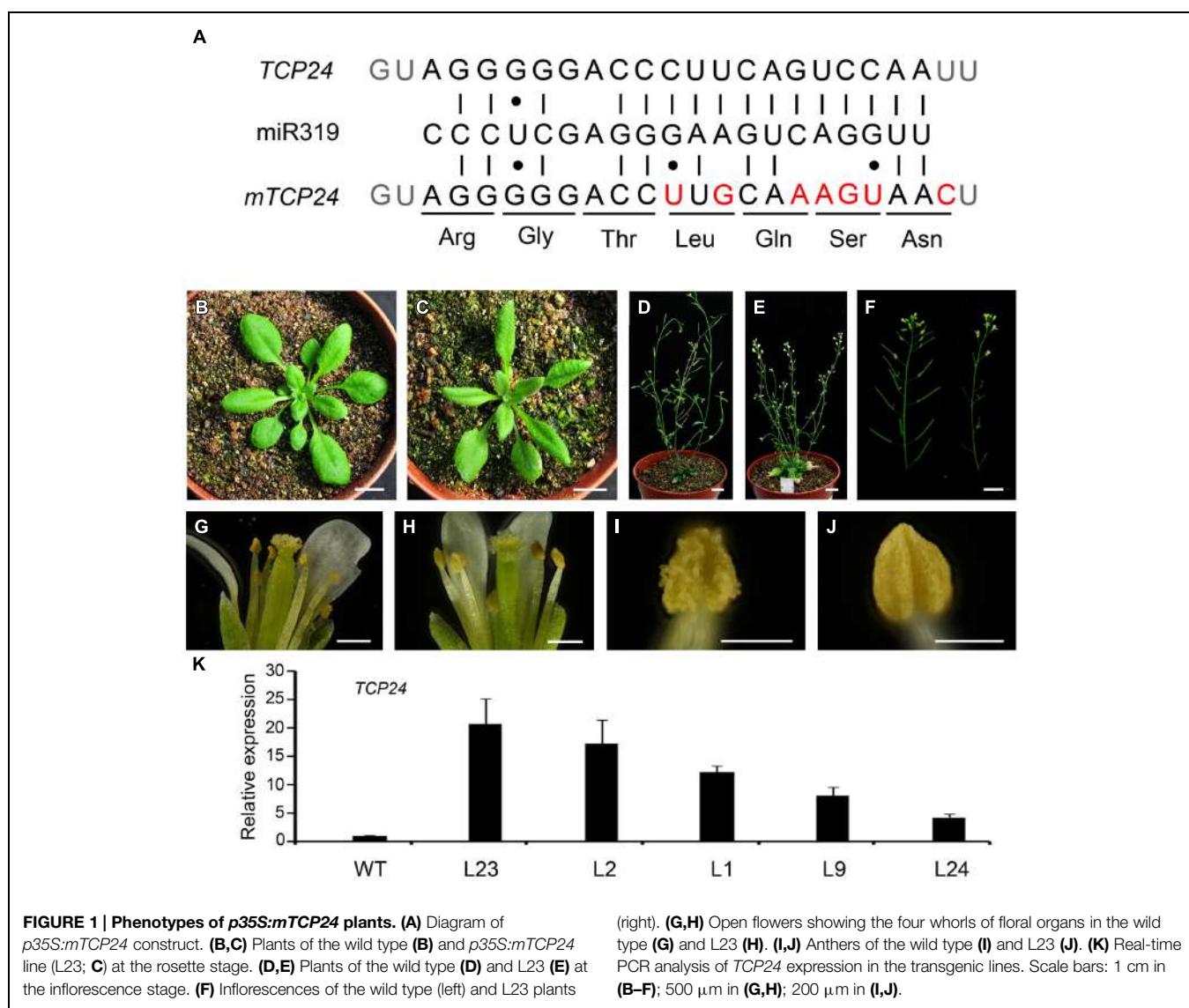
To visualize lignin deposition, plant tissues were stained with phloroglucinol-HCl solution (1.25 g of phloroglucinol dissolved in 25 ml of 95% ethanol and 10 ml of concentrated HCl), and observed under a dissecting microscope. To examine the secondary wall thickening in the endothecium, anthers were

placed onto glass slides with clearing fluid. The solution was prepared from lactic acid, chloral hydrate, phenol, clove oil and xylene in the ratio 2:2:2:1, respectively, by weight (Herr, 1971). The anthers were observed by microscopy using differential interference contrast optics.

## Results

### Overexpression of TCP24 Disrupts Anther Dehiscence in *Arabidopsis*

Mutations in single CIN-like *TCP* genes do not generate visible phenotypes owing to the redundancy among these genes (Koyama et al., 2007). To characterize the role of *TCP24* during plant development, we first constructed *mTCP24*, the miR319a-resistant version of *TCP24*, which contains nucleotide substitutions in the miR319a-binding region that do not change the encoded amino acid sequence (Figure 1A), as they did for



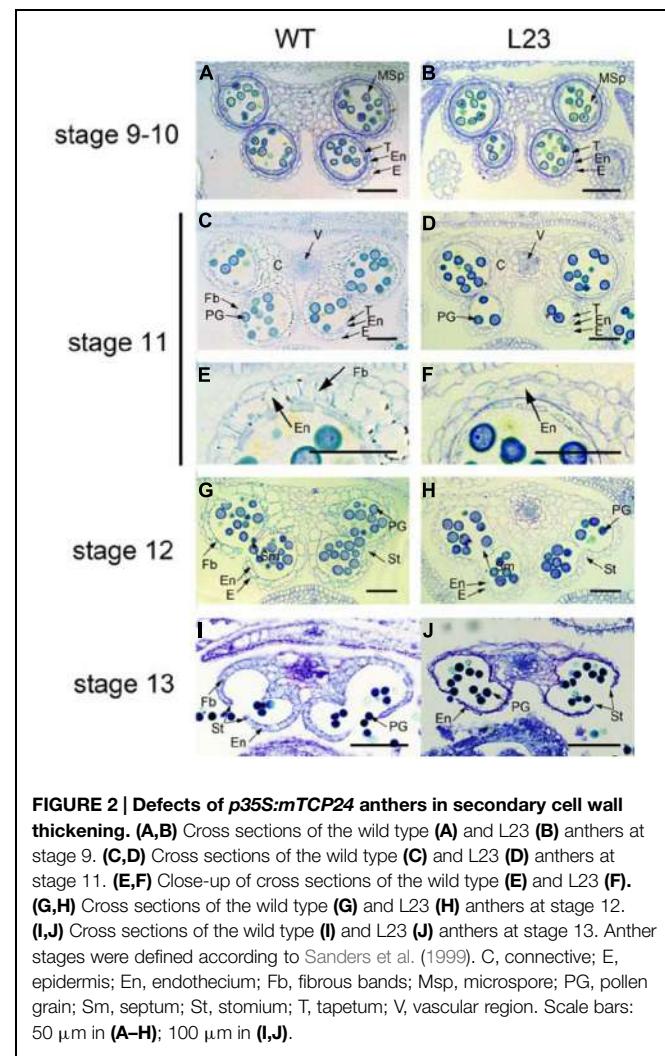
*mTCP2*, *mTCP3*, and *mTCP4* (Palatnik et al., 2003; Koyama et al., 2007). In the *p35S:mTCP24* plants, the rosette leaves were turned slightly downward (Figures 1B,C), the number of branches increased compared with the wild type (Figures 1D,E), and importantly, the flowers were partially or completely sterile (Figure 1F). Under optical microscopy, anther dehiscence was arrested (Figures 1G–J), albeit to different extents between the transgenic lines. Transcripts of *TCP24* were elevated in these independent transgenic lines compared with wild type (Figure 1K). Among these transgenic lines, the higher the *TCP24* expression was, the higher the male sterility was, indicating a correlation between the expression levels of *TCP24* and the severity of the sterile phenotypes (Supplementary Figure S1). The L2 and L23, two completely sterile lines, set seeds when they were pollinated with the wild type pollen, indicating that *p35S:mTCP24* did not affect female fertility.

### TCP24 Suppresses Secondary Wall Thickening of the Anther Endothecium

Anther dehiscence requires the degeneration of some tissues and subsequent differentiation of other tissues, including epidermis, stomium, endothecium, and septum (Goldberg et al., 1993). To verify the defects in anther dehiscence in *p35S:mTCP24* plants, we examined the anther endothecium of line L23. During stages 9–10 (Sanders et al., 1999) when microspores were formed, L23 anthers were indistinguishable from those of the wild type (Figures 2A,B). At stage 11, the tapetum of the wild type anthers was degenerated and the endothecium thickened, forming bands of striated spring-like structures (Figures 2C,E). However, secondary thickening in L23 anthers was not observed, as fibrous bands were absent in the endothecium although the tapetum was degenerated (Figures 2D,F). Secondary cell wall thickening was necessary to create the shearing force required for anther dehiscence through the stomium (Dawson et al., 1999). At later stages, the septa of L23 anthers were degraded as in the wild type (Figures 2G,H). While the stomium broke in L23, the anthers did not open (Figures 2I,J). Alexander staining showed that the pollen grains in L23 anthers were viable (Supplementary Figure S2). These observations indicate that non-dehiscence in *p35S:mTCP24* anthers is due to the defect in secondary wall thickening in the anther endothecium.

We examined the accumulation of lignin, which was the major component of secondary walls according to phloroglucinol staining. The deep red staining of lignified materials by phloroglucinol was clearly observed in the endothecium layer in the wild type anthers (Figure 3A). However, no staining was observed in L23 anthers (Figure 3B). We also treated the anthers with clearing fluid. The thickened cell walls appeared in the wild type endothecium (Figures 3C,E) but were absent in the transgenic plants (Figures 3D,F). These results indicate that *TCP24* negatively regulates secondary wall thickening in the anther endothecium.

To exclude the effect of the 35S promoter on ectopic expression, we expressed *TCP24* under the control of its native promoter (2.7 kb 5' upstream of the *TCP24* transcriptional start site) (Figure 4A). Among the *pTCP24:mTCP24* lines, some had a complete loss of fertility because seed set was not observed, while

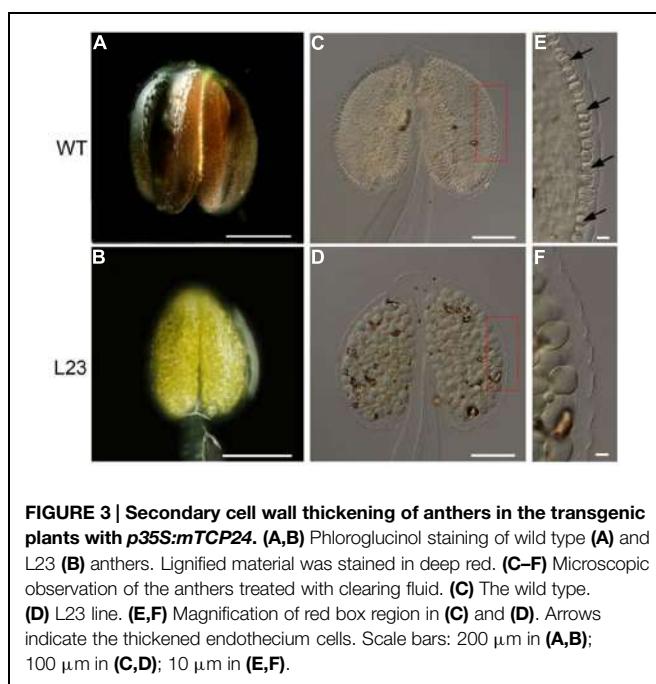


**FIGURE 2 | Defects of *p35S:mTCP24* anthers in secondary cell wall thickening.** (A,B) Cross sections of the wild type (A) and L23 (B) anthers at stage 9. (C,D) Cross sections of the wild type (C) and L23 (D) anthers at stage 11. (E,F) Close-up of cross sections of the wild type (E) and L23 (F). (G,H) Cross sections of the wild type (G) and L23 (H) anthers at stage 12. (I,J) Cross sections of the wild type (I) and L23 (J) anthers at stage 13. Anther stages were defined according to Sanders et al. (1999). C, connective; E, epidermis; En, endothecium; Fb, fibrous bands; Msp, microspore; PG, pollen grain; Sm, septum; St, stomium; T, tapetum; V, vascular region. Scale bars: 50  $\mu$ m in (A–H); 100  $\mu$ m in (I,J).

most showed a reduced fertility compared with the wild type (Figure 4B). In the transgenic line 24-2 which was sterile, there was no pollen on the stigmas, indicating that pollen grain release from the anthers was arrested (Figures 4C,D), and no pollen was observed being released from the anthers (Figures 4F,G). Under the microscope, a few pollen grains were found to be outside the stomium on 24-5 plants (Figures 4E,H). Using phloroglucinol staining, we observed no staining in the anthers of 24-2 plants (Figure 4J) and very weak red staining in the anthers of 24-5 plants (Figures 4I,K). The cell walls of the endothecium were thickened uniformly in the wild type (Figure 4L), but they were not observed in 24-2 anthers (Figure 4M). On the transgenic line 24-5 which had reduced fertility, secondary cell walls occurred in some positions (Figure 4N). These observations confirmed that the overexpression of *TCP24* inhibited secondary wall thickening in the anther endothecium.

### TCP24 Gene Expression Became Weak at the Anther Endothecium Initiation Stage

To examine the temporal and spatial expression of *TCP24* during anther development, *in situ* hybridization was performed using



the wild type anthers. The anther development was divided into 14 stages (Sanders et al., 1999). At stage 2, the *TCP24* signal was strong in the whole region (Figure 5A). At stage 3, the signal was preferential in the epidermal, parietal layer and sporogenous cells (Figure 5B). At stages 4 to 5, when four clearly defined locules were established, *TCP24* was strongly expressed in the epidermis, endothecium, middle layer, tapetum, vascular tissue, and microspore mother cell (Figures 5C–E). At stage 6, the signal became weak, and was clearly localized in the tapetum, microspore, and vascular region (Figures 5F–H). From stage 11, when secondary cell wall thickening begins, the *TCP24* signal disappeared in the endothecium, but was still present in the vascular region (Figures 5I–L). The expression domains of miR319a were similar as those of *TCP24* (Supplementary Figure S3). To address whether *TCP24* overexpression causes the ectopic distribution of *TCP24* in endothecium, we detected *TCP24* in L23 anthers. The *TCP24* expression pattern was the same as that of the wild type at stage 5, although the signal was much stronger than in the wild type (Supplementary Figure S4A). There was no ectopic signal in the endothecium during the secondary wall thickening process (Supplementary Figures S4B–D). These observations suggested that the lack of secondary wall thickening in the anthers of p35S:mTCP24 plants was due to the high level of *TCP24* rather than its misexpression.

### TCP24 Regulates the Genes Linked to Secondary Cell Wall Thickening

Secondary walls in the anther endothecium are composed of lignin and cellulose. Mutations of the genes involved in these biosynthesis processes cause non-dehiscent anthers (Brown et al., 2005; Thevenin et al., 2011). We examined the expression profiles of the genes involved in the biosynthesis of lignin (*C4H*, *4CL1*, *CCoAOMT*, and *PAL4*) and cellulose (*IRX1*, *IRX3*, and *IRX5*)

(Boerjan et al., 2003; Somerville, 2006). All of these genes were down-regulated in the flower buds of p35S:mTCP24 plants (Figure 6A). It was reported that mutations in *MYB26*, *NST1*, and *NST2*, as well as the overexpression of *AHP4*, resulted in the failure of anther dehiscence and that these genes act upstream to regulate secondary wall biosynthesis genes (Dawson et al., 1999; Steiner-Lange et al., 2003; Mitsuda et al., 2005; Yang et al., 2007). We found that *TCP24* overexpression significantly reduced the expression of *NST1* and *NST2*, but not *MYB26*. Unexpectedly, *AHP4* was greatly up-regulated (Figure 6B). These results indicate that *TCP24* negatively regulates secondary wall biosynthesis genes and is possibly upstream of these genes in the pathways.

### TCP24 Silencing is Helpful for Thickening Anther Endothecium Secondary Cell Walls

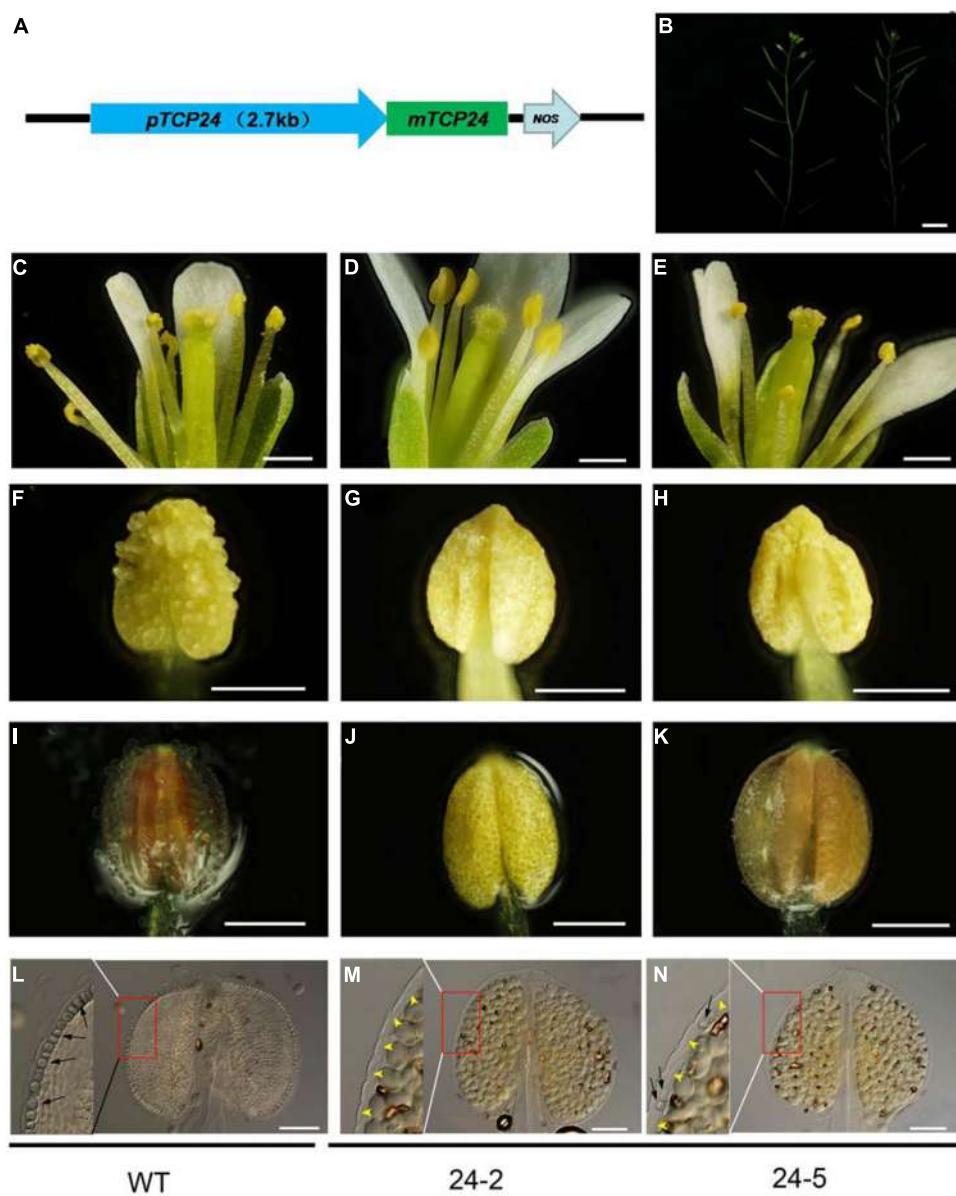
To investigate whether secondary wall thickening was affected by *TCP24*, we carefully observed the anthers of *jaw-D* mutant plants in which miR319a-targeted *TCP* genes were down-regulated (Palatnik et al., 2003). In the flower buds of *jaw-D*, these *TCP* genes were down-regulated (Figure 7A). The epidermal tissues and the anther endothecium were particularly affected in the *jaw-D* mutant. The periclinal cell walls were thicker than the wild type (Figures 7B,C).

To exclude the redundant effects of the other *TCP* genes, we created p35S:*TCP24SRDX* plants by fusing *TCP24* with the SRDX repression domain. This approach converted transcription factors into dominant repressors, even in the presence of redundant genes (Hiratsu et al., 2003), and has been extensively used to study the functions of *TCP* genes (Koyama et al., 2007, 2010; Guo et al., 2010; Kieffer et al., 2011; Uberti-Manassero et al., 2012). A total of 48 independent transgenic lines were obtained. Their anthers were wider than the wild type (Figures 7D,E) and some had protuberances on their surface (Figure 7F). Phloroglucinol staining showed that lignification was enhanced and the endothecium layers were much thicker in the anthers of p35S:*TCP24SRDX* plants compared with the wild type (Figures 7G–I). This result indicates that the posttranscriptional silencing of *TCP24* promotes the thickening of secondary cell walls in the anther endothecium.

Besides the anther endothecium, secondary wall thickening was observed in the other tissues using phloroglucinol staining. In the wild type roots, lignified secondary wall thickening was observed in vascular bundles but not in the parenchymatous cells (Figure 8A) as observed (Herve et al., 2009). In p35S:*TCP24SRDX* roots, however, it was seen in the parenchymatous cells as well (Figure 8B). In vascular bundles of the mature sepals and petals the transgenic plants exhibited stronger signals of lignified secondary wall thickening compared with the wild type plants (Figures 8C,D). These results indicate that *TCP24* repression influences the ectopic thickening of the secondary walls in various tissues.

### Discussion

miR319a-targeted *TCP* genes may play important roles in controlling cell division and differentiation during leaf



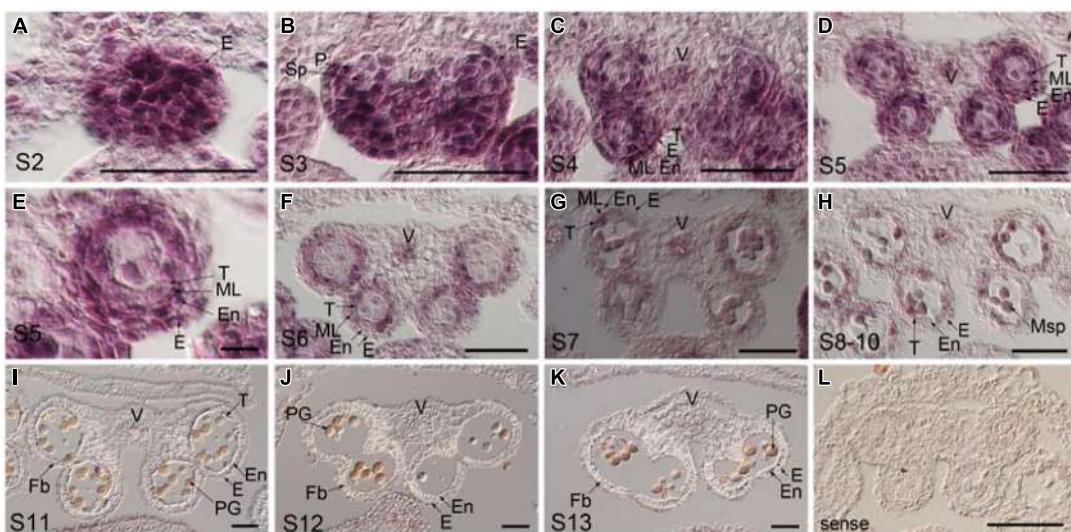
**FIGURE 4 |**The floral organs and anthers of the transgenic plants with *pTCP24:mTCP24*. **(A)** Diagram of *pTCP24:mTCP24* construct. **(B)** Inflorescences of the wild type (left) and *pTCP24:mTCP24* (24-5; right) plants. **(C–E)** Open flowers in the wild type (**C**), 24-2 (**D**) and 24-5 (**E**) lines. **(F–H)** Anthers in the wild type (**F**), 24-2 (**G**), and 24-5 (**H**) lines. **(I–K)** The wild type (**I**), 24-2 (**J**), and 24-5 (**K**)

anthers with phloroglucinol staining. **(L–N)** The wild type (**L**), 24-2 (**M**), and 24-5 (**N**) anthers treated with clearing fluid. Images in the left of each figures is magnified from the red boxes. Black arrows indicate the thickened endothecium cells, and yellow arrowheads indicate no thickened endothecium cells. Scale bars: 1 cm in **(B)**; 500  $\mu$ m in **(C–E)**; 200  $\mu$ m in **(F–K)**; 100  $\mu$ m in **(L–N)**.

development (Palatnik et al., 2003; Koyama et al., 2007, 2010; Ori et al., 2007; Efroni et al., 2008; Li et al., 2012b). In this study, we found that miR319a-targeted *TCP24* negatively regulates secondary cell wall thickening in the anther endothecium. This result suggests that miR319a-targeted *TCP* genes are multifunctional in their regulation of cell development. It has also been reported that *TCP4* can bind to the *LOX2* promoter, regulating leaf senescence by controlling the expression of jasmonic acid biosynthesis genes (Schommer et al., 2008). The

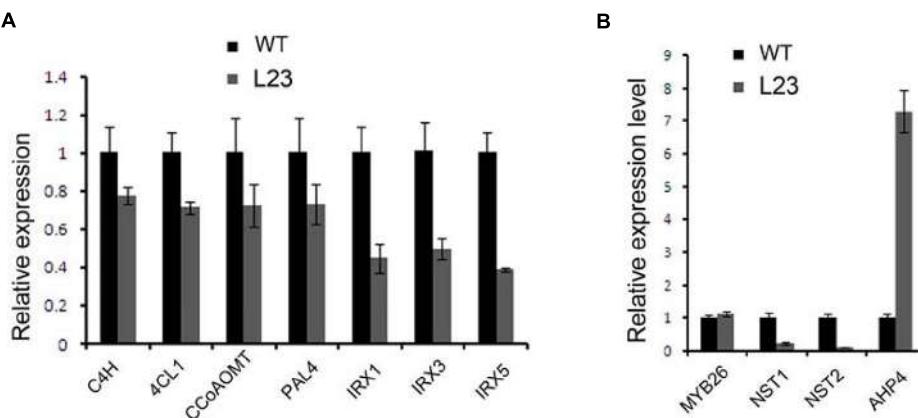
proper level of active *TCP4* is critical for petal and stamen development (Nag et al., 2009), and *TCP2* and *TCP3* interact with components of the core circadian clock (Giraud et al., 2010). Additionally, *TCP3* interacts with R2R3-MYB proteins and participates in the flavonoid biosynthesis pathway (Li and Zachgo, 2013).

In transgenic plants containing *p35S:mTCP24*, secondary cell wall thickening does not occur in the anther endothecium. Overexpression of *TCP24* under its native promoter also exhibits



**FIGURE 5 | Temporal and spatial expression patterns of *TCP24* gene during anther development.** *In situ* hybridization using *TCP24* probe. (A–K) Transverse sections of anthers at the stages 2 (A), 3 (B), 4 (C), 5 (D,E), and 6 (F), 7 (G), 8–10 (H), 11 (I), 12 (J), and 13 (K) antisense probes.

(E) Magnified picture from the top right region in (D). (L) Sense probe of *TCP24*. E, epidermis; En, endothecium; Fb, fibrous bands; ML, middle layer; Msp, microspore; P, parietal cell; PG, pollen grain; Sp, sporogenous; T, tapetum; V, vascular region. Scale bars: 50  $\mu$ m in (A–D, F–L); 5  $\mu$ m in (E).



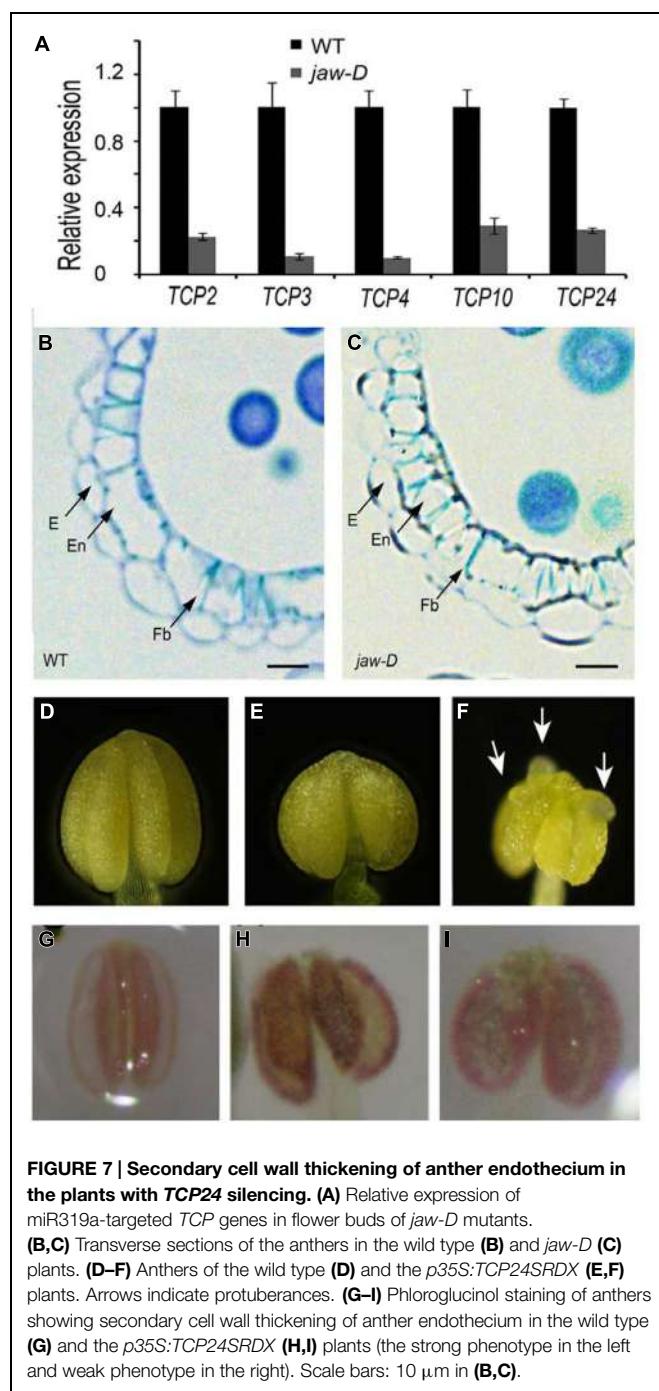
**FIGURE 6 | Regulation of *TCP24* to the genes linked to secondary cell walls.** (A) Relative expression levels of genes involved in the biosynthesis of lignin (*C4H*, *4CL1*, *CCoAOMT*, *PAL4*) and cellulose (*IRX1*, *IRX3*, and *IRX5*). (B) Relative expression levels of the genes that regulate the secondary wall thickening. Error bars represent SD of three replicates.

a similar phenotype. However, silencing *TCP24* by enhanced miR319a expression or the repression of *TCP24* using the SRDX repressor domain causes increased lignification and the deposition of secondary cell walls in the anther endothecium. Apparently, *TCP24* represses the secondary cell wall thickening in the anther endothecium. In the wild type anthers, *TCP24* strongly expresses in the endothecium when this cell layer is formed, and the expression weakens and eventually disappears when secondary wall thickening occurs. Clearly, *TCP24* acts as a repressor of secondary wall thickening at the early stage of endothecium development.

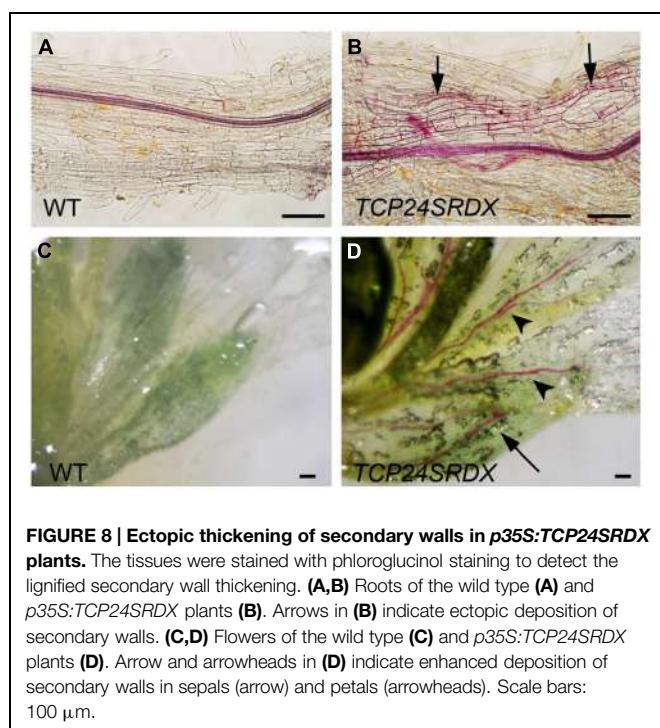
Secondary wall thickening in the anther endothecium is important for anther dehiscence. Several processes, such as

degeneration of the tapetum, septum, and breakage of stomium cells that affect dehiscence, occur normally in the transgenic plants. Pollen grains are fertile but they remain locked into the non-dehiscent anthers. Microscopic observation and histological staining suggest that this defect is due to the lack of secondary wall thickening in the anther endothecium. The importance of this process has been verified in several studies (Dawson et al., 1999; Steiner-Lange et al., 2003; Mitsuda et al., 2005; Yang et al., 2007; Jung et al., 2008; Kim et al., 2012).

A few genes have been linked to secondary thickening in anther endothecium. *MYB26*, *NST1*, and *NST2* positively control secondary thickening by regulating the expression of secondary wall biogenesis genes, and *AHP4* negatively regulates this process



(Steiner-Lange et al., 2003; Mitsuda et al., 2005; Yang et al., 2007). Meanwhile, mutations in the genes that encode secondary wall biogenesis, such as *IRX*, *4CL3*, *CCR*, and *CAD*, also result in failed secondary thickening, resulting in the non-dehiscent phenotype (Brown et al., 2005; Gui et al., 2011; Thevenin et al., 2011; Hao et al., 2014). Other mechanisms exist that can be illustrated by studying the function of mitochondrial gamma CA2 and CBSX2. *p35S:CA2* plants cause a dramatic decrease in the reactive oxygen species production in anthers, which may



impair  $H_2O_2$ -dependent lignin polymerization and deposition in the anther endothecium, resulting in a lack of secondary wall thickening in the endothecium (Villarreal et al., 2009). CBSX2 modulates the  $H_2O_2$  status and may be linked to the jasmonic acid response, which in turn controls secondary wall thickening of the anther endothelial cells (Jung et al., 2013). In this study, we demonstrate that several genes linked to secondary wall biogenesis are down-regulated in *TCP24*-overexpressing plants. *NST1* and *NST2* are down-regulated and *AHP4* is up-regulated in the transgenic plants. These results indicate that *TCP24* acts upstream of the genes that promote secondary wall thickening. It has been reported that *MYB26* is an upstream regulator of *NST1* and *NST2* (Yang et al., 2007). However, *MYB26* transcripts are not changed in *TCP24* overexpressing plants. We speculate that *TCP24* functions in a *MYB26*-independent manner.

The deregulation of *TCP24* causes defects not only in the anther endothecium, but also in roots and flower tissues. It has been reported that TCP proteins form homo- and heterodimers, and the latter bind DNA more efficiently than the former (Kosugi and Ohashi, 2002; Danisman et al., 2012). TCP4 can recognize the GGACCA motif, while TCP3 can activate downstream gene expression by directly binding the GGnCCC motif in the respective promoter (Schommer et al., 2008; Koyama et al., 2010). TCP24 may interact with other proteins and bind to the corresponding motifs in the targeted genes to execute its function. Further work is necessary to elucidate the molecular mechanisms of secondary cell wall thickening in plants.

Our results expand the classical roles of *TCP24* in cell division. *TCP24* regulates the genes that encode the enzymes responsible

for secondary cell wall biogenesis, which modify cell walls. It will be interesting to determine whether *TCP24* is involved in the relationship between cell division and cell wall development. Further studies on the *TCP* genes will provide insights into gene regulation pathways in cell differentiation.

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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.2015.00436>

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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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# OsSDS is essential for DSB formation in rice meiosis

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SDS is a meiosis specific cyclin-like protein and required for DMC1 mediated double-strand break (DSB) repairing in *Arabidopsis*. Here, we found its rice homolog, OsSDS, is essential for meiotic DSB formation. The *Ossds* mutant is normal in vegetative growth but both male and female gametes are inviable. The *Ossds* meiocytes exhibit severe defects in homologous pairing and synapsis. No γH2AX immunosignals in *Ossds* meiocytes together with the suppression of chromosome fragmentation in *Ossds-1 Osrad51c*, both provide strong evidences that OsSDS is essential for meiotic DSB formation. Immunostaining investigations revealed that meiotic chromosome axes are normally formed but both SC installation and localization of recombination elements are failed in *Ossds*. We suspected that this cyclin protein has been differentiated pretty much between monocots and dicots on its function in meiosis.

**Keywords:** rice, OsSDS, meiosis, DSB formation

## INTRODUCTION

Meiosis is one of the key processes in sexual reproduction for all sexually propagating eukaryotic organisms. Meiosis includes one single round of DNA replication but followed by two successive rounds of nuclear segregations (meiosis I and II), and finally produces four haploid gametes with halved chromosomes. The meiotic prophase I is a complicated and prolonged stage, which can be divided into five substages, like leptotene, zygotene, pachytene, diplotene, and diakinesis, based on chromosome characterizations (Ashley and Plug, 1998; Dawe, 1998). During meiotic prophase I, pairing, synapsis and recombination of homologous chromosomes are coordinately accomplished. These events make sure the precise segregation of homologs, and generate both genetic conservation and diverse individuals in the future generations (Zickler and Kleckner, 1999; Page and Hawley, 2003).

In meiosis, DSBs are purposely produced to initiate homologous recombination. The formation of DSBs in meiosis is catalyzed by a type-II topoisomerase-like enzyme Spo11 (Bergerat et al., 1997; Keeney et al., 1997). Meanwhile, a series of cofactors are also required for this process. In budding yeast, the formation of meiotic DSBs requires at least nine other proteins

(Rec102, Rec104, Rec114, Mei4, Mer2, Rad50, Mre11, Xrs2, and Ski8) for the cleavage mediated by SPO11 and further broken end resection (Paques and Haber, 1999; Keeney, 2001, 2008). The budding yeast Mre11-Rad50-Xrs2 (MRX) complex is homologous with the mammalian Mre11-Rad50-Nbs1 (MRN), which is required to incise the 5' end of the break and then form 3' single-strand tails (Symington, 2002; Mimitou and Symington, 2009). After that, one 3' free single-strand DNA end recruits two RecA homologs, Rad51 and Dmc1, to mediate the single-end invasion (SEI) with its homologous duplex DNA (Bishop, 1994; Hunter and Kleckner, 2001), and the other 3' free single-strand DNA end on the other side of the nick is captured simultaneously to form the Double Holliday Junction (DHJ). And then, the DHJ is exclusively processed into crossovers (COs), which represents the accomplishment of homologous recombination (Allers and Lichten, 2001; Bishop and Zickler, 2004; Borner et al., 2004). Consequently, meiotic DSBs are finally repaired during this process.

The function of SPO11 initiating meiotic recombination seems to be widely conserved within eukaryotes, as more and more homologs of SPO11 were identified in a wide range of organisms covering yeasts, flies, mice, humans, and plants

(Dernburg et al., 1998; McKim and Hayashi-Hagihara, 1998; Celerin et al., 2000; Hartung and Puchta, 2000; Romanienko and Camerini-Otero, 2000; Grelon et al., 2001; Yu et al., 2010). Unlike animals and fungi where a single SPO11 is sufficient for meiotic DSBs formation, higher plants always possess multiple SPO11 homologs (Keeney et al., 1997; Grelon et al., 2001; Hartung et al., 2007; Shingu et al., 2012; Sprink and Hartung, 2014). But not every SPO11 homolog has the function to cleavage double-strand DNA and generate DSBs in plants. *Arabidopsis* owns three SPO11 homologs and they appear to function in two distinct processes, AtSPO11-1 and AtSPO11-2 in DSB formation, while AtSPO11-3 in DNA replication (Stacey et al., 2006; Shingu et al., 2012). While in monocot rice, there are five SPO11 homologs have been identified (Jain et al., 2006, 2008). Among them, only OsSPO11-4 has been proved to be with double-strand DNA cleavage activity (An et al., 2011). OsSPO11-1 is essential for homologous pairing, recombination and SC installation (Yu et al., 2010; Luo et al., 2014). So, it seems that the formation of meiotic DSBs is more complicated in plants.

Besides SPO11, several other DSB formation proteins have been identified recently in multicellular eukaryotes. Mei1 and Mei4 were shown to be required for DSB formation in mice (Libby et al., 2003; Kumar et al., 2010). Using a high throughput genetic screen, AtPRD1, AtPRD2, and AtPRD3 were identified to be essential for DSB formation in *Arabidopsis* (De Muyt et al., 2009). Nevertheless, AtDFO was also found to be necessary for DSB formation in *Arabidopsis* (Zhang et al., 2012). Studies in rice revealed that CRC1 works together with PAIR1 as a complex to regulate meiotic DSB formation (Miao et al., 2013).

Studies in budding yeast demonstrated that cyclin-dependent kinase Cdc7 and Cdc28 can directly regulate the meiotic DSB formation via the phosphorylation of Mer2 (Henderson et al., 2006; Sasanuma et al., 2008; Wan et al., 2008). However, in plants, only a few cyclins have been found involved in meiosis (Bulankova et al., 2013). The meiosis specific cyclin SDS was first found in *A. thaliana*, which play a specific role in regulating synapsis in prophase I (Azumi et al., 2002; Wang et al., 2004). In a recent study, SDS was found to be required for DMC1-mediated DSB repair (De Muyt et al., 2009). Although the rice SDS-RNAi plants showed the similar meiotic defects with those in *Arabidopsis* (Chang et al., 2009), the molecular mechanism of SDS in rice meiosis remains to be clear. Here, we identified the SDS homolog in rice by map-based cloning. Surprisingly, we found OsSDS is essential for DSB formation during rice meiosis, which is much different from that in *Arabidopsis*. We suspected this cyclin protein had been differentiated pretty much between monocots and dicots on its function in meiosis.

## MATERIALS AND METHODS

### PLANT MATERIALS

The rice (*Oryza sativa* L.) spontaneous mutant *Ossds-1* was isolated from an *indica* rice, Zhongxian 3037. The F2 and F3 mapping populations were generated by crossing the *Ossds-1<sup>±</sup>* heterozygous plants with a *japonica* cultivar, Zhonghua 11. The other two mutant alleles, *Ossds-2* and *Ossds-3*, both were spontaneous mutants arose in tissue culture of Nipponbare. The meiotic

mutant *Osrad51c* has been reported previously (Tang et al., 2014). The *Ossds-1 Osrad51c* double mutant was generated by crossing the two heterozygous *Ossds-1<sup>±</sup>* and *Osrad51c<sup>±</sup>*, and further identified from the F2 progeny. All plant materials were grown in paddy fields in the summer in Beijing or in the winter in Hainan.

### MOLECULAR CLONING OF *OsSDS*

Total 861 sterile plants segregated from the F2 and F3 mapping populations were used for isolation the target gene. Sequence-tagged site (STS) markers were developed according to sequence differences between the *japonica* variety Nipponbare and the *indica* variety 9311, using the data published on the NCBI website (<http://www.ncbi.nlm.nih.gov>). All primers are listed in Supplemental Table 1.

### RNAi ANALYSIS

In the first exon, a 336-bp fragment of *OsSDS* cDNA sequence was chosen and amplified with the primers SDS-RNAi-F (adding a *Bam*H site) and SDS-RNAi-R (adding a *Sall* site) (Supplemental Table 1). RNAi vector construction and transformation were performed as described (Wang et al., 2009).

### COMPLEMENTATION TEST

The complementary plasmid was constructed by cloning the 10.8 kb OSJNBa0081P02 genomic DNA fragment containing the entire *OsSDS* coding region into the pCAMBIA-1300 vector. A control plasmid, containing 7.8 kb of the truncated *OsSDS* gene was also constructed. Both of these plasmids were transformed into EHA105 and then into embryonic calli of *OsSDS<sup>±</sup>* plants. The genotypes of the transgenic plants were further identify using the Primers SDS-JD (Supplemental Table 1).

### REAL-TIME PCR FOR TRANSCRIPT EXPRESSION ASSAY

Total RNA was extracted from the root, internode, leaf and panicle of Zhongxian 3037. Real-time PCR analysis was performed using the Bio-Rad CFX96 real-time PCR instrument (Bio-Rad, <http://www.bio-rad.com/>) and EvaGreen (Biotium, <http://www.biotium.com/>). The RT-PCR was carried out using the gene-specific primer pairs SDS-RT-F and SDS-RT-R for *OsSDS*. The primers Ubi-RT-F and Ubi-RT-R for ubiquitin were used as an internal control for the normalization of RNA sample. The results were analyzed using OPTICON MONITOR 3.1 (Bio-Rad). Each experiment had three replicates.

### CLONING THE FULL-LENGTH *OsSDS* cDNA

Total RNA was extracted from the panicle of Zhongxian 3037. The 3' RACE and 5' RACE were performed according to the protocol of the kit (3'-Full RACE Core Set and 5' -Full RACE Core Set; Takara, <http://www.takara-bio.com/>). 3' RACE was carried out using primers 3R-1F, 3R-2F, 3R-3F, and adaptor primer (P-ada). During 5'RACE, the RNA was reverse transcribed with 5' (P)-labeled primer (SDS-4Rb); the first and second PCRs were performed using two sets of *OsSDS* specific primers (5R-1 and 5R-2). The 3' RACE-PCR and 5' RACE-PCR products were cloned and sequenced. *OsSDS* amino acid sequence translation and alignment were completed with the Vector NTI 11.5

(Invitrogen). *OsSDS* gene structure diagram was generated from GSDS (<http://gsds.cbi.pku.edu.cn/index.php>).

### MEIOTIC CHROMOSOME PREPARATION

Young panicles with appropriate size of both *Ossds* mutants and wild type were collected, fixed in Carnoy's solution (ethanol:glacial acetic, 3:1) and stored at -20°C. Microsporocytes at the appropriate meiotic stage were squashed and stained with acetocarmine. After washing the chromosome preparations with 45% acetic acid and freezing them in liquid nitrogen, the coverslips were quickly removed with a razor blade and the slides harbored with samples were dehydrated through an ethanol series (70, 90, and 100%) for 5 min each and finally air-dried. Chromosomes on slides were counterstained with 4, 6-diamidinophenylindole (DAPI) in an anti-fade solution (Vector Laboratories, Burlingame, CA, USA). Finally, images were captured under the ZEISS A2 fluorescence microscope with a micro CCD camera (Zeiss, <http://www.zeiss.de/en>).

### FLUORESCENCE IMMUNOLocalIZATION

Fresh young panicles (40–60 mm) were fixed with 4% (w/v) paraformaldehyde for 30 min at room temperature. Anthers with appropriate stage were squashed into one drop of 1×PBS solution added on a slide. Then, covering the slide with a coverslip and pressing it with appropriate strength, the slide together with the coverslip was frozen thoroughly in liquid nitrogen. After quickly prizing up the coverslip, the slide was dehydrated through an ethanol series (70, 90, and 100%). The following immunolocalization procedure was performed as described (Tang et al., 2014).

The polyclonal antibodies against γ-H2AX, OsMSH5, OsREC8, PAIR2, PAIR3, ZEP1, OsMER3, and OsZIP4 used in this study have been described previously (Wang et al., 2009, 2010; Shao et al., 2011; Shen et al., 2012; Zhang et al., 2012; Luo et al., 2013; Miao et al., 2013).

## RESULTS

### CHARACTERIZATION OF A STERILE MUTANT

We identified a spontaneous mutant exhibiting complete sterility in a rice field of Zhongxian 3037. From the heterozygous plant progeny related to this mutation, the normal fertile plants and the sterile plants were segregated in a 3:1 ratio, indicating that it was a single recessive mutation ( $\chi^2 = 0.57$ ;  $P > 0.05$ ). The mutant plant was normal during vegetative growth and could not be distinguished from the wild type based on plant morphology (Supplemental Figure 1A). However, when come into reproductive stage, its spikelets exhibited complete sterility (Supplemental Figure 1B). So we further examined the mature pollen viability of the mutant by staining with 1% iodine potassium iodide solution (I<sub>2</sub>-KI) (Supplemental Figures 1C,D). Only empty and shrunken pollen grains were observed in the mutant plant, indicating that microspores of the mutant are all abnormal and inviable. Moreover, when pollinated with wild-type pollens, the mutant spikelets still did not set any seeds, indicating that its female gametes were also affected.

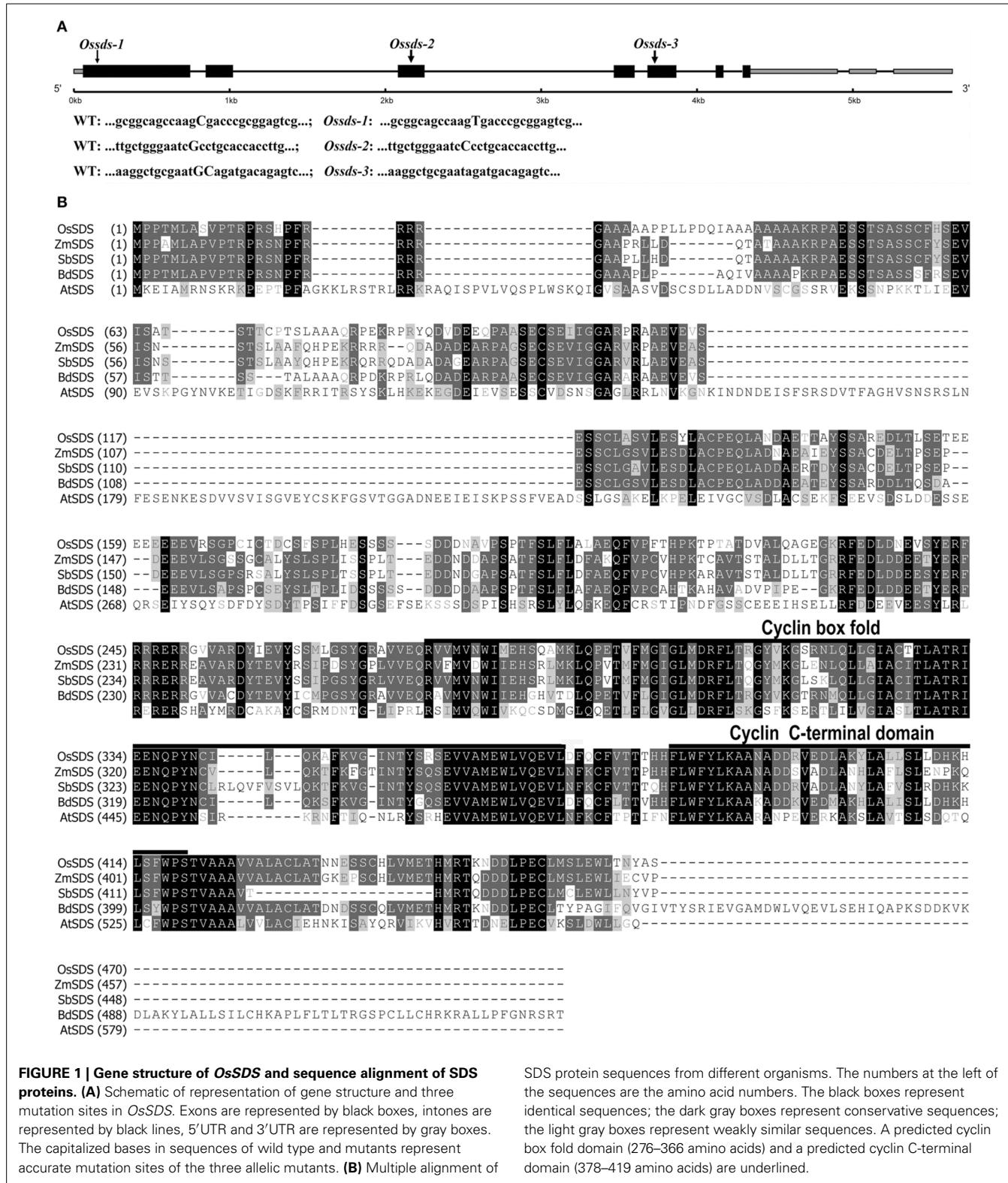
### MAP-BASED CLONING OF *OsSDS*

To isolate the mutated gene, we constructed a population by crossing heterozygous plants with a japonica cultivar Zhonghua 11. A total of 861 sterile plants segregated from the F2 and F3 populations were used for mapping the target gene. Linkage analysis initially mapped the target gene onto the long arm of chromosome 3, which subsequently further delimited to a 130 kb region. Within this region, we found one candidate gene (*Os03g12414*) annotated as a putative cyclin with high similarity with *Solo Dancers* (SDS) gene in *Arabidopsis*. Thus, this candidate gene was chosen to be amplified and sequenced. Sequencing analysis revealed that there was a transversion happened from base C to T in the first exon, which produces a premature termination codon (TGA) and causes early termination (Figure 1A). We named the mutant here *Ossds-1* and suspected the mutation of *Os03g12414* leading to the sterile phenotype. We also obtained two other alleles arose from tissue culture of Nipponbare, named *Ossds-2* and *Ossds-3*, respectively. They all showed the same defects as *Ossds-1*. Sequencing analysis showed that there were a transversion from base G to C at the third exon causing corresponding amino acid A replaced by P in *Ossds-2* and two bases (GC) deletion at the fifth exon resulting in frame-shift mutation causing a premature termination codon (TGA) in *Ossds-3* (Figure 1A). As earlier termination close to the start codon in *Ossds-1* compared with the other mutants, it was selected for the subsequent experiments described below. To further confirm the mutant phenotype was resulted by the mutation of *OsSDS* gene, RNA interference (RNAi) approach was carried out to down-regulate SDS in rice variety Yandao 8. We got 27 transgenic plants with 19 plants exhibited complete sterility. Additionally, the transformation of plasmid pCAMBIA-1300 containing the whole *OsSDS* gene was successful in rescuing the sterility of the mutant plants, just as expected. These results strongly confirmed that the mutation of *OsSDS* gene led to the sterile phenotype as described above.

Expression of *OsSDS* was also analyzed by real-time PCR (RT-PCR). As shown in Supplemental Figure 2, transcripts were all detected at root, internode, leaf and panicle, indicating that *OsSDS* is not a meiosis-specific gene.

### FULL-LENGTH cDNA CLONING AND DEDUCED PROTEIN SEQUENCE OF *OsSDS*

The full-length cDNA of *OsSDS* gene was obtained by performing RT-PCR combined with 5' and 3' rapid amplification of cDNA ends PCR (RACE-PCR) using specific primers. We found that the *OsSDS* cDNA is comprised of 2786 bp containing an open reading frame (ORF) of 1410 bp and 1376 bp 5' and 3' untranslated regions (UTRs). The *OsSDS* cDNA sequence obtained is consistent with one mRNA (GenBank: AK065907.1) from the public network database (<http://www.ncbi.nlm.nih.gov/>). As shown in Figure 1A, the *OsSDS* gene contains seven exons and six introns. The predicted 469 amino acid protein of *OsSDS* shares as low as 30.6% identity with SDS in *Arabidopsis*, but with high similarity at the C-terminal (Figure 1B). Compared with the dicots *Arabidopsis*, *OsSDS* shares more than 60% identity with those in monocots, such as *Zea mays*, *Sorghum bicolor*, and *Brachypodium*. Conserved domain searching in NCBI revealed there are two conserved domains in SDS, namely, cyclin box fold domain



(residues 276–366) and cyclin C-terminal domain (residues 378–419) (**Figure 1B**). Blast searching in NCBI revealed that SDS is a plant specific protein and owns one single copy in the plant kingdom.

SDS protein sequences from different organisms. The numbers at the left of the sequences are the amino acid numbers. The black boxes represent identical sequences; the dark gray boxes represent conservative sequences; the light gray boxes represent weakly similar sequences. A predicted cyclin box fold domain (276–366 amino acids) and a predicted cyclin C-terminal domain (378–419 amino acids) are underlined.

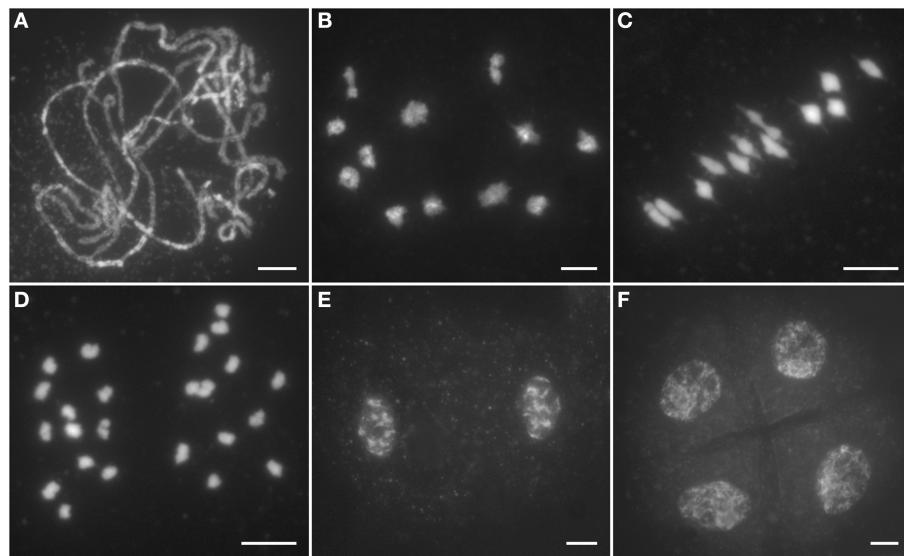
### MEIOTIC DEFECTS IN *Ossds*

To clarify whether the sterile phenotype of *Ossds* is caused by meiosis defects, chromosome behaviors at different stages of pollen mother cells (PMCs) from both wild type and *Ossds-1*

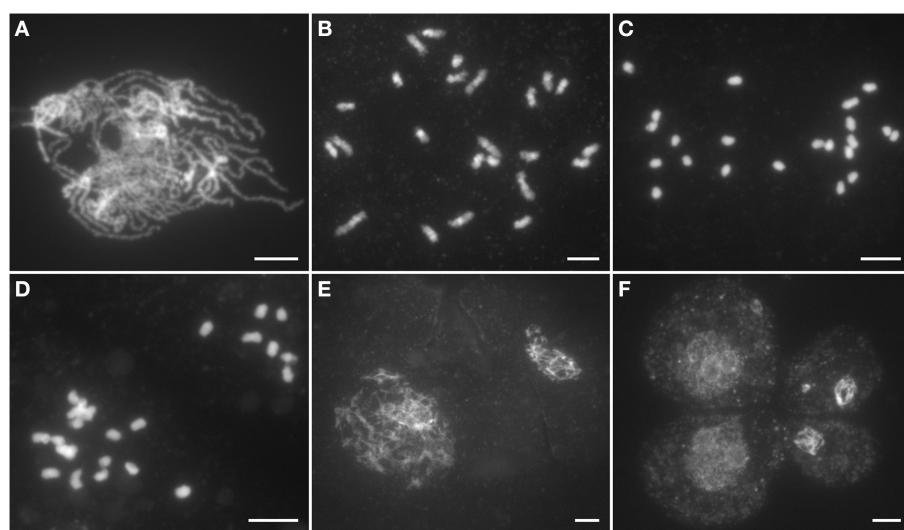
mutants were investigated. In wild type, chromosomes began to condense and became visible as thin thread-like structures at leptotene. After that, homologous chromosomes started to pair at zygotene. Fully synapsis between homologs formed at pachytene (**Figure 2A**). After further chromosome condensation, 12 bivalents were clearly observed at diakinesis (**Figure 2B**). Accompanying with the spindle installation, these bivalents aligned on the equatorial plate at metaphase I (**Figure 2C**). Then, homologous chromosomes separated and moved to the two opposite poles from anaphase I to telophase I (**Figures 2D,E**). During meiosis II, sister chromatids of each chromosome

separated from each other and finally tetrads formed (**Figure 2F**).

In *Ossds-1* PMCs, chromosome behaviors were similar to the wild type from leptotene to zygotene. However, obvious abnormalities were first observed at pachytene stage, where the *Ossds-1* mutant shows severely defects in homologous chromosome pairing and synapsis, and fully synapsed homologs were never observed (**Figure 3A**). Due to the lack of chromosome pairing, only univalents were observed at diakinesis (**Figure 3B**). During metaphase I, the univalents were unable to align on the equator plate (**Figure 3C**). From anaphase I to



**FIGURE 2 | Male meiosis of the wild type. (A)** Pachytene; **(B)** Diakinesis; **(C)** Metaphase I; **(D)** Anaphase I; **(E)** Dyad; **(F)** Tetrad. Chromosomes stained with 4,6-diamidino-2-phenylindole (DAPI). Bars = 5 μm.



**FIGURE 3 | Male meiosis of the *Ossds-1* mutant. (A)** Pachytene; **(B)** Diakinesis; **(C)** Metaphase I; **(D)** Anaphase I; **(E)** Dyad; **(F)** Tetrad. Chromosomes stained with DAPI. Bars = 5 μm.

telophase I, they randomly segregated into two daughter cells (**Figure 3D**). In meiosis II, dyads and tetrads always exhibited different sizes caused by uneven chromosome segregation (**Figures 3E,F**). Cytological observation of meiocytes from the other two alleles, *Ossds-2* and *Ossds-3*, as well as *OsSDS* RNAi plants showed the same meiotic defects as described in *Ossds-1* (Supplemental Figure 3). Therefore, we proposed that the sterility of *Ossds* was caused by uneven segregation of homologous chromosomes without pairing and recombination during propose I.

#### **OsSDS IS ESSENTIAL FOR MEIOTIC DSB FORMATION**

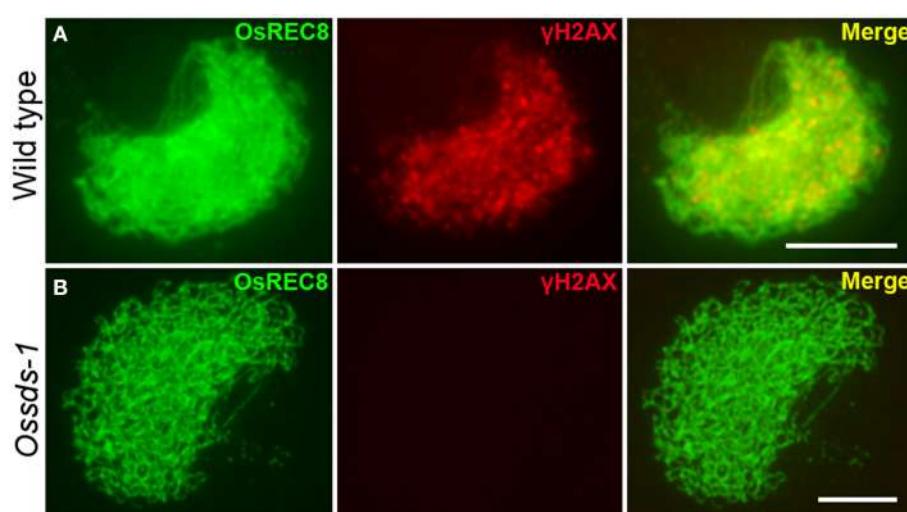
The above cytological observation indicated *Ossds* showed similar defects with the loss of function of CRC1, a meiotic DSB formation protein in rice (Miao et al., 2013). We wondered whether *OsSDS* is also required for meiotic DSB formation. Phosphorylation of histone H2AX occurs within a few minutes after a DSB initiated in mitosis (Banath et al., 2010), and the same kind phosphorylation takes place during the meiotic DSB formation (Dickey et al., 2009). To verify this hypothesis, a dual-immunostaining experiment was carried out utilizing antibodies specific for  $\gamma$ H2AX and OsREC8 raised from rabbit and mouse, respectively, in the meiocytes both from wild type and *Ossds-1*. OsREC8, one of the key cohesion proteins in rice meiosis, is localized onto meiotic chromosomes from leptotene to metaphase I (Wang et al., 2009). Here, we used it as a biomarker to indicate the rice meiotic chromosomes in the prophase I specifically. Results showed that numerous dot and patchy immunosignals of  $\gamma$ H2AX were detected at zygote in wild type (**Figure 4A**). However, no  $\gamma$ H2AX immunosignals was detected in *Ossds-1* meiocytes at the corresponding stage (**Figure 4B**), showing that *OsSDS* is essential for meiotic DSB formation.

During meiosis, all DSBs will be repaired by the repair system for maintaining genome stability. The loss function of repair proteins always results in chromosome fragmentation. OsRAD51C

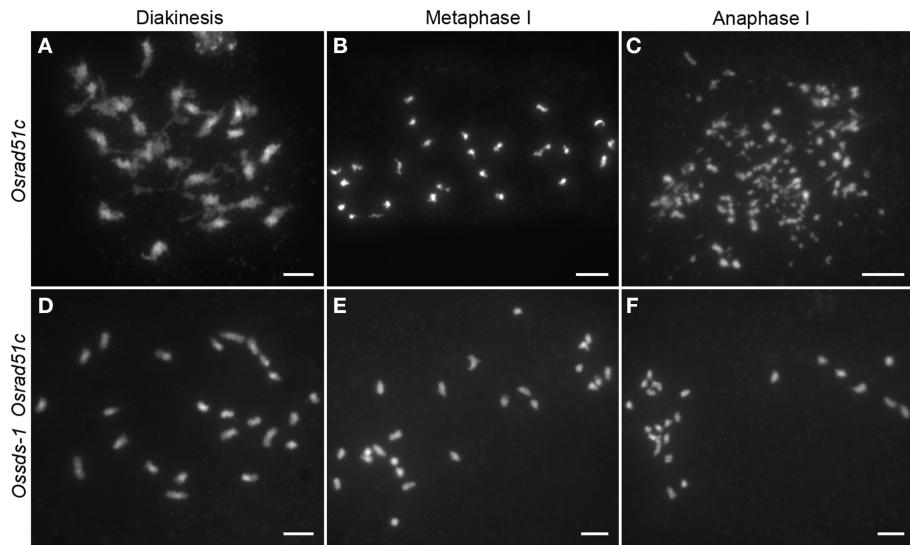
has been proved to be involved in meiotic DSB repair in rice (Tang et al., 2014). To verify this speculation, the *Ossds-1 Osrad51c* double mutant was generated by crossing the two heterozygous *Ossds-1 $\pm$*  and *Osrad51c $\pm$* , and further identified from their F2 progeny. In the *Osrad51c* mutant meiocytes, cytological observation shows 24 irregularly univalents appeared at diakinesis (**Figure 5A**). These univalents did not align well along the equatorial plate, and several chromosome fragments started to be shown at metaphase I (**Figure 5B**). Numerous chromosome fragments detained at the equatorial plate, while those massive chromosome bodies with centromeres moved into the two opposite poles at anaphase I (**Figure 5C**). While in the *Ossds-1 Osrad51c* meiocytes, chromosome behaviors were very much similar to *Ossds-1* at the corresponding stages (**Figures 5D–F**). Together with the above  $\gamma$ H2AX immunostaining data, we demonstrated very strong evidence that *OsSDS* is essential for DSB formation during rice meiosis.

#### **MEIOTIC CHROMOSOME AXES NORMALLY FORMED BUT SC INSTALLATION FAILED IN *Ossds***

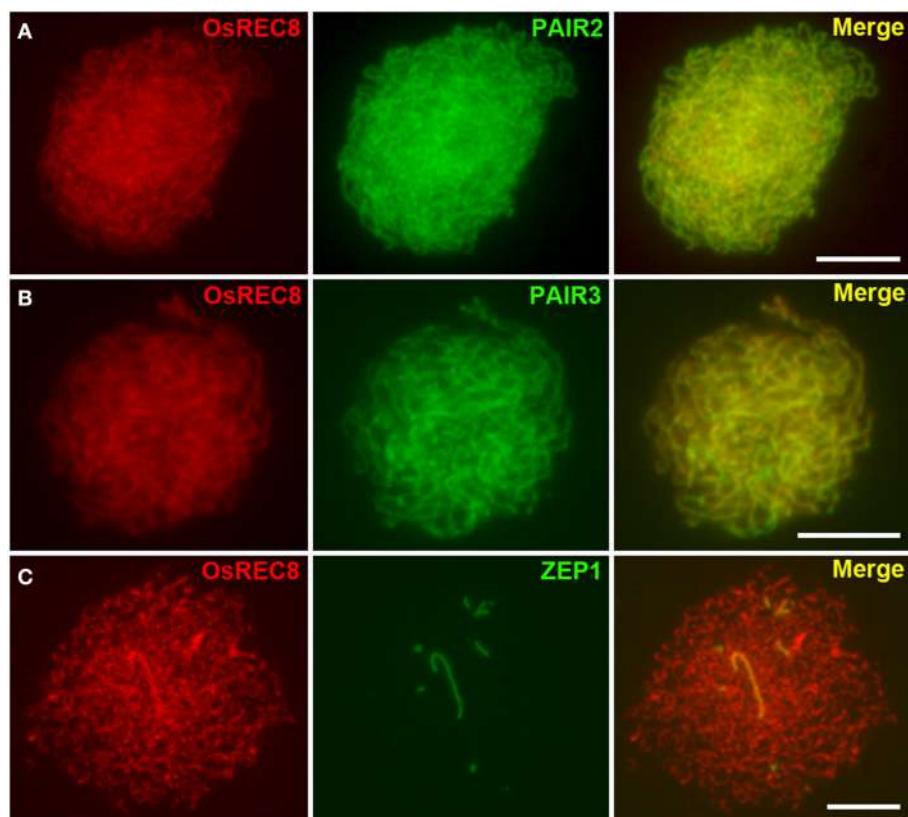
In rice, there are three axis-associated proteins OsREC8, PAIR2 and PAIR3 have been reported playing important roles in SC assembly (Nonomura et al., 2006; Shao et al., 2011; Wang et al., 2011). Except for those axial elements, ZEP1, the central element of SC, has also been identified (Wang et al., 2010). To investigate what kind of SC installation defects happened due to the loss function of *OsSDS*, we conducted immunodetection experiments using antibodies against PAIR2, PAIR3, and ZEP1 in *Ossds-1* microsporocytes. The results showed that OsREC8 was normally localized onto chromosomes in *Ossds-1* meiocytes. Moreover, both PAIR2 and PAIR3 were overlapped very well with OsREC8 at zygote, indicating their normal localization along the chromosome axis (**Figures 6A,B**). However, ZEP1 signals always appeared as short dots or discontinuous lines at early prophase I (**Figure 6C**), showing the deficient central element



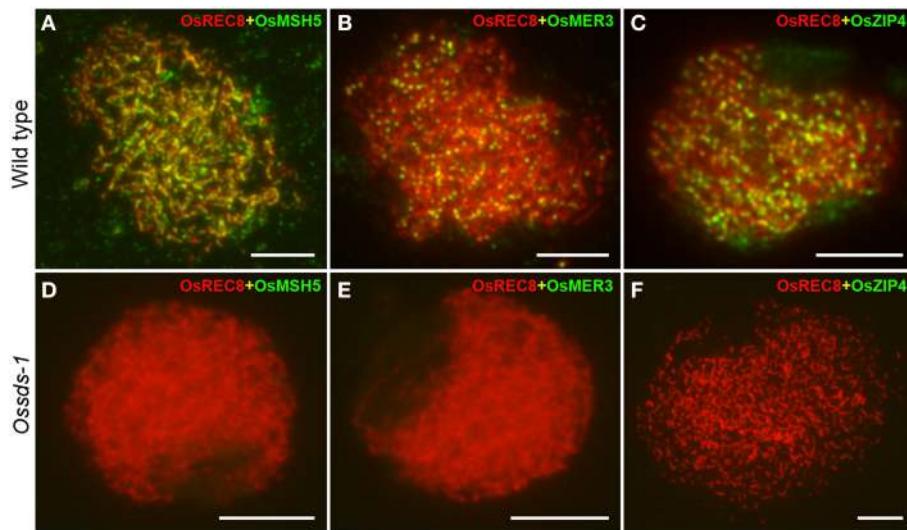
**FIGURE 4 |** Immunostaining of  $\gamma$ -H2AX at zygote in the wild type and *Ossds-1* mutant. OsREC8 signals were used to indicate the chromosome axes. Bars = 5  $\mu$ m.



**FIGURE 5 | Comparison of chromosome behaviors between *Osrad51c* and the *Ossds-1 Osrad51c* double mutant.** Chromosomes were stained with DAPI. Bars = 5  $\mu$ m.



**FIGURE 6 | Dual immunostaining detection of several meiotic proteins in the *Ossds-1*.** **(A)** OsREC8 (red) and PAIR2 (green) signals at late zygotene; **(B)** OsREC8 (red) and PAIR3 (green) signals at pachytene; **(C)** OsREC8 (red) and ZEP1 (green) signals at pachytene. Bars = 5  $\mu$ m.



**FIGURE 7 | Immunostaining detection of three ZMM proteins in the wild type and *Ossds-1*.** (A–C) Immunostaining for OsMSH5, OsMER3, and OsZIP4 at zygotene in the wild type.

(D–F) Immunostaining for OsMSH5, OsMER3, and OsZIP4 at zygotene in *Ossds-1*. OsREC8 was used indicating chromosome axes. Bars = 5  $\mu$ m.

installation of SC in the mutant. Thus, the meiotic chromosome axes normally formed but SC installation failed in *Ossds*.

#### OsSDS IS CRITICAL FOR FAITHFUL LOCALIZATION OF RECOMBINATION ELEMENTS ONTO MEIOTIC CHROMOSOMES

Meiotic homologous recombination finally falls into two recombination pathways by forming two type crossovers, interference-sensitive COs (class I) and interference-insensitive COs (class II). ZMM proteins are closely associated with class I crossovers formation (Chen et al., 2008; Shinohara et al., 2008). In rice, OsMSH5, OsMER3 and OsZEP4 are three ZMM proteins participating in the class I COs formation (Wang et al., 2009; Shen et al., 2012; Luo et al., 2013). To determine whether the defective OsSDS also affects the localization of OsMSH5, OsMER3, and OsZEP4, dual immunolocalization were carried out using antibodies against OsMSH5, OsMER3, and OsZEP4. In the wild-type microsporocytes, immunostaining experiments showed that OsMSH5, OsMER3, and OsZEP4 were observed as punctuate foci on chromosomes at zygotene (Figures 7A–C), and these foci persisted until late pachytene stage (Wang et al., 2009; Shen et al., 2012; Luo et al., 2013). However, no obvious signal foci of OsMSH5, OsMER3, and OsZEP4 were observed on chromosomes in *Ossds-1* microsporocytes at the corresponding stage (Figures 7D–F), indicating that OsSDS is critical for the localization of OsMSH5, OsMER3 and OsZEP4.

#### DISCUSSION

During meiosis, Spo11-catalyzed DSB formation is a highly conserved biological process among eukaryotes (Li and Ma, 2006). As increased data on plant meiosis research, several DSB formation proteins have been identified in higher plants, such as PRD1, PRD2, PRD3, and AtDFO in *Arabidopsis* (De Muyt et al., 2009; Zhang et al., 2012). And in rice, CRC1 was also reported to be involved in meiotic DSB formation besides those OsSPO11

homologs (Miao et al., 2013; Luo et al., 2014). Deficiency of these proteins always cause severe defects in homologous chromosome pairing, synapsis and nondisjunction.

MRE11 is known as an important DSB processing protein. The *Atmre11 sds* double mutant exhibited similar phenotype with the *Atmre11* single mutant (De Muyt et al., 2009), proposing that SDS may be not required for meiotic DSB formation in *Arabidopsis*. Moreover, in *sds* mutant, the localization of DMC1 was abnormal while RAD51 was normally loaded. Thus, the role of SDS in *Arabidopsis* was thought to be necessary for DMC1-mediated DSB repair utilizing the homologous chromosome (De Muyt et al., 2009). While in rice, we provided evidences that OsSDS is essential for meiotic DSB formation. SDS is a plant specific cyclin protein. Amino acid sequences alignment revealed that SDS orthologs between dicots and monocots showed very low identities, *Arabidopsis* SDS sharing 30.6% identity with OsSDS, and as low as 26.1% with BdSDS. While among monocots, they share high identities. For example, ZmSDS shares 69.1% with OsSDS, and 83.1% with SbSDS. The diverged sequences between dicots and monocots suggest that SDS function in meiosis has been differentiated a lot during its evolution.

To date, the precise mechanisms of DSB formation are still unclear. It has been reported that the NBS1 protein is recruited to the end of the DSB soon after a DSB formed, which then initiates the formation of the NBS1/MRE11/RAD50 complex (Kobayashi, 2004). After that, the ATM protein phosphorylates the serine 139 residue of H2AX through its auto-phosphorylation and leading to  $\gamma$ H2AX formation (Kinner et al., 2008). Studies revealed that  $\gamma$ H2AX plays dual role in the DSB triggered signaling pathway: one is recruiting more MRN complex to the DSB site thus enhancing the signalization by a positive feedback loop; the other one is binding the DNA damage repair proteins (Paull et al., 2000; Minter-Dykhouse et al., 2008). The process of H2AX

phosphorlation takes place just within a few minutes after a DSB occurrence (Banath et al., 2010). We did not detect any  $\gamma$ H2AX immunosignals in *Ossds*, indicating OsSDS is required for DSB formation during rice meiosis.

It has been reported that cyclin-dependent kinase Cdc7 and Cdc28 can directly regulate the meiotic DSB formation via the phosphorylation of Mer2 in budding yeast (Henderson et al., 2006; Sasanuma et al., 2008; Wan et al., 2008). However, we still do not know the molecular mechanism of how rice cyclin OsSDS being involved in meiotic DSB formation.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2015.00021/abstract>

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# MeioBase: a comprehensive database for meiosis

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Meiosis is a special type of cell division process necessary for the sexual reproduction of all eukaryotes. The ever expanding meiosis research calls for an effective and specialized database that is not readily available yet. To fill this gap, we have developed a knowledge database MeioBase (<http://meiosis.ibcas.ac.cn>), which is comprised of two core parts, *Resources* and *Tools*. In the *Resources* part, a wealth of meiosis data collected by curation and manual review from published literatures and biological databases are integrated and organized into various sections, such as *Cytology*, *Pathway*, *Species*, *Interaction*, and *Expression*. In the *Tools* part, some useful tools have been integrated into MeioBase, such as *Search*, *Download*, *Blast*, *Comparison*, *My Favorites*, *Submission*, and *Advice*. With a simplified and efficient web interface, users are able to search against the database with gene model IDs or keywords, and batch download the data for local investigation. We believe that MeioBase can greatly facilitate the researches related to meiosis.

**Keywords:** meiosis, MeioBase, knowledge database, meiotic genes, protein-protein interaction, eukaryotes, sexual reproduction

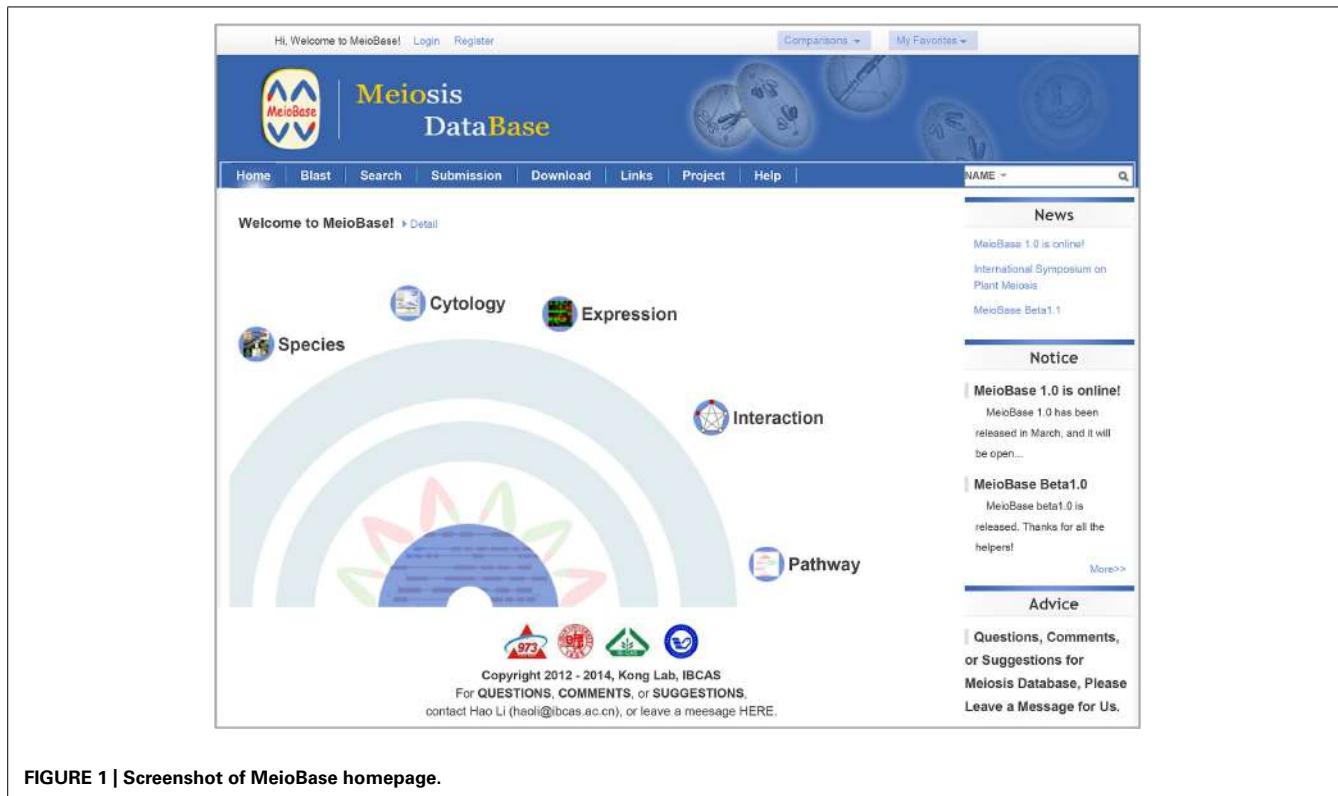
## INTRODUCTION

Meiosis is a specialized cell division process essential for all sexually reproducing organisms. During meiosis, a single round of DNA replication is followed by two successive rounds of nuclear division, meiosis I and meiosis II. Meiosis I is unique and involves the segregation of homologous chromosomes (homologs), whereas meiosis II is similar to mitosis and results in the segregation of sister chromatids. The function of meiosis is to generate four haploid gametes, which are able to develop into germ cells. Fertilization of the germ cells, then, can restore the offspring to the chromosome number and complexity level of their parents (Zickler and Kleckner, 1998; Hamant et al., 2006). Meiosis not only ensures the stability of chromosome numbers between generations, but also provides genetic materials for biodiversity.

Studies of meiosis have been carried out extensively for over 100 years (Hamant et al., 2006). Chromosome behaviors in some species have been examined in detail by using cytological approaches (Orr-Weaver, 1995; Zickler and Kleckner, 1999; Ma, 2005; Birchler and Han, 2013). Over the last two decades, much efforts have been devoted to understanding the genetic basis and molecular mechanisms of meiosis in model species, such as nematode (*Caenorhabditis elegans*), budding yeast (*Saccharomyces cerevisiae*), *Arabidopsis* (*Arabidopsis thaliana*), rice (*Oryza sativa*), and maize (*Zea mays*). Genes regulating meiosis, especially those involved in homologous chromosome paring, synapsis, recombination and separation in prophase I, have also been cloned and

characterized in terms of their functions (Hollingsworth et al., 1990; Sym et al., 1993; Keeney et al., 1997; Klimyuk and Jones, 1997; Yang et al., 1999; Li et al., 2004; Tang et al., 2014). Recent advances in transcriptomics, protein-protein interactions (PPIs), and phylogenetic analyses of genes and gene families related to meiosis have improved our understanding of this complex process dramatically (Kee and Keeney, 2002; Wang et al., 2004; Lin et al., 2006; Vignard et al., 2007; Chen et al., 2010; Tang et al., 2010; Yang et al., 2011; Dukowic-Schulze et al., 2014).

The ever expanding studies of meiosis and the data accumulated, which are scattered in tremendously diverse literatures and a few of large databases, such as NCBI, Ensemble, and TAIR, call for an integrative and encyclopedia-like platform for meiosis (Hamant et al., 2006; Handel and Schimenti, 2010; Luo et al., 2014). Recently, two databases related to reproductive development, GermOnline and Plant Male Reproduction Database (PMRD), have been established. GermOnline is a cross-species microarray expression database focusing on germline development, reproduction and cancer (Lardenois et al., 2010). PMRD is a comprehensive resource for browsing and retrieving knowledge about genes and mutants related to plant male reproduction (Cui et al., 2012). Notwithstanding, neither databases provide comprehensive information about meiosis, because data related to meiosis is heavily fragmented. Therefore, researchers are in great need of effective tools or databases to quickly obtain precise meiotic data from the exponentially increasing amount of information.



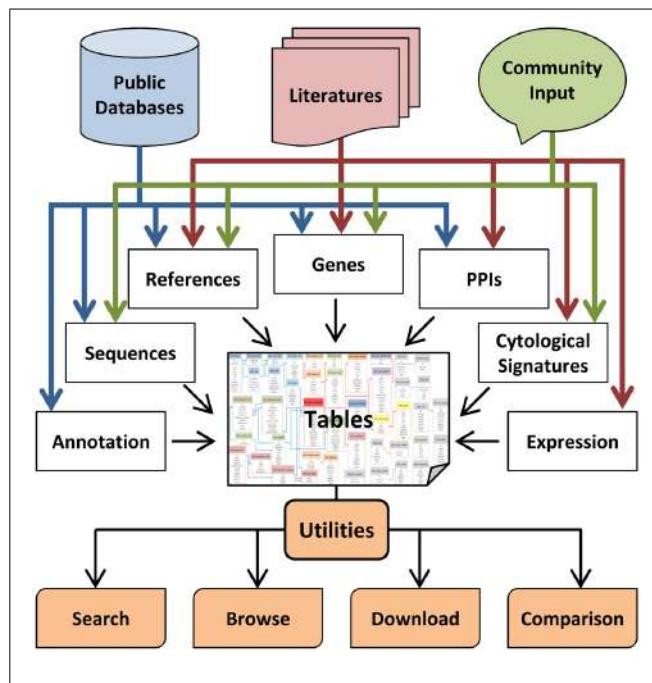
**FIGURE 1 | Screenshot of MeioBase homepage.**

Here, by collecting and integrating all sorts of information related to meiosis, as well as including and developing powerful tools for search, comparison and analysis, we established a comprehensive and specialized database for meiosis, MeioBase. It will not only serve the meiosis research community, but also help any users who need an easy and efficient access to various kinds of information related to meiosis.

## DATABASE STRUCTURE AND WEB INTERFACE

The database intends to provide all necessary resources and tools for meiosis-related researches (Figure 1). In the *Resources* part, information related to meiosis are categorized and integrated into five major sections (i.e., *Cytology*, *Pathway*, *Species*, *Interaction*, and *Expression*). In the *Tools* part, useful functions for searching, analyzing, uploading, and downloading data are included in seven sections (i.e., *Search*, *Download*, *Blast*, *Comparison*, *My Favorites*, *Submission*, and *Advice*). This structure can provide a centralized and user-friendly web portal for meiosis-related studies.

The website is built on a Linux, Apache, MySQL, and PHP (LAMP) stack. MySQL is used for storage, maintenance, and operation of the database, and 44 data sheets have been designed to form a network storing all the data (Figure 2). The front-end interface is implemented in PHP, which is a popular scripting language for dynamic web page. A well-defined and packaged JavaScript called jQuery is used to enhance the interface of the website and improve user experience. A navigation tool bar containing search box and links, such as *Home*, *Blast*, *Submission*, *Download*, and *Help*, are also included in each web page.



**FIGURE 2 | The flowchart for construction of MeioBase.** MeioBase is a comprehensive database for browsing and retrieving knowledge about meiosis, meiotic genes, and related data. MeioBase brings together three main sources of knowledge: (1) basic information about genes from public databases; (2) detailed curation of meiosis-related studies from the literatures; (3) public contributions from research community and other users. All of the information is stored in relational database tables that could be accessed by the utilities of MeioBase through any web browser.

## RESOURCES

Cytological features of meiosis have been described in detail for many species, which laid a solid foundation for the study of the molecular basis of meiosis (Zickler and Kleckner, 1999; Ma, 2005). Here, in the *Cytology* section, we provide an overview of the cytological process of meiosis, with special emphasis on the chromosome behaviors at different stages. To help understand the conservation and variability of the meiotic process during evolution, cytological features in model species are summarized and compared. Important advances related to meiosis can also be retrieved through clicking the *Updated Advances* links from the overview page.

During meiotic prophase I, several critical events related to meiotic chromosome structure and interaction occur, including pairing, synapsis, recombination, and segregation. Consistent with this, genes regulating meiosis have also been grouped into pathways or networks, each of which corresponds to one of the meiotic events (Gerton and Hawley, 2005; Chang et al., 2011). For this reason, we have established the *Pathway* section, in which genes with similar or related functions, as well as the complex regulatory relationships between them, are visualized by diagrams.

During the last 20 years, many genes involved in meiosis have been discovered and functionally characterized. By literature mining, 483 meiotic genes have been collected (Table 1). In the *Species* section, users can find the genes of a certain species and go into the details. In the *Gene Detailed Information* page, we have integrated useful information from references, databases and web servers. Data are organized by different aspects, such as *General Information*, *Featured Domains*, *Protein Signatures*, *Gene Ontology*, *Protein Sequence*, and *References*. *General Information* contains species name, gene name, gene family name, a brief description, gene model ID, and the coding sequence (CDS) of the gene.

*Featured Domains* provides protein domains predicted by Pfam. *Protein Signatures* and *Gene Ontology* provide more information of the functions of the proteins. *References* includes literatures with PMIDs in PubMed (Figure 3).

We also include 11,201 pieces of PPI data of the collected meiotic genes of *Arabidopsis*, nematode, and budding yeast in MeioBase, and display them in the *Interaction* section (Table 1). The PPI data are retrieved from 283 literatures and 11 databases, such as BIND, BioGRID, DIP, IntAct, and STRING. Users can search for partners of the proteins of interest, for which corresponding sources and references are provided.

To provide more comprehensive information on meiosis, references related to gene expression patterns are collected and displayed in the *Expression* section. Until now, important references of various species, such as *Arabidopsis*, rice, maize, petunia, wheat, mouse, rat, budding yeast, and fission yeast, have been listed in this section and more expression data of meiosis are being collected.

In addition to the aforementioned five sections, MeioBase provides many other resources in the *Links* section, such as the commonly used databases, powerful web servers for molecular and genomic analysis, and experimental protocols for meiosis research, etc. An introduction to our “Plant Meiosis Project” can be found in the *Project* section. Important progresses and events on meiosis research are available in the *News* section. Release notes of MeioBase are announced in the *Notice* section. To provide an overview of our database, we also include a detailed introduction in the *Help* section.

## TOOLS

MeioBase provides various ways to retrieve the data that users are interested in. By using the search box, users can search genes by gene names, gene model IDs or MeioBase IDs. In the *Search*

**Table 1 | Data status in the current MeioBase.**

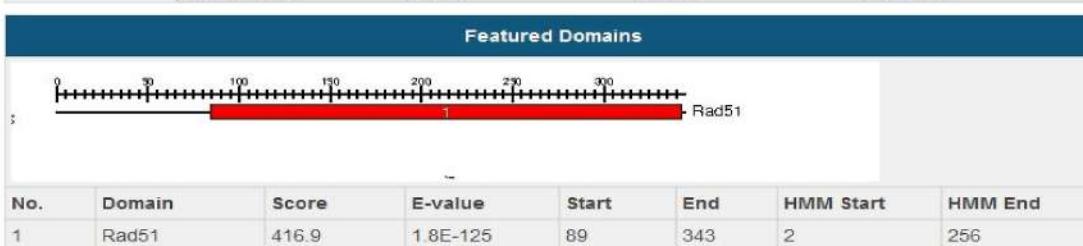
| Species                          | Common name   | Number of meiotic genes <sup>1</sup> |                         |                         |                              |                           |              | PPIs <sup>2</sup> |
|----------------------------------|---------------|--------------------------------------|-------------------------|-------------------------|------------------------------|---------------------------|--------------|-------------------|
|                                  |               | Total                                | Initiation &<br>Pairing | Synaptonemal<br>complex | Chromosomal<br>recombination | Chromosome<br>segregation | Unclassified |                   |
| <i>Arabidopsis thaliana</i>      | Arabidopsis   | 88                                   | 17                      | 8                       | 37                           | 11                        | 15           | 445               |
| <i>Oryza sativa</i>              | Rice          | 32                                   | 8                       | 4                       | 18                           | 2                         | 0            | NA                |
| <i>Zea mays</i>                  | Maize         | 8                                    | 4                       | 1                       | 2                            | 1                         | 0            | NA                |
| <i>Caenorhabditis elegans</i>    | Nematode      | 178                                  | 16                      | 11                      | 31                           | 62                        | 58           | 675               |
| <i>Mus musculus</i>              | Mouse         | 10                                   | 0                       | 6                       | 4                            | 0                         | 0            | NA                |
| <i>Saccharomyces cerevisiae</i>  | Budding yeast | 162                                  | 18                      | 9                       | 71                           | 22                        | 42           | 10081             |
| <i>Schizosaccharomyces pombe</i> | Fission yeast | 5                                    | 1                       | 0                       | 3                            | 1                         | 0            | NA                |
| Total                            |               | 483                                  | 64                      | 39                      | 166                          | 99                        | 115          | 11201             |

<sup>1</sup>The number of functionally characterized meiotic genes that have been integrated into MeioBase.

<sup>2</sup>The number of PPI data that have been integrated into MeioBase.

**A**

|                    |  | General Information |                                 |  |                                 |  |  |  |
|--------------------|--|---------------------|---------------------------------|--|---------------------------------|--|--|--|
| Species            | <i>Arabidopsis thaliana</i>  |                     |                                 |  |                                 |  |  |  |
| MDB ID             | Ath000138  |                     |                                 |  |                                 |  |  |  |
| Gene Family        | RAD51  |                     |                                 |  |                                 |  |  |  |
| Gene Name          | DMC1   |                     |                                 |  |                                 |  |  |  |
| Protein Properties | Length: 344 aa MW: 37512.8 Da PI: 5.4905   |                     |                                 |  |                                 |  |  |  |
| Description        | <b>Functionally-known meiotic gene.</b> DISRUPTION OF MEIOTIC CONTROL 1 (DMC1); FUNCTIONS IN: in 6 functions; INVOLVED IN: DNA repair, meiosis, chiasma assembly, reciprocal meiotic recombination, DNA metabolic process; LOCATED IN: nucleus; EXPRESSED IN: 25 plant structures; EXPRESSED DURING: 12 growth stages; CONTAINS InterPro DOMAIN/s: DNA recombination/repair protein RecA/RadB, ATP-binding domain (InterPro:IPR020588), DNA repair Rad51/transcription factor NusA, alpha-helical (InterPro:IPR010995), ATPase, AAA+ type, core (InterPro:IPR003593), Meiotic recombinase Dmc1 (InterPro:IPR011940), DNA recombination and repair protein, RecA-like (InterPro:IPR016467), DNA recombination/repair protein RecA, monomer-monomer Interface (InterPro:IPR020587), DNA recombination and repair protein Rad51, C-terminal (InterPro:IPR013632); BEST Arabidopsis thaliana protein match is: RAS associated with diabetes protein 51 (TAIR:AT5G20850.1); Has 11998 Blast hits to 11924 proteins in 3797 species: Archae - 689; Bacteria - 8026; Metazoa - 742; Fungi - 447; Plants - 503; Viruses - 22; Other Eukaryotes - 1569 (source: NCBI BLINK) |                     |                                 |  |                                 |  |  |  |
| Gene Model         | <b>Gene Model ID</b><br>AT3G22880.1<br>NM_113188.2   |                     | <b>Type</b><br>genome<br>refseq |  | <b>Source</b><br>TAIR<br>Refseq |  | <b>Coding Sequence</b><br><a href="#">View CDS</a><br><a href="#">View CDS</a> |  |

**B****C**

| Protein Signatures |                |         |       |     |             |                |
|--------------------|----------------|---------|-------|-----|-------------|----------------|
| Database           | Entry ID       | E-value | Start | End | InterPro ID | Description    |
| PIR-PSD            | PIRSF005856    | 0       | 1     | 344 | IPR016467   | Rad51          |
| PANTHER            | PTHR22942:SF13 | 0       | 9     | 344 | nolPR       | PTHR22942:SF13 |
| PANTHER            | PTHR22942      | 0       | 9     | 344 | nolPR       | PTHR22942      |

**D**

| Gene Ontology |                    |                                  |
|---------------|--------------------|----------------------------------|
| GO Term       | GO Category        | GO Description                   |
| GO:0006259    | Biological Process | DNA metabolic process            |
| GO:0007131    | Biological Process | reciprocal meiotic recombination |

**E**

| Protein Sequence   |                                   |                               |
|--|-----------------------------------|-------------------------------|
| Length: 344 aa   | <a href="#">Download sequence</a> | <a href="#">Send to blast</a> |
| MMASLKAEEET SQMLQLVEREE NDEDDELDEM IDKLIAQGIN AGDVVKLQEA GIHTCNGLMM HTKKNLTGK GLSEAKVDKI 80<br>CAAEKIVNF GYMTGSDALI KRKSVVKIT GCQALDDLLG GGIETSAITE AFGEFRSGKT QLAHTLCVIT QLPTNMKGNN 160<br>GKVAVIDTEG TFRDRIVPI AERFGMDPGE VLDDNIIYARA YTYEHQYNLL LGLAAKMSEE PFRILIVDSI IALFRVDFTG 240<br>RGELADRQQK LAQMLSRLIK IAEEFNVAVY MTNQVIADPG GGMFISDPKK PAGGHVLAHA ATIRLLFRKKG KGDTRVCKVY 320<br>DAPNLAEAEA SFQTQGGIA DAKD |                                   |                               |

**F**

| References |   |
|------------|---|
| 1          | Klimyuk VI, Jones JD.   |
|            | AtDMC1, the <i>Arabidopsis</i> homologue of the yeast DMC1 gene: characterization, transposon-induced allelic variation and meiosis-associated expression |
|            | PMID: 9025299   |

**FIGURE 3 | Screenshot of gene information.** The Gene Detailed Information page of a meiotic gene consists of six aspects. **(A)** General Information; **(B)** Featured Domains; **(C)** Protein Signatures; **(D)** Gene Ontology; **(E)** Protein Sequence; **(F)** References.

section, users can search genes not only by inputting their model IDs but also by providing keywords describing them. PPI data can also be searched with keywords. All data can be downloaded in the *Download* section, which links to the FTP site.

Blast search against all the data in MeioBase is provided to facilitate users of finding similar sequence of a given sequence. In the *Comparison* section, users can add any two genes in the gene list and compare them. Users can also add any ten genes into the *My Favorites* section for fast checking afterward.

Moreover, we have integrated sections specifically for contributing meiosis data or suggestions to MeioBase. In the *Submission* section, users can first download and fill in a customized excel file as directions with meiotic genes, PPI data, and other data not yet included in the database, and then upload it to the database. After checking the uploaded data, we will add the qualified ones into MeioBase timely. In *Advice* section in homepage and at the foot of every page, users could ask any questions or give us comments or suggestions about this database. We will appreciate every user for improving MeioBase and reply as soon as possible.

## FUTURE PLANS

MeioBase is the first web database providing comprehensive information on meiosis. It is only a start of establishing a large and well-known database on meiosis. We will reiterate the process of database structure and user interface development to enhance the data content and functionality. The major data content enhancement will come from elaboration of the gene annotation and incorporation of more meiotic genes in other species, various expression data from references and other databases, PPI networks of different species, and other vital pathways during meiosis.

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# Pollination triggers female gametophyte development in immature *Nicotiana tabacum* flowers

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In *Nicotiana tabacum*, female gametophytes are not fully developed at anthesis, but flower buds pollinated 12 h before anthesis produce mature embryo sacs. We investigated several pollination-associated parameters in *N. tabacum* flower buds to determine the developmental timing of important events in preparation for successful fertilization. First, we performed hand pollinations in flowers from stages 4 to 11 to study at which developmental stage pollination would produce fruits. A Peroxidase test was performed to correlate peroxidase activity on the stigma surface, indicative of stigma receptivity, with fruit set. Pollen tube growth and female gametophyte development were microscopically analyzed in pistils of different developmental stages. Fruits were obtained only after pollinations of flower buds at late stage 7 and older; fruit weight and seed germination capacity increased as the developmental stage of the pollinated flower approached anthesis. Despite positive peroxidase activity and pollen tube growth, pistils at stages 5 and 6 were unable to produce fruits. At late stage 7, female gametophytes were undergoing first mitotic division. After 24 h, female gametophytes of unpollinated pistils were still in the end of the first division, whereas those of pollinated pistils showed egg cells. RT-qPCR assay showed that the expression of the *NtEC1* gene, a marker of egg cell development, is considerably higher in pollinated late stage 7 ovaries compared with unpollinated ovaries. To test whether ethylene is the signal eliciting female gametophyte maturation, the expression of ACC synthase was examined in unpollinated and pollinated stage 6 and late stage 7 stigmas/styles. Pollination induced *NtACS* expression in stage 6 pistils, which are unable to produce fruits. Our results show that pollination is a stimulus capable of triggering female gametophyte development in immature tobacco flowers and suggests the existence of a yet undefined signal sensed by the pistil.

**Keywords:** stigma receptivity, pollen tube growth, pollination signal, female gametophyte development, fruit weight, seed germination capacity

## Introduction

Angiosperms correspond to a group of plants with distinct characteristics, including the presence of ovules enclosed in a maternal organ, known as the pistil. The pistil is generally composed of a stigma, a style, and an ovary that develops into a fruit after fertilization. The fertilization process begins with the deposition of pollen grains onto a receptive stigma surface. When pollen grain recognition and acceptance occurs, it will hydrate and germinate, producing a pollen tube that grows through the style in the direction of the ovary until reaching an embryo sac, known as the angiosperm female gametophyte. Next, the pollen tube bursts, releasing two sperm cells in the interior of the embryo sac. One of the sperm cells fuses to the egg cell to produce a diploid embryo, whereas the second sperm cell fuses to the central cell, generating a triploid endosperm (Lersten, 2004).

Successful reproduction in angiosperms depends on a series of cell-cell interactions between male gametophytes and the specialized tissues of the pistil and female gametophytes (Beale and Johnson, 2013). Over the last few years, new information and discoveries have increased our knowledge about the signals produced by the female gametophytes to attract and direct the pollen tubes (Beale and Johnson, 2013). However, less is known about signals produced by the pollen tube or pistil in response to pollen tube growth, establishing communication with female gametophytes. Some evidence of this male-female directional signaling comes from studies of orchid species, in which ovule differentiation and development are pollination-dependent (Zhang and O'Neill, 1993; O'Neill, 1997). Once inside the ovule, the orchid pollen tube waits for the female gametophyte to complete development before releasing sperm cells to promote fertilization (O'Neill et al., 1993). In other plants, such as almond, the ovule is partially developed at anthesis and reaches full maturation only after pollination (Pimienta and Polito, 1983). This pattern of female gametophyte development triggered by pollination stimulus has also been observed for sweet pepper (Ofosu-Anim et al., 2006) and maize (Mól et al., 2000).

In breeding programs, researchers may perform bud pollination to overcome incongruity in interspecific crosses or self-incompatibility. Hand pollination using mature pollen can be performed on immature flowers (Haring et al., 1990) in an attempt to bypass the effects of self-incompatibility on species of Brassicaceae (Hiscock and Dickinson, 1993), as well as Solanaceae species (Chalivendra et al., 2013). In *Nicotiana tabacum*, a species with a Polygonum-type embryo sac (Huang and Russell, 1992), the ovule is not fully developed at anthesis, and the egg cell is not usually observed at the embryo sacs (Tian and Russell, 1997; Lobanova and Enaleeva, 1998; De Martinis and Mariani, 1999; Chen et al., 2012). In this species, the effects of pollination on ovule development have mainly been examined at stages close to anthesis. Hand-pollinated tobacco flower buds 12 h before anthesis reach the mature embryo sac stage earlier than flower buds emasculated and not pollinated (Tian and Russell, 1997), suggesting the existence of a male-female directional signaling. De Martinis and Mariani

(1999) noted that pollinations in young flower buds (stage 6) do not induce embryo sac formation and seed production, but they did not investigate this aspect in detail. Thus, little is known regarding the developmental timing of important events in preparation for successful fertilization in *N. tabacum* flowers.

Our hypothesis is that pollinations performed on young flower buds will be effective and produce fruits, despite the fact that *N. tabacum* female gametophytes are not fully developed at anthesis. At which flower developmental stage pollination will be productive? We have examined several parameters related to reproductive success (Calixto et al., 2009), such as fruit formation, seed production and germination capacity, and correlated them with stigma receptivity based on peroxidase activity, microscopy analysis of pollen tube growth, embryo sac development, and *NtEC1* (Egg Cell 1) gene expression. Our results represent a detailed analysis of the effects of pollination on *N. tabacum* flower buds at stages prior to anthesis and shows that preparation for successful fertilization is a gradual process in which the necessary requirements are achieved in phases. This work provides evidence for the existence of male-female signaling produced by pollination and considers whether ethylene could be this signal through an investigation of ACC synthase expression. We have shown that a yet undefined pollination signal is sensed by the *N. tabacum* pistil throughout half of its development and is sufficient to trigger cellular and molecular female gametophyte maturation.

## Materials and Methods

### Plant Material

Seeds from *N. tabacum* cv. Petit Havana SR1 were sown in expanded polystyrene trays containing PlantMax commercial substrate (Eucatex, Brazil). After germination and growth to a height of approximately 3 cm, plantlets were transferred to plastic bags and later to 20 L vases. During germination and growth, plants were cultivated in standard greenhouse conditions and irrigated by aspersión. The stages of tobacco flower development were determined using parameters previously described by Koltunow et al. (1990).

### Controlled Pollinations and Fruit Analyses

Tobacco pistils from stages 4 to 11 of flower development were emasculated and hand pollinated with mature pollen grains from flowers at anthesis (stage 12). Stage 12 flowers were not included in this work because they are naturally pollinated at this stage. For each analyzed stage, a minimum of eight pistils (from at least six independent plants) were hand pollinated and labeled with sewing threads of different colors. Approximately 20 days after pollination, the pollinated pistils were analyzed for the presence or absence of fruits. The obtained fruits were collected individually and dried at room temperature for approximately 2 days. On the third day, fruits were separately weighed on a precision balance (Acculab - L series). The data obtained were analyzed statistically

using an analysis of variance (ANOVA) of PROC GLM (software SAS version 9). When variation between two stages was detected, differences with  $p < 0.05$  were considered significant.

### Analysis of Seed Germination

To establish the germination capacity of the seeds produced, 300 seeds (from fruits obtained at each developmental stage) were placed in sterile wet filter paper (100 seeds per plate). Two weeks later, the number of germinated seeds was counted, and the results were analyzed using Student's *t*-test ( $p \leq 0.05$ ).

### Determination of Stigma Receptivity

To study the stigma receptivity, we used special peroxidase test papers (Peroxtesmo KO, Macherey-Nagel – Düren, Germany) as proposed by Dafni and Maués (1998). For this purpose, four stigmas from tobacco flowers at stages 4 to 11 were pressed against peroxidase test-paper and were regarded as positive when blue coloration developed.

### Analyses of Pollen Tube Growth

Controlled hand pollinations were performed with stages 4–11 tobacco pistils, as described above. According to De Graaf et al. (2003), pollen tubes reach the tobacco ovary 24 h after pollination. Thus, pollinated pistils were excised 24 h after hand pollination. Stigmas/styles and ovaries were separated and immediately fixed in FPA 50 [2.5 mL of 37% formaldehyde (Sigma), 2.5 mL of propionic acid (Vetec – Brazil), and 45 mL of 50% ethanol (Merck)]. The samples were subjected to 15 mmHg vacuum for 15 min in the presence of the fixative. This procedure was repeated four times, and the material was left on the fixative overnight. The fixative was substituted by 50% ethanol, and the material was incubated at 8°C overnight. The next day, 50% ethanol was substituted by 70% ethanol. Longitudinally hand-opened stigmas/styles and ovaries were placed on a glass slide and stained in a 0.1% solution of aniline blue in 0.1 N K<sub>3</sub>PO<sub>4</sub> (Kho and Bera, 1968). The samples were carefully squashed between a glass slide and coverslip in the aniline blue solution, revealing the pollen tube callose plugs. Visualization and documentation were performed with a Zeiss Axiolab epifluorescence microscope (HBO 103W/2 lamp) using an excitation wavelength of 450/90 nm and an emission wavelength of 520 nm. Images were taken using a Zeiss AxioCam Color 412-312 and AxioVision LE4.8 software.

### Microscopic Analyses of Pollinated and Unpollinated Ovaries

Late stage 7 flowers (35 mm) were emasculated and kept unpollinated or were pollinated with pollen grains from stage 12 flowers (anthesis). Ovaries from both pollinated and unpollinated flowers were harvested after 24 h and fixed in FAA 50 [5 mL of glacial acetic acid (Merck), 5 mL of 37% formaldehyde (Sigma), and 90 mL of 50% ethanol (Merck)] for 24 h (Johansen, 1940). Then, the samples were transferred to 50% ethanol and subsequently to 70% ethanol, in which they were stored. The ovaries were dehydrated

in a graded ethanol/xylol series and embedded in paraffin. The embedded material was sliced into 6-μm sections, mounted on microscope slides and stained with 0.05% of toluidine blue pH 6.8. The pictures were taken using a Leica DM50 microscope equipped with a Leica DFC 320 digital camera.

### RNA Extraction, cDNA Synthesis, and RT-qPCR Analysis

As described above, stage 6 and late stage 7 flowers were emasculated and kept unpollinated or were pollinated with mature pollen. After 24 h, stigmas/styles and ovaries were collected in liquid nitrogen and stored at –80°C (three biological replicates for each condition, each replicate containing three pistil samples). The RNA of each sample was extracted using Trizol (Invitrogen®) according to the manufacturer's protocol. RNA integrity was checked by electrophoresis in 1.2% agarose and 20 mM guanidine isothiocyanate gel. RNA samples were treated with RNase-free DNase (Promega®) following the manufacturer's instructions and an aliquot was used to check for genomic DNA contamination in a standard qPCR with GAPDH (glyceraldehyde 3-phosphate dehydrogenase) primers (see below). DNA-free RNA was cleaned using *Clean up – Rneasy Mini Kit* (Qiagen) and quantified in a NanoDrop 2000 (Thermo Scientific). SuperScript III reverse transcriptase (Invitrogen®) was used to generate cDNA from 1 μg of the RNA samples. qPCR experiments were carried out in three technical replicates on an Applied Biosystems 7500 Fast Real-Time PCR System. Each reaction was composed of 5 μL of GoTaq qPCR Master Mix (Promega), 1 μL of sterile Milli-Q purified water, 1 μL (2.5 μM) of each adequate primer (GAPDH forward GCATCTTGATGCCAAGGCTGGAA and GAPDH reverse TCGAGTGCTGTA GCCCATTCGTT; RPL2 forward CGGGTGTGTCACTTTCCG TTACCCG and reverse ATACCCTCAGCAGGCCACGAAC; NtEC1 forward CTGTTGGCCTTCTATGCTTACT and reverse GGTTGAGGTGATGGAGTTC; and NtACS forward TTCAG AGCCTGGTTGGTTAG and reverse GACTCCTCCTTCAAT CCCTTAC), and 2 μL of cDNA. The cycling conditions consisted of a initial step of 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. RPL2 (Ribosomal Protein L2) and GAPDH were previously validated by our group as the best reference genes for different experimental conditions and pistil samples (unpublished results). The efficiency of primer pairs was determined from the slope of the standard curve using the formula Efficiency ( $E$ ) = 10 ( $-1/\text{slope}$ ) and then converted to percentage efficiency, where % of efficiency =  $(E-1) \times 100\%$ . Confirmation of amplicon specificity was based on the dissociation curve at the end of each run (ramp time 55–95°C). qPCR reactions in the absence of template were also performed as negative controls for each pair of primers. The expression levels of NtEC1 and NtACS were determined using the formula:  $2^{-\Delta Ct}$ , where  $\Delta Ct = (C_{tag} - C_{ref})$ , Ct = threshold cycle, tag = tag gene, and ref = reference gene, derived from the  $2^{-\Delta \Delta Ct}$  method originally published by Livak and Schmittgen (2001). Relative expression was determined by comparing the NtEC1 transcript expression level between unpollinated, considered as 1, and pollinated ovaries.

For *NtACS*, the expression ratios were determined comparing pollinated with unpollinated pistils for each stage (6 or late 7). Statistical analysis using expression data were performed using the REST tool (Pfafll et al., 2002) and data are available in Supplementary Table S1. The accession numbers are: GAPDH – KR007670; RPL2 – X62500; NtEC1 – KP987452; NtACS – X98492.

## Results

### *Nicotiana tabacum* Fruit Weight and Size are Dependent on the Flower Developmental Stage in Which Pollination Occurs

Controlled pollinations with mature pollen (see Materials and Methods) were performed on stigmas at stages 4–11 of flower development. Pollinations performed on stigmas at stages 4–6 did not produce fruits (Table 1). Fruit formation was only observed as a result of pollinations performed at later stages (7–12). However, although fruits were produced in 100% of pollinations performed at stages 8–12, at stage 7, fruit formation was dependent on the specific size of the flower bud (Table 1). Fruits were produced only when the pollinated flower buds were 34 mm or longer. Therefore, we divided the stage 7 initially described by Koltunow et al. (1990) into early stage 7, with flower buds with sizes between 28 and 33 mm, and late stage 7, with flower buds with sizes between 34 and 38 mm. Fruits obtained by this analysis were weighted and photographed. The mean fruit weight increased in accordance with the flower developmental stage in which pollination was performed, i.e., the fruit was heavier at later stages (Table 1). ANOVA and contrast comparison statistical analyses were performed and demonstrated no significant difference in relation to the mean fruit weight produced among pollinations performed at late stage 7 and stages 8 and 9 (Table 1). No statistically significant differences were observed in the mean fruit weight obtained by pollinations at stages 8, 9, 10, and 11 (Table 1). However, the mean fruit weight corresponding to late stage 7 was significantly different from the mean fruit weight of pollinations conducted at stages 10 and 11 (Table 1).

**Figure 1** shows that in addition to fruit weight, fruit size was also influenced by pollinations performed in flower buds at different developmental stages, with size increasing from late stage 7 to stage 11 (Figure 1) in parallel with their increasing fruit weight. The most likely explanation for the differences observed in fruit size and weight is the number of seeds successfully produced as a result of pollinations conducted at the different developmental stages.

### Pollination at Earlier Flower Developmental Stages Affects Seed Germination Capacity

To study the germination capacity of seeds produced by pollinations at different developmental stages, we used triplicates of 100 seeds from fruits produced at late stage 7 and onward and placed them in wet filter paper. Two weeks later, the germinated seeds were counted, and the numbers were statistically analyzed (Student's *t*-test with  $p \leq 0.05$ ). Seeds from late stage 7 pollinations showed the lowest germination capacity ( $65\% \pm 5.7\%$ ). As shown in Figure 2, the germination capacity increased among seeds produced by pollinations at stage 8 and later toward anthesis. The highest germination capacity ( $94\% \pm 1.5\%$ ) was verified with seeds of stage 11 pollinations, the latest stage analyzed in this study. Significant differences were observed in the germination capacity of seeds from fruits produced at all developmental stages, except in seeds from stages 9/10, and 10/11 fruits (Figure 2). There were seeds capable of germination in all fruits obtained by controlled hand pollinations. However, under our experimental conditions, not all seeds produced were able to germinate, suggesting they were malformed, or physiologically immature.

### Peroxidase Activity Correlates with Stigma Ability to Sustain Pollen Tube Growth

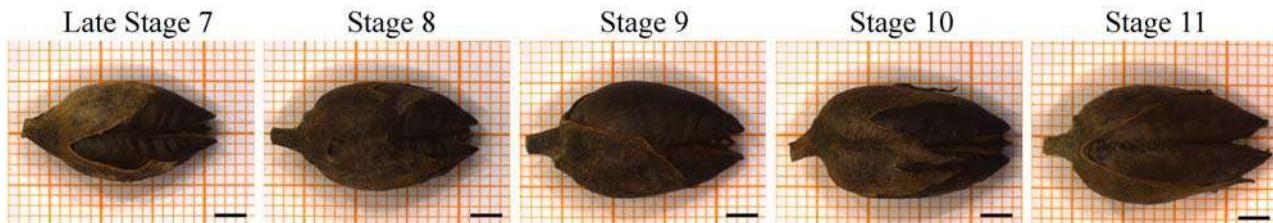
According to Dafni and Maués (1998), the Peroxtesmo KO peroxidase test is the most reliable method for establishing stigma receptivity. Therefore, we have used this test on four stigmas of each flower developmental stage (from 4 to 11) of *N. tabacum*. The peroxidase activity test was negative in all stage 4 stigmas

**TABLE 1 | Analysis of fruit formation as a result of hand pollinations performed at different stages of *N. tabacum* flower development.**

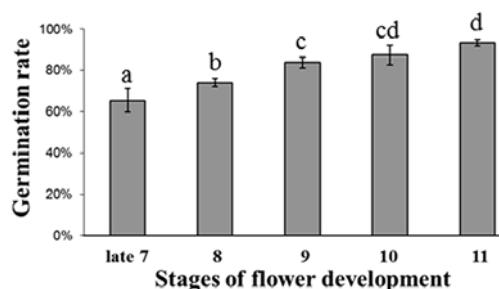
| Flower developmental stages | Length of floral bud (mm)* | Number of flower buds pollinated | Presence of fruit | Amount of fruits formed (%) | Average fruit weight (mg)** |
|-----------------------------|----------------------------|----------------------------------|-------------------|-----------------------------|-----------------------------|
| 4                           | 16–19                      | 15                               | No                | 0 (0%)                      | —                           |
| 5                           | 20–21                      | 15                               | No                | 0 (0%)                      | —                           |
| 6                           | 22–27                      | 16                               | No                | 0 (0%)                      | —                           |
| Early 7                     | 28–33                      | 09                               | No                | 0 (0%)                      | —                           |
| Late 7                      | 34–38                      | 11                               | Yes               | 11 (100%)                   | $5.88 \pm 2.9^a$            |
| 8                           | 39–42                      | 17                               | Yes               | 17 (100%)                   | $9.20 \pm 4.9^{ab}$         |
| 9                           | 43–44                      | 12                               | Yes               | 12 (100%)                   | $12.13 \pm 4.7^{ab}$        |
| 10                          | 45–46                      | 13                               | Yes               | 13 (100%)                   | $14.13 \pm 7.6^b$           |
| 11                          | 47                         | 12                               | Yes               | 12 (100%)                   | $14.75 \pm 7.8^b$           |

\*Data originally published by Koltunow et al. (1990) and used in the present work to establish the stages of tobacco flower development.

\*\*Different letters indicate significant differences of fruit weight. Statistical analyses were performed using analysis of variance (ANOVA) and Contrast comparison with  $p \leq 0.05$ .



**FIGURE 1 | Representative fruits produced as a result of hand pollinations performed at different stages of *Nicotiana tabacum* flower development.**  
There is a gradual increase in fruit size from late stage 7 to stage 11.



**FIGURE 2 | Tobacco seed germination capacity evaluated as the percentage of germinated seeds.** For each developmental stage, three replicates of 100 seeds each were used. Bars indicate the SD, and different letters represent statistically significant differences by Student's *t*-test ( $p \leq 0.05$ ).

analyzed. In contrast, all stigmas from stages 5 and later showed positive results on the peroxidase activity test, suggesting that stigmas were receptive to pollen grains at developmental stages earlier than anthesis.

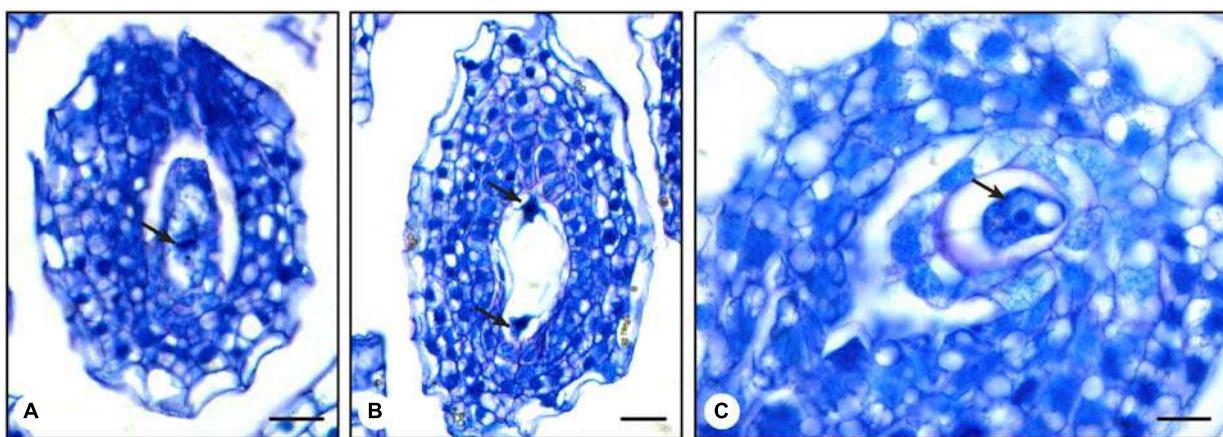
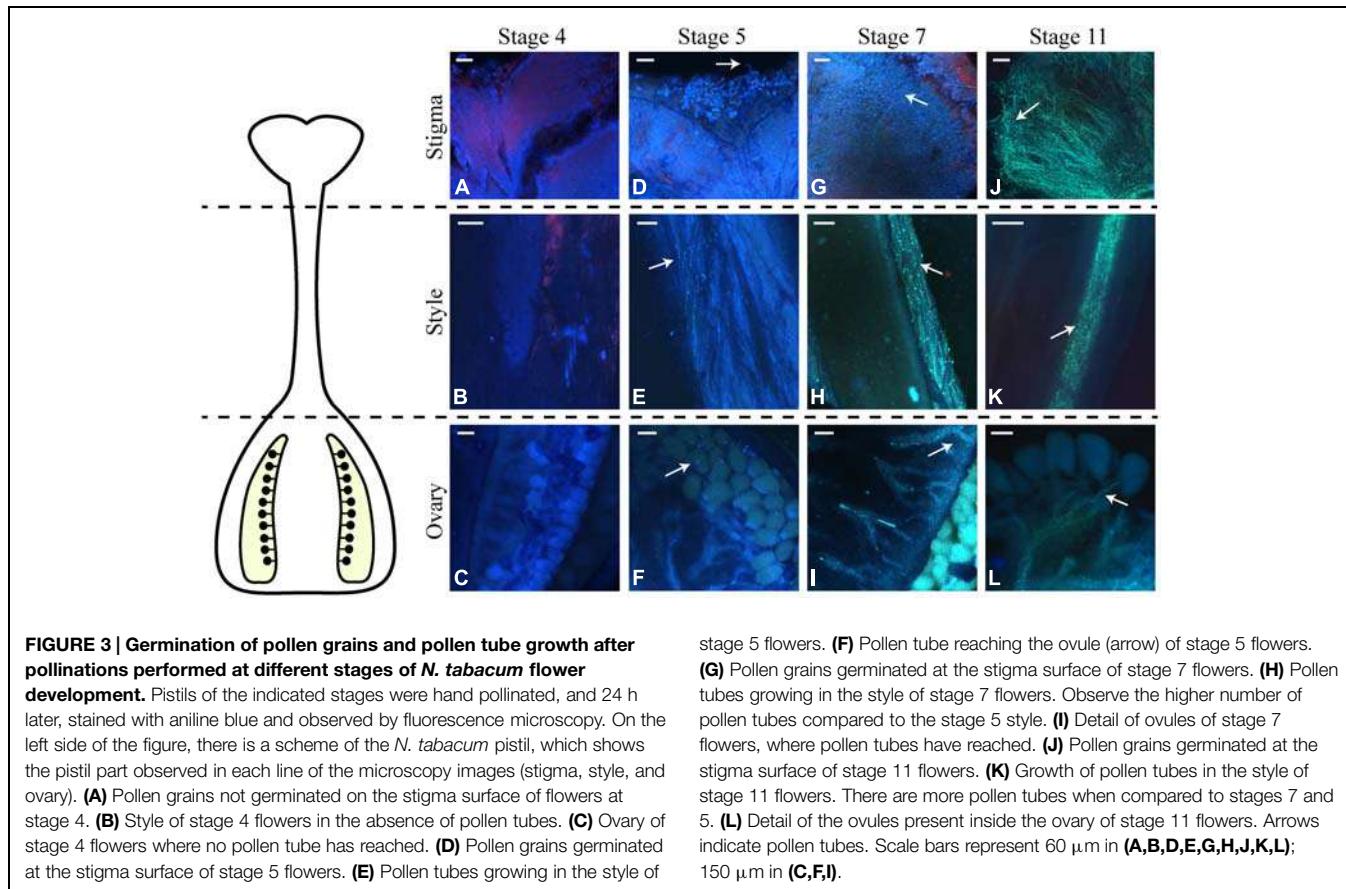
Effective stigma receptivity was assessed by the ability to sustain pollen germination and pollen tube growth. For this purpose, pistils from different development stages (4 to 11) were hand pollinated with mature pollen grains obtained from open flowers (stage 12). Pistils were collected 24 h after pollination, a period of time sufficient for pollen tubes to reach the ovary (De Graaf et al., 2003). After aniline blue staining, the pollen tubes were observed under fluorescence microscopy. In pollinations performed on stage 4 pistils, the pollen grains tended not to remain on the stigma surface, and no pollen hydration was observed. Consequently, no growing pollen tubes were detected on the stigma or style or at the ovary (Figures 3A–C). For pollinations performed on pistils of stage 5 flower buds, the pollen grains on the stigma surface hydrated and emitted pollen tubes (Figures 3D,E). It was also possible to visualize pollen tubes growing through the entire style length, reaching the ovary (Figure 3F) and ovules (Supplementary Figure S1). Pollinations performed at stage 6 and later resulted in an increasing number of hydrated pollen grains and pollen tubes growing through the style. This is clearly shown for the pollination of stages 7 and 11 pistils, shown in Figures 3G–L. Therefore, the inability to produce fruits in pollinations performed in stages 5 and 6 pistils

is not due to incompetence of the stigmas/styles to sustain pollen tube growth.

### Pollination Stimulates *N. tabacum* Ovule Maturation Prior to Anthesis

In *N. tabacum*, the ovules are not fully developed at anthesis (Tian and Russell, 1997; De Martinis and Mariani, 1999). Therefore, how do late stage 7 pollinated pistils produce fruits? To answer this question, we analyzed the effect of pollination on female gametophyte development. Late stage 7 flower buds (35 mm long) were emasculated and either hand pollinated with mature pollen or left unpollinated. This developmental stage was chosen because it is the earliest stage in which fruit formation was observed. After 24 h, the ovaries were collected and prepared for histological analysis. Unpollinated late stage 7 ovaries, 0 h after emasculation, showed young female gametophytes at the beginning of the first mitotic division, in which the formation of the metaphasic plate was observed (Figure 4A). In unpollinated late stage 7 ovaries, 24 h after emasculation, we expected to find structures similar to those described in flower buds at stage 8 (De Martinis and Mariani, 1999). As anticipated, it was possible to observe the end of the first mitotic division (Figure 4B). In contrast, late stage 7 ovaries 24 h after hand pollination clearly displayed formed egg cells (Figure 4C). Under natural conditions, *N. tabacum* egg cells are only found at anthesis or later (Tian and Russell, 1997; De Martinis and Mariani, 1999). Ovule differentiation is not synchronized in *N. tabacum* ovaries, and ovules at the top of the ovary (next to the style) are typically more advanced than ovules at the base of the ovary.

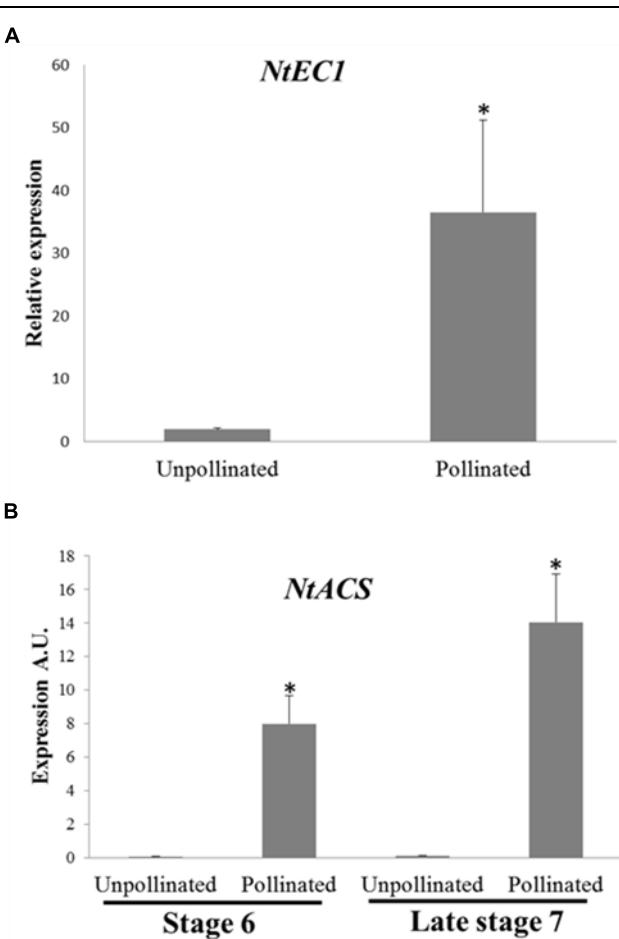
To confirm the effects of pollination on female gametophyte development at the molecular level, we analyzed the expression of the *NtEC1* gene in late stage 7 ovaries 24 h after emasculation (unpollinated) and 24 h after emasculation and pollination (pollinated). *EC1* is a gene specifically expressed in the egg cell (Sprunck et al., 2012; Rademacher and Sprunck, 2013). Therefore, we searched the available databases using the *Arabidopsis EC1.2* sequence (AT2G21740) as a query to find the *N. tabacum EC1* homolog (*NtEC1* – Supplementary Figure S2) and designed specific primers for RT-qPCR. The expression of *NtEC1* was more than 30-fold higher in pollinated compared to unpollinated late stage 7 ovaries (Figure 5A). Taken together, these results demonstrate that pollination stimulus is able to accelerate female ovule maturation even in early developmental stages prior to anthesis.



**FIGURE 4 | Histological analysis of the pollination effect on *N. tabacum* late stage 7 ovules.** **(A)** Ovule from non-pollinated flower bud at late stage 7 (35 mm in size). The picture shows an ovule at the beginning of the first mitotic division, where it is possible to visualize the metaphasic plate (arrow). **(B)** Ovule from non-pollinated flower bud harvested 24 h after being emasculated at late stage 7 (35 mm in size). **(C)** Ovule from pollinated flower bud harvested 24 h after pollination at late stage 7 (35 mm in size). This picture shows the egg cell (arrow). This structure is typically observed only in ovules of stage 12 flowers 24 h after anthesis (Tian and Russell, 1997; De Martinis and Mariani, 1999). Bars represent 20  $\mu$ m in the first two pictures **(A,B)** and 10  $\mu$ m in the last picture **(C)**.

To further clarify the nature of the pollination signal necessary for ovule maturation, we investigated whether pollination would induce ACC synthase expression in young pistils. Flowers at stage 6 and late stage 7 were emasculated and either hand

pollinated with mature pollen or left unpollinated. After 24 h, the stigmas/styles were collected and used for RNA extraction. RT-qPCR experiments have shown that unpollinated young pistils do not express *NtACS*, whereas pollination is capable of inducing



**FIGURE 5 | Expression of *NtEC1* and *NtACS* genes in unpollinated and pollinated pistils of stage 6 and late stage 7 flower buds. (A)** Expression of *NtEC1* gene in unpollinated and pollinated ovaries of late stage 7 flower buds. Expression was normalized using as reference genes GAPDH and RPL2. Relative expression was determined by comparing the *NtEC1* transcript level between unpollinated, considered as 1, and pollinated ovaries. **(B)** Expression of *NtACS* gene in unpollinated and pollinated stigmas/styles of stage 6 and late stage 7 flower buds. The relative expression levels are represented in arbitrary units (A.U.) normalized to the expression level of the GAPDH gene, used as a reference, in each RNA sample. REST statistical analysis indicated a difference in *NtEC1* gene expression among unpollinated and pollinated ovaries **(A)** and differences between unpollinated and pollinated stigma/styles in each stage (6 and late 7; **B**). (Bars represent the SE and \* indicates statistically significant difference).

its expression in stigmas/styles at both developmental stages (**Figure 5B**). There is a difference between *NtACS* expression in pollinated stage 6 and late stage 7 samples; however, this difference is not statistically significant. These results suggest that if ethylene and/or ACC contribute to the pollination signal, they are not sufficient to guarantee successful fertilization and fruit set.

## Discussion

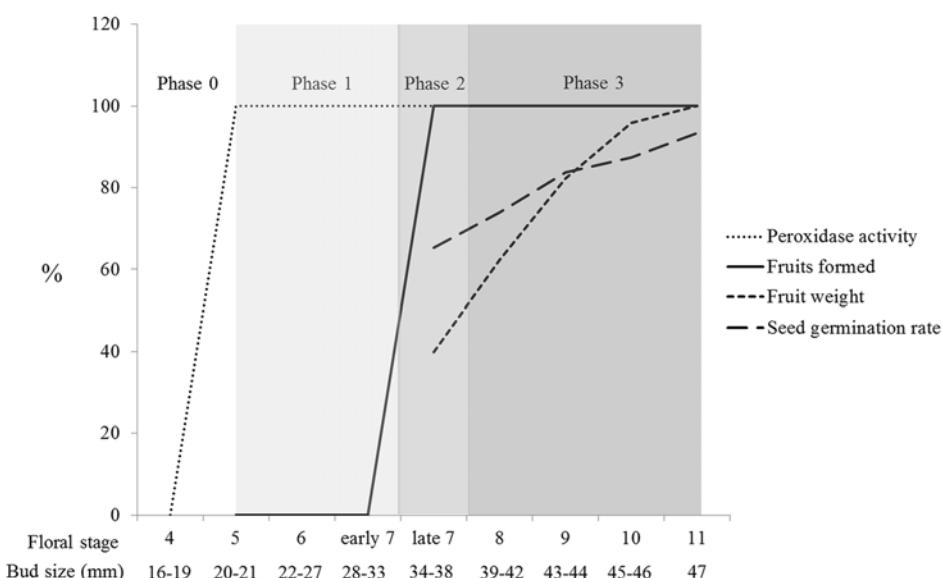
The present study was designed to investigate the developmental timing of important events in preparation for successful

fertilization. As a major achievement, we determined the effects of pollination in *N. tabacum* ovule development in flower buds prior to anthesis. At stage 5, the stigma is already receptive, displaying positive peroxidase activity and consistently supporting pollen tube growth through the style. Controlled hand pollinations at different developmental stages demonstrated that fruit formation occurred only at late stage 7 (34–38 mm) onward. Pollinations at stages 5, 6 and early stage 7 did not result in fruits despite stigma receptivity. Based on the developmental and physiological differences concerning fruit formation between 28–33 mm and 34–38 mm stage 7 flower buds, we propose to divide stage 7, previously described by Koltunow et al. (1990), into early stage 7 and late stage 7, respectively.

Effective pollination, which results in fruit set, is mainly determined by stigma receptivity, pollen tube kinetics, and ovule development (Sanzol and Herrero, 2001). Our results on positive peroxidase activity correlated with the stigma's ability to sustain pollen tube growth or, in other words, stigma receptivity. However, the developmental stages of stigmas/styles influenced the number of growing pollen tubes (**Figure 3**); later developmental stages exhibited higher capacities to sustain a larger number of growing pollen tubes. In some species, the low number of pollen tubes is directly related to fruit abortion (Sutherland, 1987; Björkman, 1995; Niesenbaum, 1999). Our results show that in *N. tabacum*, there is a direct correlation between the developmental stage of the pollinated pistil, the amount of growing pollen tubes, and fruit set (**Figures 3 and 6**; **Table 1**). Therefore, an additional important parameter to be considered for effective pollination is the number of pollen tubes growing through the style.

Which developmental changes in the pistil can affect the number of growing pollen tubes? Pollen tube growth is heterotrophic, and stages 5, 6 and early stage 7 tobacco pistils may not have sufficient carbohydrates to sustain a large number of pollen tubes. An alternative explanation is that styles younger than late stage 7 may not have the necessary intercellular spaces between cells of the transmitting tissue. Tobacco pollen tubes grow in the intercellular spaces of the specialized tissues of the stigma/style, and, at anthesis, these cells are loosely arranged and easily separated (Cresti et al., 1986). The developmentally regulated expression of a pistil-specific pectin acetyl esterase gene, which is important for decreasing cell adhesion among these cells (Quiapim et al., 2009) and some other morphological and/or physiological factors, may limit the number of pollen tubes growing in pistils younger than late stage 7. A sharp developmental threshold exists: tobacco flower buds with 33 mm or less in length do not have the necessary characteristics to sustain enough pollen tubes and thus to produce a sufficient number of fertilized ovules. Tobacco flower buds of 34 mm and longer are capable of expressing or have already accumulated all the factors required for growth of a minimum number of pollen tubes and thus for fruit production.

Concerning ovule development, our results clearly show that at late stage 7, ovules are still immature and the female gametophytes are at the beginning of the first mitotic division. Therefore, it is surprising that pollinations at this developmental stage produce fruits and seeds. Our detailed analyses of



**FIGURE 6 | Phases of tobacco pistil development in preparation for successful pollination and fertilization.** Phase 0 represents the initial developmental stages in which no preparation for pollination exists. Phase 1 was defined as the onset of stigma receptivity (positive peroxidase activity at the stigma surface and sustainable pollen tube growth through the style) until the

developmental moment in which pollination results in fruit set. Phase 2 represents the turning point, in which pollinations become productive and are restricted to late stage 7. Phase 3 is comprised of the developmental stages 8–11, in which gradual improvements in fruit weight and seed germination capacity occur.

pollinations performed on late stage 7 pistils demonstrated the effect of accelerating female gametophyte maturation. The pollination effect in triggering ovule maturation was demonstrated both by histological analysis as well as expression of the *NtEC1* gene. In this context, a signal is clearly produced in response to pollen tube growth through the stigma/style and reaches the ovules. What is the nature of this signal? Several types of signals have already been identified as mediators during plant reproduction, such as ethylene (O'Neill et al., 1993; De Martinis and Mariani, 1999; Jones and Woodson, 1999) and its precursor ACC (Jones and Woodson, 1999), gamma-amino butyric acid (Palanivelu et al., 2003), IAA (Chen and Zhao, 2008), jasmonic acid and its derivatives (Avanci et al., 2010; Stitz et al., 2014), calcium (Tian and Russell, 1997; Ge et al., 2009), and peptides (Higashiyama, 2010; Chae and Lord, 2011). Pollination acts as a stimulus and increases the concentration of calcium and ethylene in *N. tabacum* flowers (Tian and Russell, 1997; De Martinis and Mariani, 1999). ACC oxidase-silenced transgenic plants and with impaired ethylene synthesis are unable to complete female gametophyte development (De Martinis and Mariani, 1999). In addition, application of ethylene restores female gametophyte development in these transgenic flowers (De Martinis and Mariani, 1999). Furthermore, the concentrations of enzymes related to ethylene synthesis are altered in response to pollination in Solanaceae species (Llop-Tous et al., 2000; Weterings et al., 2002). These studies were mainly conducted with flowers at developmental stages close to anthesis. However, no detailed study was previously performed to investigate the effect(s) of pollination in young tobacco flower buds, and little is known about the signal(s) produced at these earlier stages.

We investigated the expression of the ACC synthase gene as an attempt to identify the pollination signal that triggers female gametophyte maturation at young pistils. The results show that stage 6 pistils, which are unable to produce fruits, induce *NtACS* expression in a pollination-dependent manner. This result is consistent with the literature (Weterings et al., 2002) and suggests that ACC and/or ethylene is not the primary signal necessary for ovule maturation, at least at early stages of flower development. An alternative explanation is that the ACC and/or ethylene produced at stage 6 does not reach a threshold level and is thus insufficient to trigger the maturation observed at late stage 7. Additionally, ovule maturation is dependent on perception of the pollination signal. Therefore, it is possible that although stage 6 stigmas/styles produce the pollination signal (e.g., ACC and/or ethylene), ovules do not express the signal receptor yet and are incompetent to respond. We remain unable to define the nature of the pollination signal, but it can travel fast and/or act as a long-range signal, reaching embryo sacs located a few centimeters away. Additionally, this pollination signal is so strong that it is capable of overwriting the natural developmental program within the tobacco ovule and accelerates female gametophyte maturation in anticipation of fertilization.

Tobacco pistil development occurs in multiple stage-specific phases along the pistil path (stigma, style, and ovary) in preparation for pollination and fertilization. We propose that phase 0 comprises the initial developmental stages (stages 1–4), from stigma differentiation until the onset of stigma receptivity. Phase 1 represents the first pistil indicator of a preparation for pollination: the onset of peroxidase activity at the stigma surface, which parallels the capacity of sustaining pollen tube

growth or stigma receptivity (stages 5 to early stage 7). At this phase, a few pollen tubes reach the ovules but do not penetrate them (Supplementary Figure S1). Phase 2 could be defined as a turning point based on an array of physiological acquisitions that allow a large number of pollen tubes to grow a long distance, reaching, and penetrating the ovules. Thus, late stage 7 corresponds to a critical developmental moment, in which sufficient pollination signal(s) is(are) produced and embryo sacs are capable of perceiving and responding. Hence, female gametophyte maturation is triggered; fruits and seeds are produced. However, the fruits are small, and not all seeds are able to germinate (the germination rate was  $65\% \pm 5.7\%$  for late stage 7 pollination), suggesting that pollination is sufficient but that a parallel mechanism (secondary signals or cellular development) should take place for proper seed formation (stages 11 and 12). Phase 3 is comprised of intermediate developmental stages (stages 8–11) in which gradual improvements take place, resulting in increasingly larger and heavier fruits (which is correlated with the number of seeds), containing seeds with progressively higher germination capacity (Figure 6). Phase 4, likely representing the best pollination and fertilization conditions, should occur at anthesis (stage 12), a developmental moment not analyzed in this work.

In recent years, knowledge has increased considerably concerning the responses elicited at the pollen tube in response to its growth along the pistil path and the signals produced by the pistil which are perceived by the pollen (Qin et al., 2009; Palanivelu and Johnson, 2010; Palanivelu and Tsukamoto, 2012; Beale and Johnson, 2013). However, little is known about the pollination signal and the responses triggered at the stigma/style and ovary (the female responses during pollen–pistil interactions). Our results indicate the existence of a powerful program that guarantees the coordinated and synchronized

development of male and female gametophytes, ensuring successful reproduction. It would be interesting to perform RNASeq studies to identify genes expressed at different parts of the style, as well as in ovaries, in pollinated, and unpollinated pistils at different developmental stages and at different time points after pollination. These studies would help establish the time necessary for induction/commitment, its physiological basis and, eventually, identification of the pollination signal receptor. Additionally, investigation of the nature of the pollination signal and pistil requirements to perceive it could be useful for the success of self-incompatible and interspecific crosses performed in evolutionary studies and breeding programs.

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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.2015.00561>

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# Loss of the *Arabidopsis thaliana* P4-ATPases ALA6 and ALA7 impairs pollen fitness and alters the pollen tube plasma membrane

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Members of the P4 subfamily of P-type ATPases are thought to create and maintain lipid asymmetry in biological membranes by flipping specific lipids between membrane leaflets. In *Arabidopsis*, 7 of the 12 Aminophospholipid ATPase (ALA) family members are expressed in pollen. Here we show that double knockout of ALA6 and ALA7 (*ala6/7*) results in siliques with a ~2-fold reduction in seed set with a high frequency of empty seed positions near the bottom. Seed set was reduced to near zero when plants were grown under a hot/cold temperature stress. Reciprocal crosses indicate that the *ala6/7* reproductive deficiencies are due to a defect related to pollen transmission. *In-vitro* growth assays provide evidence that *ala6/7* pollen tubes are short and slow, with ~2-fold reductions in both maximal growth rate and overall length relative to wild-type. Outcrosses show that when *ala6/7* pollen are in competition with wild-type pollen, they have a near 0% success rate in fertilizing ovules near the bottom of the pistil, consistent with *ala6/7* pollen having short and slow growth defects. The *ala6/7* phenotypes were rescued by the expression of either an ALA6-YFP or GFP-ALA6 fusion protein, which showed localization to both the plasma membrane and highly-mobile endomembrane structures. A mass spectrometry analysis of mature pollen grains revealed significant differences between *ala6/7* and wild-type, both in the relative abundance of lipid classes and in the average number of double bonds present in acyl side chains. A change in the properties of the *ala6/7* plasma membrane was also indicated by a ~10-fold reduction of labeling by lipophilic FM-dyes relative to wild-type. Together, these results indicate that ALA6 and ALA7 provide redundant activities that function to directly or indirectly change the distribution and abundance of lipids in pollen, and support a model in which ALA6 and ALA7 are critical for pollen fitness under normal and temperature-stress conditions.

**Keywords:** pollen, temperature stress tolerance, lipid flippases, phosphatidic acid, phosphatidylinositol

## Introduction

Biological membranes are highly organized structures and often have a non-random distribution of lipid species between their constituent leaflets (Van Meer, 2011). Members of the P4 subfamily

of P-type ATPases (P4-ATPases) have been shown to catalyze the flipping of phospholipids across biological membranes (Coleman et al., 2009; Zhou and Graham, 2009) and are thought to help create and maintain lipid asymmetry between membrane leaflets (Paulusma and Elferink, 2010; Sharom, 2011; Tanaka et al., 2011; Coleman et al., 2013; Hankins et al., 2015; López-Marqués et al., 2015). Lipid asymmetry between membrane leaflets, and its dissipation, have been linked to a wide variety of cellular processes including: cell-to-cell signaling, regulation of membrane permeability, vesicular trafficking, enzyme regulation, and apoptosis (Verhoven et al., 1995; Tannert et al., 2003; Fernandis and Wenk, 2007; Muthusamy et al., 2009; Paulusma et al., 2009; Sebastian et al., 2012; Xu et al., 2013). Studies in yeast and plants have implicated P4-ATPases in vesicular trafficking and tolerance to varied temperature (Ripmaster et al., 1993; Chen et al., 1999; Gomès et al., 2000; Gall et al., 2002; Hua et al., 2002; Pomorski et al., 2003; Poulsen et al., 2008; McDowell et al., 2013). However, the mechanistic relationship between these functions and flippase activity has not been determined.

Evidence indicates that P4-ATPases have different substrate preferences. For example, in yeast, Drs2p transports PS (phosphatidylserine) and PE (phosphatidylethanolamine) (Natarajan et al., 2004; Zhou and Graham, 2009), whereas Dnf1p transports both PC (phosphatidylcholine) and PE (Kato et al., 2002; Pomorski et al., 2003). Although many P4-ATPases require interaction with a CDC50-family protein for ER export and flippase activity, evidence from *Arabidopsis* shows that subcellular localization and substrate specificity are determined by the P4-ATPase (López-Marqués et al., 2010). Recently, studies of yeast and mammalian P4-ATPases have identified residues that contribute to their substrate specificities (Baldridge and Graham, 2012, 2013; Vestergaard et al., 2014).

The P4-ATPase family in *Arabidopsis thaliana* consists of 12 proteins: ALA 1 to ALA12 (Axelsen and Palmgren, 2001; Baxter et al., 2003; Pedersen et al., 2012). Flippase activity has been reported for ALA2 and ALA3 when co-expressed with a beta-subunit in a yeast mutant deficient for its endogenous plasma membrane (PM) localized P4-ATPases (*dnf1Δdnf2Δ*) (Poulsen et al., 2008; López-Marqués et al., 2010). ALA2 specifically transports PS, whereas ALA3 transports PE, PC, and PS (Poulsen et al., 2008; López-Marqués et al., 2010). Additionally, ALA1 has been shown to localize to the PM (López-Marqués et al., 2012), ALA2 to the PVC (López-Marqués et al., 2010) and ALA3 to the *trans*-Golgi network (Poulsen et al., 2008). Of the 12 ALA isoforms, knockout phenotypes have only been reported for *ala3* mutants (Poulsen et al., 2008; Zhang and Oppenheimer, 2009; McDowell et al., 2013). Loss of ALA3 results in pleiotropic phenotypes affecting root, shoot, and reproductive development. Additionally, *ala3* mutants are highly sensitive to growth conditions such as temperature and soil. A cold-sensitive reduction in plant size has also been observed for plants expressing an RNAi construct against ALA1 (Gomès et al., 2000).

Here, we present evidence that the *Arabidopsis* P4-ATPases ALA6 and ALA7 are important for rapid, sustained pollen tube growth and are essential for temperature stress tolerance. Genetic evidence indicates that the activities of ALA6 and ALA7 are highly redundant. Pollen defects could be rescued by the

expression of fluorescently-tagged ALA6 fusion proteins, which localized simultaneously to both the plasma membrane and highly mobile endomembrane vesicles. Mass spectrometry analysis of mature pollen grains revealed significant differences in lipid composition between *ala6-1/7-2* and wild-type. We also show that the ability of lipophilic FM dyes to stain *ala6-1/7-2* pollen tubes is reduced by ~10-fold relative to wild-type, indicating altered properties associated with the plasma membrane. Together, these results suggest a model in which ALA6 and ALA7 directly or indirectly change the distribution and concentration of lipids in pollen, and that these flippases are critical for pollen fitness under normal and temperature-stress conditions.

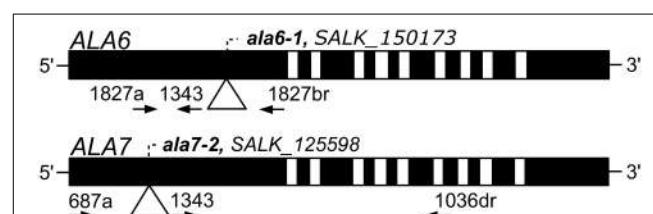
## Materials and Methods

### T-DNA Insertional Mutants

Two T-DNA insertional mutants were used in this study: *ala6-1* (SALK\_150173, ss757) and *ala7-2* (SALK\_125598, ss733) (Alonso et al., 2003). Mutants were obtained from the *Arabidopsis* Biological Resource Facility at Ohio State University (<http://abrc.osu.edu/>) and were identified using PCR-based screening techniques. Both mutants are in the Col-0 background. The locations of the T-DNA insertions and PCR primers are indicated in Figure 1. Sequences for the PCR primers can be found in File S2. The individual mutant lines were crossed to create the double mutant *ala6-1/7-2* (ss1351).

### Plant Growth Conditions

Seeds were sown on 0.5× Murashige and Skoog medium (pH 5.7) containing 1% agar and 0.05% MES. Following 48 h of stratification (4°C, dark), seedlings were grown at room temperature (~23°C) under 24 h light for 7–10 d before being transplanted to soil. The soil used was Sunshine SMB-238 (SunGro Horticulture, Agawam, MA) supplemented with 10-10-10 fertilizer and Marathon pesticide following the manufacturer's instructions. Plants were grown until maturity in a growth chamber (Percival Scientific, Perry, IA) under a long-day photoperiod (16 h light at



**FIGURE 1 | Diagrams of ALA6 and ALA7 showing locations of T-DNA disruptions.** Filled boxes represent exons and open boxes represent introns. T-DNA insertions are represented with triangles and identified by *ala* allele numbers and T-DNA allele accessions. Primers used for PCR genotyping are represented by arrows and point in the 5' to 3' direction. Primer 1343 corresponds to the T-DNA left border. The left-border junctions are as follows: *ala6-1*, TGGGACTCCGGCTAACGCACCGatgccttaatgccttaatccgt; and *ala7-2* LB: atttgtttacccacaatatcttGAACATCAAATGTGAAGATCCAA. Capital letters represent ALA genomic DNA and lowercase letters represent T-DNA.

20°C/8 h dark at 18°C, 70% humidity, and ~125  $\mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity).

## Plasmid Construction

For expression in plants, two ALA6 genomic DNA fragments were PCR amplified from BAC clone F20D21 using the primer pairs 1035a+br (C-terminal tag) and 1035a+brs (N-terminal tag) and Phusion High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA). An ALA1 genomic DNA fragment was amplified from *Arabidopsis* genomic DNA (Col-0 ecotype) using the primer pair 1030a+br (C-terminal tag) and Pfu-Turbo DNA Polymerase (Agilent, Santa Clara, CA). PCR primer sequences can be found in File S2. Poly-A tails were added to the PCR products using ExTaq (Takara, Mountain View, CA) and the resultant DNA fragments were T/A cloned into the pGEM-T Easy vector ( $\text{Amp}^r$  in bacteria) using the pGEM-T Easy Vector System kit ( $\text{Amp}^r$  in bacteria) (Promega, Madison, WI). All PCR-derived fragments were sequence verified to be error free in the pGEM-T Easy vector. The ALA6 and ALA1 fragments were subcloned from the pGEM vector into a derivative of the pGreenII plant vector ( $\text{kan}^r$  in bacteria,  $\text{hyg}^r$  in plants) (Hellens et al., 2000). Junction sites were sequence verified to be error free. All fusion proteins expressed in this study were under the control of the *Arabidopsis thaliana* ACA9 promoter, which drives a moderate expression preferentially in pollen (Schiøtt et al., 2004). Internal stock numbers for each plasmid are: ps1730, ACA9p-NTAP2(G)-ALA6; ps1728, ACA9p-i-ALA6-TAP2(Y); ps1729, ACA9p-i-ALA1-TAP2(Y); and ps779, ACA9p-i-TAP2(Y). Sequence information for each plasmid can be found in File S3.

For expression in yeast, two ALA6 cDNA fragments were amplified using the primer pairs oli4300+oli4301 (untagged ALA6) and oli4299+oli4301 (RGSH10-ALA6). PCR primer sequences can be found in File S2. The ALA6 cDNA template was created from total RNA extracted from Col-0 pollen. The PCR fragments were cloned into the yeast plasmids pRS423-GAL (untagged) (Burgers, 1999) and pMP4062 (His tag, RGSH10) (López-Marqués et al., 2012) using homologous recombination. PCR fragments and corresponding plasmids were transformed into the *S. cerevisiae* strain ZHY709 (*MATα his3 leu2 ura3 met15 dnf1Δ dnf2Δ drs2::LEU2*) (Hua et al., 2002) using the lithium acetate method (Gietz and Woods, 2002). Positive transformants were identified after 4 d of growth at 28°C using synthetic complete media (SCD) plates without histidine (0.7% Yeast Nitrogen base, 2% glucose, 1× drop out media supplement) (Rose and Broach, 1990). All PCR-derived fragments were sequence verified to be error free.

## Plant Transformation

Plants were transformed with *Agrobacterium tumefaciens* strain GV3101 carrying the pSOUP helper plasmid using the floral dip method (Clough and Bent, 1998; Hellens et al., 2000). T1 seedlings were grown on 0.5× Murashige and Skoog (MS) medium (pH 5.7) containing 1% agar, 0.05% MES, and 25  $\mu\text{g/ml}$  hygromycin to identify successful transformants.

## In-Vitro Pollen Tube Growth

The pollen tube growth medium was based on the method described by Boavida and McCormick, and contained: 5 mM CaCl<sub>2</sub>, 0.01% H<sub>3</sub>BO<sub>3</sub>, 5 mM KCl, 10% sucrose, 1 mM MgSO<sub>4</sub>, pH 7.5–7.8, and 1.5% low melting agarose (Boavida and McCormick, 2007). Pollen from stage 13 to 14 flowers was placed on pistils, either from the corresponding genotype or from surrogate *ms-1* plants, and the pistils were then placed on ~400  $\mu\text{L}$  of pollen tube growth medium layered over a microscope slide. The slides were incubated at room temperature (~23°C) in a square petri dish containing water-soaked paper towels to maintain high humidity. Pollen tubes were grown for 2–6 h prior to analysis, unless being used for a time course. For the time course analysis of pollen tube length, pollen tubes were photographed with a Hamamatsu Orca ER camera attached to a Leica DM-IRE2 microscope (JH Technologies, Fremont, CA). Length measurements were done using the Fiji software package (Schindelin et al., 2012).

## Confocal Microscopy

Images were collected using an Olympus IX81 FV1000 confocal microscope run by the Olympus Fluoview 1.07.03.00 software package (Olympus, Center Valley, PA). A 60× objective (numerical aperture 1.42) was used throughout. Excitation at wavelengths of 488 nm (GFP, FM4-64, and FM1-43) and 515 nm (YFP) was provided with an Argon-Ion laser. A spectral emission range of 500–600 nm was used for GFP, 545–595 nm for YFP, 670–726 nm for FM-4-64, and 575–605 nm for FM1-43.

## FM Dye Staining

Pollen tubes were stained with the lipophilic dyes FM4-64 and FM1-43 (Invitrogen - Molecular Probes, Eugene, OR). For both dyes, 10  $\mu\text{M}$  staining solutions were prepared by dissolving the individual dye in liquid pollen tube growth medium. When needed, sodium azide (NaAz) was added to the staining solution to a final concentration of 0.05%. Staining solution was directly applied to pollen tubes growing on solid medium layered over a microscope slide (see above section: *In-vitro* Pollen Tube Growth). Images were captured 1–45 m after the addition of the staining solution, as described in the Confocal Microscopy Section. Fluorescence was quantified in terms of average pixel intensity using the Fiji software package (Schindelin et al., 2012).

## Lipid Profiling

Pollen for lipid analysis was collected from independent, parallel-grown groups of ~75 plants. Total lipid extracts were obtained from pollen using chloroform/methanol extraction, described below. To deactivate phospholipases prior to lipid extraction, pollen samples were immersed for 15 m in 3 mL of 75°C isopropanol + 0.01% butylated hydroxytoluene (BHT). The first extraction step was done by adding 1.5 mL chloroform and 0.6 mL of water to the pollen/isopropanol mixture. Four subsequent extraction steps were done using chloroform/methanol (2:1) + 0.01% BHT. Each extraction was done for 1 h; except for the last extraction, which was done overnight (~12 h). The five extracts for each sample were combined and washed twice: first with 1 mL of 1 M KCl and second with 2 mL of water.

Lipid samples were then evaporated to 1 mL and sent to the Kansas Lipidomics Research Center (<http://www.k-state.edu/lipidomics/>).

edu/lipid/lipidomics) for routine plant polar lipid analysis by tandem mass spectrometry. The two mass spectrometers used were an Applied Biosystems API 4000 and an Applied Biosystems Q-TRAP, separated by a collision cell. Samples were introduced by electrospray ionization, with no pre-analysis separation. Analysis was done using both precursor and neutral loss scans.

### Lipid Translocation Assays

Lipid translocation was assayed as previously described (López-Marqués et al., 2010). ALA6-containing yeast (see Plasmid Construction) was transformed with the yeast plasmid pRS426-GAL (Burgers, 1999); either empty, or containing the putative beta-subunits ALA interacting subunit 1 (ALIS1), ALIS3, or ALIS5 (Poulsen et al., 2008). As controls, the wild type BY4741 (*MATα his3 leu2 ura3 met15*; EUROSCARF) (positive control) and the mutant ZHY709 (negative control) were transformed with empty plasmids pRS423-GAL and pRS426-GAL. Flow cytometry was performed on a FACSCalibur cell analyzer (BD Biosciences, San Jose, CA) equipped with an argon laser using Cell Quest software. Thirty thousand cells were analyzed without gating during the acquisition, and live cells were selected based on forward/side-scatter gating and propidium iodide exclusion. A histogram of the green (NBD) fluorescence of living cells was used to calculate the mean fluorescence intensity of total cells.

### Yeast Membrane Fractionation and Immunolabeling

Fractionation of yeast membranes in sucrose gradients, quantification of protein contents, and Western blot analysis were carried out as described in López-Marqués et al. (2012). Fractions enriched in ER (30% sucrose) and PM (48% sucrose) were collected by sucrose density gradient fractionation and used for Western blot analysis. Immunodetection of RGSH6- and RGSH10-tagged proteins was performed using a commercial BSA-free RGS-His<sup>TM</sup> antibody produced in mouse (Qiagen, Valencia, CA). We used a monoclonal anti-dolichol phosphate mannose synthase (Dpm1p) antibody (Molecular Probes, Eugene, OR) as a marker for the endoplasmic reticulum and a polyclonal antibody against the C-terminal end of the yeast proton ATPase (Pma1p) (Monk et al., 1991) as a marker for the

plasma membrane. Golgi fractions were detected with an affinity purified anti-Sed5p polyclonal antibody (Sapperstein et al., 1996).

## Results

Among the seven ALAs that are most highly expressed in *A. thaliana* pollen, *ALA6* (At1g54280) and *ALA7* (AT3g13900) are two of the most closely related (89% amino acid identity) and account for approximately 56% of the *ALA* subfamily mRNA in pollen grains or growing tubes (Lorraine et al., 2013) (Figure S1a). To determine if these genes have redundant functions in pollen development, we isolated the *ala6-1* and *ala7-2* T-DNA gene disruption lines from the SALK collection (Alonso et al., 2003) and crossed them to make the double knockout *ala6-1/7-2* (Figure 1). The T-DNA insertions for both *ala6-1* and *ala7-2* are in the first exon and are predicted to disrupt the production of functional proteins.

### **ALA6 and ALA7 are Important for Pollen Fitness**

A pollen transmission defect was observed for double knockout combinations of *ala6-1* and *ala7-2* in which only one allele was segregating (e.g., *ala6-1(-/-)/ala7-2(+/)* or *ala6-1(+/+)/ala7-2(-/-)*) (Table 1). In plants allowed to self-fertilize, double-homozygous progeny were observed at frequencies of 5.3–8.3%, representing a ~4-fold decrease from the expected 25%. In pollen outcrosses, transmission of the *ala6-1/7-2* allele was reduced to 2.9–3.7%, representing a ~15-fold decrease from the expected 50%. Transmission of *ala6-1/7-2* through the female gametophyte appeared normal, indicating that the observed segregation distortion was the result of a pollen-autonomous defect.

Plants heterozygous for *ala6-1(+/)* or *ala7-2(+/)* individually showed no evidence of a segregation distortion when allowed to self-fertilize (Table 1). However, manual pollen outcrosses showed a ~2-fold decrease in *ala6-1* transmission, indicating the loss of ALA6 alone can result in a detectable phenotype. A similar deficiency was not seen for *ala7-2*.

Evidence that the pollen transmission defects were caused by loss of ALA6 and ALA6/7 was corroborated by rescuing mutant pollen with transgenes encoding either ALA6-YFP, or GFP-ALA6 (Table 2). Both transgenes were expressed under the control of the ACA9 promoter, which drives a moderate level of expression

**TABLE 1 | Segregation analysis shows a defect in transmission through male gametophytes carrying *ala6-1* and *ala6-1/7-2* mutations.**

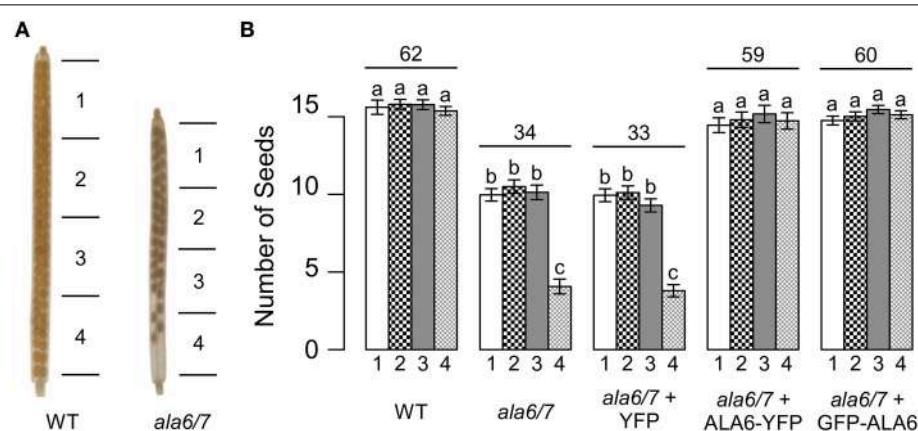
| <i>♂ × ♀</i>                     | Cross Description | Assay            | Expected (%) | Observed (%) | <i>n</i> | <i>p</i> -value |
|----------------------------------|-------------------|------------------|--------------|--------------|----------|-----------------|
| <i>ala6-1(+/−)/ala7-2 × same</i> | Selfed            | <i>ala6(−/−)</i> | 25           | 5.3          | 94       | <0.0001         |
| <i>ala6-1/ala7-2(+/−) × same</i> | Selfed            | <i>ala7(−/−)</i> | 25           | 8.3          | 144      | <0.0001         |
| <i>ala6-1/ala7-2(+/−) × WT</i>   | Male Outcross     | <i>ala7(−)</i>   | 50           | 3.7          | 301      | <0.0001         |
| WT × <i>ala6-1/ala7-2(+/−)</i>   | Female Outcross   | <i>ala7(−)</i>   | 50           | 44.7         | 152      | 0.1944          |
| <i>ala6-1(+/−) × same</i>        | Selfed            | <i>ala6(−/−)</i> | 25           | 20           | 90       | 0.4803          |
| <i>ala7-2(+/−) × same</i>        | Selfed            | <i>ala7(−/−)</i> | 25           | 27.3         | 132      | 0.5465          |
| <i>ala6-1(+/−) × WT</i>          | Male Outcross     | <i>ala6(−)</i>   | 50           | 25.6         | 238      | <0.0001         |
| <i>ala7-2(+/−) × WT</i>          | Male Outcross     | <i>ala7(−)</i>   | 50           | 50.2         | 221      | 0.9464          |

WT is an abbreviation for wild-type. The observed results were compared to an expected Mendelian segregation. Statistical significance was determined by the Pearson's Chi-Squared test.

**TABLE 2 | Segregation analysis shows that the pollen transmission defects of *ala6-1* and *ala6-1/7-2* can be rescued by ALA6-YFP or GFP-ALA6 fusion proteins.**

| $\delta \times \varphi$           | Cross Description | Transgene (TG)     | Expected TG (%) | Observed TG (%) | n          | p-Value        |
|-----------------------------------|-------------------|--------------------|-----------------|-----------------|------------|----------------|
| <i>ala6-1/7-2 + TG(+-) × same</i> | Selfed            | ALA6-YFP, GFP-ALA6 | 75              | 93.2, 91.5      | 869, 1509  | Both <0.0001   |
| <i>ala6-1/7-2 + TG(+-) × WT</i>   | Male Outcross     | ALA6-YFP, GFP-ALA6 | 50              | 97.5, 99.1      | 475, 875   | Both <0.0001   |
| <i>ala6-1 + TG(+-) × WT</i>       | Male Outcross     | ALA6-YFP, GFP-ALA6 | 50              | 77.7, 82.8      | 282, 331   | Both <0.0001   |
| WT × <i>ala6-1/7-2 + TG(+-)</i>   | Female Outcross   | ALA6-YFP, GFP-ALA6 | 50              | 51.7, 51.3      | 487, 567   | 0.4411, 0.5287 |
| WT × <i>ala6-1 + TG(+-)</i>       | Female Outcross   | ALA6-YFP, GFP-ALA6 | 50              | 51.4, 51.6      | 140, 221   | 0.7353, 0.6377 |
| <i>ala6-1/7-2 + TG(+-) × same</i> | Selfed            | YFP                | 75              | 72.5            | 1601       | 0.0218         |
| <i>ala6-1/7-2 + TG(+-) × same</i> | Selfed            | ALA1-YFP           | 75              | 75.6            | 324        | 0.7975         |
| WT + TG(+-) × same                | Selfed            | ALA6-YFP, GFP-ALA6 | 75              | 68.3, 71.4      | 1854, 2735 | Both <0.0001   |

Lines containing a single copy of the transgene were maintained through outcrosses. WT is an abbreviation for wild-type and TG is an abbreviation for transgene. The observed results were compared to an expected Mendelian segregation. Statistical significance was determined by the Pearson's Chi-Squared test. For the GFP-ALA6 rescue construct: 6/6 lines showed equivalent rescue of *ala6-1/7-2* (ss1878–1881, ss1883–1884); 3/3 lines showed equivalent rescue of *ala6-1* (ss1889–1891); and 9/9 lines did not improve reproductive fitness in WT (ss1895–1903). For the ALA6-YFP rescue construct, 3/3 lines showed equivalent rescue of *ala6-1/7-2* (ss1885, ss1886, ss1888); 3/3 lines showed equivalent rescue of *ala6-1* (ss1892–1894); and 6/6 failed to show any changes to reproductive fitness in a WT background (ss1904–1909). For the YFP-only control construct, 7/7 lines failed to rescue *ala6-1/7-2* (ss1910–1916). For the ALA1-YFP control construct, 2/2 lines failed to rescue *ala6-1/7-2* (ss1917–1918).



**FIGURE 2 | Loss of ALA6 and ALA7 results in reduced seed set with an uneven distribution of seed.** **(A)** Representative examples of wild-type and *ala6-1/7-2* siliques cleared with 70% EtOH to show seed positions. **(B)** Graph of seed set by sector. Siliques were divided into four sectors of equal length with sector 1 at the top (stigma end) of the siliques and sector 4 at the base of the siliques. Average results ( $\pm$ SE) are reported for two independent experiments,  $n = 35$ –93 siliques. Siliques were collected from a

total of 5–13 plants for each genotype. Sector numbers appear below each column and the average total seed set for each genotype is given above the corresponding sector data. For the GFP-ALA6 rescue construct, 7/9 lines showed equivalent rescue of *ala6-1/7-2*: ss1878–1884. For the ALA6-YFP rescue construct, 4/6 lines showed equivalent rescue of *ala6-1/7-2*: ss1885–1888. a,b,c Columns sharing common labels (letters) are not significantly different from each other ( $p > 0.05$ ).

preferentially in pollen (Schiøtt et al., 2004). The transgenes were stably expressed in either an *ala6-1* or an *ala6-1/7-2* mutant background, and reciprocal crosses were done to test for an increase in the transmission efficiency of gametes harboring a transgene. Both transgenes showed an expected 50% transmission through the female, confirming that only one copy of the transgene was present in the plants used in the reciprocal crosses. In contrast, pollen transmission of the transgenes was increased to 78–83% in the *ala6-1* background and 98–99% in the *ala6-1/7-2* background. Neither transgene showed an increased pollen transmission in wild-type control plants, indicating that the improved transmission in mutants was the result of rescuing the pollen defects associated with *ala6-1* and *ala6-1/7-2* knockouts. Additional controls showed that *ala6-1/7-2* pollen was not rescued by an empty vector or a more distantly related ALA isoform,

ALA1. Since the transmission defect associated with the double mutant was more pronounced, subsequent studies were done using *ala6-1/7-2*.

### Seed Set in *ala6-1/7-2* is Reduced and Hypersensitive to Temperature Stress

In homozygous *ala6-1/7-2* mutants, seed set within each siliques was decreased to ~55% that of wild-type (Figure 2). Furthermore, seed was unevenly distributed within *ala6-1/7-2* siliques, with a high frequency of empty seed positions at the bottom of the siliques. Seed set was restored to wild-type levels in *ala6-1/7-2* plants expressing a GFP-ALA6 or an ALA6-YFP construct, but not an empty vector control, indicating that the phenotype was caused by loss of ALA6 and ALA7. A seed set phenotype was not observed when *ala6-1/7-2* pistils were manually fertilized with

wild-type pollen (Figure S2), indicating that pollen defects alone account for the mutant phenotype.

To determine if the reduction in *ala6-1/7-2* seed set is dependent upon growth conditions, plants were allowed to self-fertilize under a temperature stress that cycled between hot days ( $40^{\circ}\text{C}$  peak) and cold-nights ( $-1^{\circ}\text{C}$  low) (Figure S3). The temperature stress caused seed set in wild-type siliques to be reduced to  $\sim 38\%$  that of unstressed, whereas *ala6-1/7-2* mutants were sterile (Figure 3). Seed set was restored to wild-type levels in *ala6-1/7-2* plants expressing a GFP-ALA6 or an ALA6-YFP construct, but not an empty vector control, confirming that the sensitivity to temperature stress was caused by loss of ALA6 and ALA7.

### ***ala6-1/7-2* Pollen Tubes are Short and Slow**

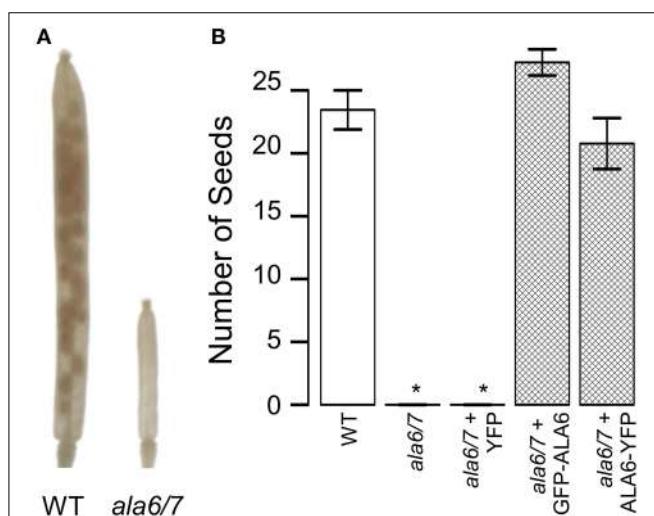
To quantify growth defects associated with *ala6-1/7-2* pollen, *in-vitro* growth assays were done over a 24 h time course (Figure 4). After the 24 h growth period, the overall length of *ala6-1/7-2* pollen tubes was  $\sim 35\%$  that of wild-type. The maximal growth rate of *ala6-1/7-2* pollen tubes was also reduced to  $\sim 45\%$  that of wild-type. Expression of two different ALA6 transgenes (ALA6-YFP and GFP-ALA6) restored the growth rate and overall length of *ala6-1/7-2* pollen tubes to nearly that of wild-type.

To evaluate the *in-vivo* relevance of *ala6-1/7-2* pollen tube growth defects, pollen from *ala6-1(-/-)/7-2(+/-)* plants was used to fertilize wild-type pistils, and the resulting mature siliques were divided into three sectors of equal length (top, middle, and

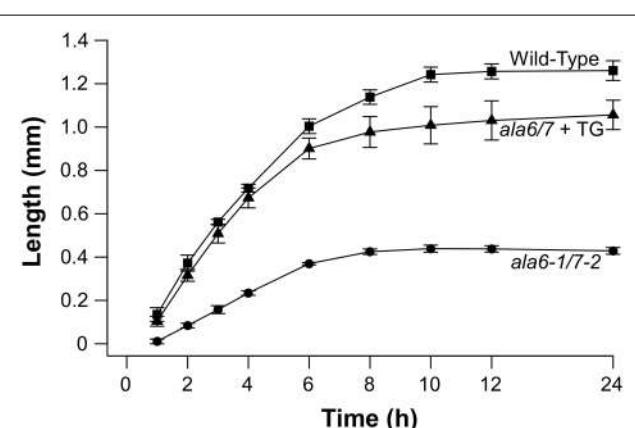
bottom). Without growth defects, the *ala6-1/7-2* allele would be expected to transmit to all three sectors equally, with 33% of the total transmission in each sector. However, 72.7% of the *ala6-1/7-2* pollen transmission was observed in the top sector, whereas no transmission of *ala6-1/7-2* was observed in the bottom sector (Table 3). These results indicate that the competitive fitness of *ala6-1/7-2* pollen relative to wild-type decreases in the distal region of the pistil, consistent with *in-vitro* growth assays showing *ala6-1/7-2* pollen tubes to be slow and short (Figure 4).

### **The Subcellular Localization of ALA6 Includes the Plasma Membrane and Endomembrane Structures**

The potential subcellular localization of ALA6 was investigated using confocal microscopy to image GFP-ALA6 and YFP-ALA6 fusion proteins in the pollen tubes of stable *ala6-1/7-2* transgenic plants (Figure 5). The transgenes encoding both fusion proteins were shown to rescue *ala6-1/7-2* pollen defects (Figures 2, 3; Table 2). In pollen tubes treated with 0.05% NaAz, both ALA6 fusion proteins showed a localization pattern consistent with a plasma membrane association, although some association with endomembrane structures was also observed (Figure 5C, Movie S1). In growing pollen tubes not treated with NaAz, the relative amount of endomembrane-associated ALA6 fusion protein was higher, with the ALA6-labeled structures engaged in cytoplasmic streaming (Figure 5D, Movie S2). These localization patterns were observed for 11 of 11 independent transgenic lines (7 GFP-ALA6, 4 ALA6-YFP), regardless of whether expression levels were high or at the lower limits of detection, providing evidence that the patterns were not associated with an over-expression artifact.



**FIGURE 3 | Loss of ALA6 and ALA7 results in sterility under hot-day/cold-night temperature stress.** Plants were allowed to self-fertilize under a temperature stress that cycled between hot-days ( $40^{\circ}\text{C}$  peak) and cold-nights ( $-1^{\circ}\text{C}$  low) with periods of intermediate temperature between the extremes for acclimation. (A) Representative examples of wild-type and *ala6-1/7-2* siliques cleared with 70% EtOH to show seed positions. (B) Graph of overall seed set. Average results ( $\pm \text{SE}$ ) are reported for two independent experiments,  $n = 15\text{--}85$  siliques. Siliques were collected from at least four plants for each genotype. For the GFP-ALA6 rescue construct, 6/6 lines showed equivalent rescue of *ala6-1/7-2*: ss1878, ss1879, and ss1881-1884. For the ALA6-YFP rescue construct, 3/3 lines showed equivalent rescue of *ala6-1/7-2*: ss1885, ss1887, and ss1888. \*Statistically significant difference between wild-type and *ala6-1/7-2* (Welch's *t*-test,  $p < 0.05$ ).

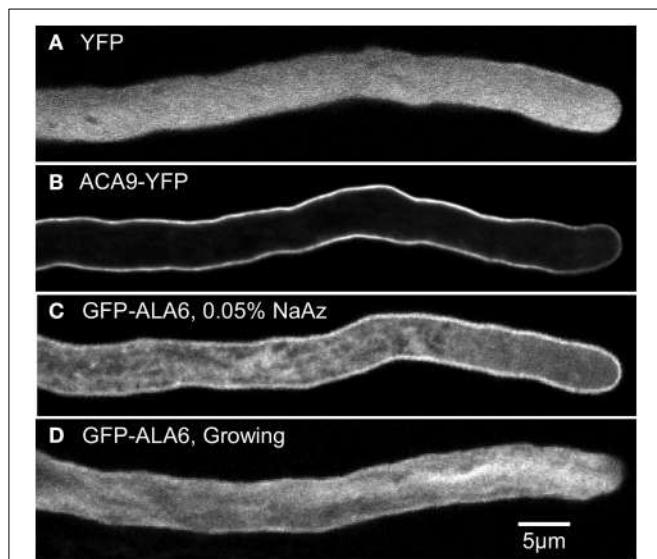


**FIGURE 4 | In-vitro assays show *ala6-1/7-2* pollen tubes are short with reduced rates of growth.** Pollen was placed on pistils, either from the corresponding genotype or from surrogate *ms-1* plants, and the pistils were placed on agar-solidified growth media. Pollen tubes growing out of the pistils were measured over a 24 h time course. Lengths were reported for each time point as the average length of the 10 longest pollen tubes. Values and error bars represent mean  $\pm \text{SE}$  for three independent experiments for all genotypes. An independent transgenic line was used for each *ala6-1/7-2* + TG experiment (ss1883, ss1886, and ss1888) with each line showing similar results.

**TABLE 3 |** The transmission of *ala6-1/7-2* through pollen is restricted to the top 2/3 of the siliques.

| ♂ × ♀                       | Assay          | % Total <i>ala6-1/7-2</i> Transmission |        |        | n   | p-Value |
|-----------------------------|----------------|--|--------|--------|-----|---------|
|                             |                | Top                                    | Middle | Bottom |     |         |
| Expected                    | n/a            | 33                                     | 33     | 33     | n/a | n/a     |
| <i>ala6-1/7-2(+/-) × WT</i> | <i>ala7(-)</i> | 72.7                                   | 27.3   | 0      | 11  | 0.01    |

Wild-type (WT) and *ms-1* pistils were fertilized with *ala6-1(-/-)/7-2(+/-)* pollen and the resulting siliques were divided into three sectors of equal length Top (stigma end), Middle, and Bottom (base of the siliques). The observed results are compared to an expected equal distribution of mutant alleles across all three sectors. Statistical significance was determined by the Pearson's Chi-Squared test.



**FIGURE 5 |** Confocal fluorescence micrographs showing GFP-ALA6 localizes to the pollen tube perimeter and endomembrane structures. **(A,B)** Growing pollen tubes expressing **(A)** YFP (ss1919) as a marker for the cytosol and **(B)** ACA9-YFP (ss471-472) as a marker for the plasma membrane (Myers et al., 2009). **(C,D)** Pollen tubes expressing GFP-ALA6 (ss1880) either **(C)** treated with 0.05% NaAz, or **(D)** growing. Constructs were expressed under the control of the ACA9 promoter in stable transgenic Arabidopsis plants. Images of GFP-ALA6 are representative of seven GFP-ALA6 (ss1878–1884) and four ALA6-YFP (ss1885–1888) transgenic lines in which the transgenes were shown to rescue the *ala6-1/7-2* phenotype. The pattern of localization was equivalent for expression levels that ranged from high to the lower limits of detection. The images shown for GFP-ALA6 represent GFP signals that were significantly above background autofluorescence, as determined by comparison with WT pollen imaged using the same exposure settings (black images not shown).

## Lipid Composition is Altered in *ala6-1/7-2* Pollen Grains

To determine if loss of ALA6/7 is correlated with a change in lipid composition, tandem mass spectrometry (MS/MS) was done on lipids extracted from *ala6-1/7-2* and wild-type pollen grains (Figure 6, File S1). The MS/MS analysis detected polar lipids from 11 head-groups (MGDG, monogalactosyldiacylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; DGDG, digalactosyldiacylglycerol; PG, phosphatidylglycerol; LPG, lysophosphatidylglycerol; LPC, lysophosphatidylcholine; LPE,

lysophosphatidylethanolamine; and PS, phosphatidylserine) and quantified the double bonds within the corresponding acyl side chain(s). In total, the abundances of 144 distinct lipids were measured. We chose to examine pollen grains instead of growing tubes because pollen grains could be more easily harvested in sufficient quantities, with the majority of cells in the same developmental and physiological state. Expression profiling data suggests that both ALA6 and ALA7 are expressed at similar levels in mature pollen grains and growing pollen tubes (Figure S1b). Significant differences between *ala6-1/7-2* and wild-type were observed, both in the concentrations of lipid head-groups (Figure 6A) and the average number of double bonds (i.e., unsaturation) within acyl side chains (Figure 6B). For example, we observed a ~2-fold decrease in PI concentration and a ~2-fold increase in PA concentration in *ala6-1/7-2* pollen relative to wild-type.

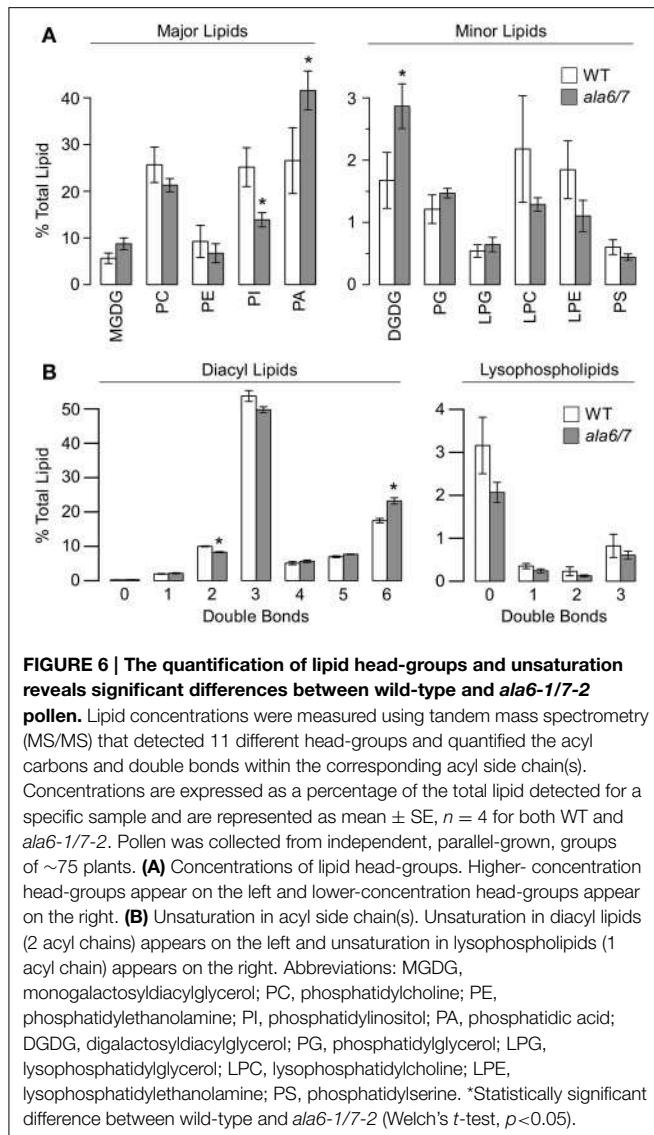
## FM-Dye Staining is Reduced in the *ala6-1/7-2* Pollen Tube Plasma Membrane

In an attempt to measure rates of endocytosis, wild-type and *ala6-1/7-2* pollen tubes were stained with the lipophilic dyes FM4-64 and FM1-43 (Figure 7). For wild-type tubes only, both FM dyes showed strong PM staining after less than 2 m, and increasing internal staining over a 45 m time period. In contrast, *ala6-1/7-2* pollen tubes failed to show any significant PM or internal staining, even after 45 m. However, both wild-type and *ala6-1/7-2* pollen tubes showed similar staining when exposed to the sterol-dye Filipin (Boutté et al., 2011) (Figure S4a), indicating that the change in the *ala6-1/7-2* plasma membrane that limits FM-dye staining does not affect all dyes. Additionally, the FM dyes were able to stain membranes throughout *ala6-1/7-2* pollen tubes that had been killed by prolonged NaAz exposure (Figure S4.3b), indicating that loss of ALA6 and ALA7 does not affect all membranes equally.

## Discussion

### ALA6 and ALA7 are Critical for Rapid, Sustained Pollen Tube Growth

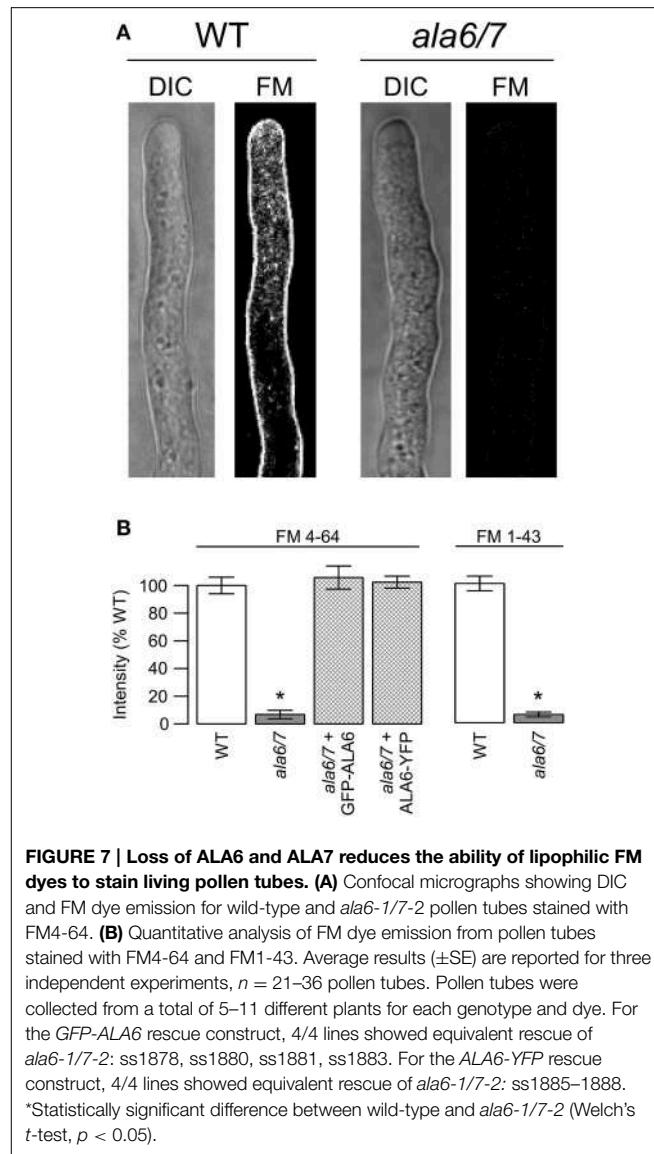
In this study, we present genetic evidence that the simultaneous loss of ALA6 and ALA7 result in a male-autonomous reproductive defect (Tables 1, 2). *In-vitro* growth assays indicate that, relative to wild-type, *ala6-1/7-2* pollen tubes have more than 2-fold reductions in both maximal growth rate and overall length (Figure 4). These defects are corroborated *in-vivo* by both the



high frequency of empty seed positions in the bottom of *ala6-1/7-2* siliques (**Figure 2**) and the 0% success rate of *ala6-1/7-2* pollen in competition with wild-type to fertilize ovules in the bottom third of a pistil (**Table 3**). Only a weak reproductive defect was observed for *ala6-1* pollen, indicating that ALA6 and ALA7 provide a significant level of redundancy (**Table 1**). The observation of a pollen-specific defect for *ala6-1/7-2* is consistent with mRNA expression profiling data, which indicate that both isoforms are expressed primarily in pollen (Figure S1b).

### The Subcellular Localization of ALA6 Includes the Plasma Membrane and Endomembrane Structures

GFP-ALA6 and ALA6-YFP fusion proteins were observed both at the perimeter of pollen tubes, consistent with plasma membrane localization, and on highly mobile endomembrane structures (**Figure 5, Movies S1, S2**). While we cannot completely rule



out the possibility of artifacts arising from over-expression or the fluorescent tags, identical localization patterns were observed at both high and low levels of protein expression, and for both N- and C-terminal tags. In addition, because GFP-ALA6 and ALA6-YFP fusion proteins rescued the *ala6-1/7-2* pollen defect, we conclude that at least a subset of the observed fusion proteins were in functional locations. This supports a working hypothesis that ALA6 and ALA7 might function in multiple membrane locations, including the plasma membrane.

### Lipid Composition is Altered in *ala6-1/7-2* Pollen

Tandem mass spectroscopy revealed a  $\sim 2$ -fold increase in PA concentration and a  $\sim 2$ -fold decrease in PI concentration in *ala6-1/7-2* pollen grains relative to wild-type (**Figure 6**, File S1). While the yeast P4-ATPase mutants  $\Delta drs2$  and  $\Delta drs2\Delta dnf1\Delta dnf2$  have been linked to altered concentrations of PE, PS, PC, and PI (Pomorski et al., 2003), the lipid changes in

*ala6-1/7-2* are distinct in two important ways. First, no change in PA concentration was observed for either yeast mutant, whereas a ~2-fold increase was observed for *ala6-1/7-2* pollen. Second, increased PI concentration was observed for the yeast mutant  $\Delta drs2\Delta dnf1\Delta dnf2$ , whereas a ~2-fold decrease was observed for *ala6-1/7-2* pollen. A parallel lipid profiling analysis was not done on growing pollen tubes because of the difficulties in obtaining sufficient sample material. Therefore, we cannot exclude the possibility that the lipid profile of *ala6/7* pollen changes during tube growth. It is also possible that the observed differences between Col-0 and *ala6/7* pollen are the result of limited changes in lipid composition at a specific subcellular location.

While the mechanisms causing altered lipid concentrations in *ala6/7* pollen are currently unknown, the correlation between an increase in PA concentration and a decrease in PI concentration could be explained by changes in either the biosynthesis or degradation of PI. For example, loss of ALA6/7 might alter the properties of one or more membrane systems, and thereby indirectly inhibit the activities of enzymes involved in PI biosynthesis, such as ER-associated PI synthase (PIS) proteins (Collin et al., 1999; Justin et al., 2002; Lofke et al., 2008). An alternate and non-exclusive possibility is that loss of ALA6/7 might result in increased degradation of PI into PA through the activities of phospholipase C (PLC) and diacylglycerol kinase (DGK) (Testerink and Munnik, 2011). PA has a well-established role in stress signaling (Testerink and Munnik, 2005, 2011), and it is possible that PA production is increased in mutant pollen as a result of a “physiological stress condition” associated with the loss of ALA6/7. It is also possible that loss of ALA6/7 might disrupt a feedback mechanism that adjusts the levels of PI metabolites, which have been shown to regulate the activity of the yeast P4-ATPase Drs2p (Azouaoui et al., 2014).

### Loss of ALA6 and ALA7 Disrupts FM Dye Staining of the Pollen Tube Plasma Membrane

A ~10-fold decrease in FM dye staining was observed for live *ala6-1/7-2* pollen tubes relative to wild-type (Figure 7). FM dyes selectively label membranes and are commonly used as tools for studying endocytosis and other vesicular trafficking processes (Betz et al., 1996; Bolte et al., 2004; Hoopmann et al., 2012). When a cell is exposed to an FM dye, the plasma membrane is stained immediately, followed by a time-dependent internalization into endomembrane structures (Bolte et al., 2004). While it is not clear how the loss of ALA6/7 reduces the ability of an FM dye to stain the PM, possible explanations include changes in the PM’s surface charge or fluidity. However, it is unclear whether the PM’s charge and fluidity could be altered enough to block FM dye staining without compromising the ability of the membrane to carry out basic biological functions. Alternate explanations include quenching or sequestration of FM dyes by an unknown molecule(s) in the extracellular matrix or plasma membrane of mutant pollen tubes.

### Lipid Transport Activity of ALA6

In an attempt to determine the lipid transport activity of ALA6, the enzyme was expressed in the yeast triple mutant

$\Delta drs2\Delta dnf1\Delta dnf2$  (Hua et al., 2002). This yeast mutant is deficient in phospholipid transport across the plasma membrane and has been successfully used to quantify the lipid transport activities of ALA2 and ALA3 (Poulsen et al., 2008; López-Marqués et al., 2010). However, ALA6 failed to show any lipid translocation activity for NBD-labeled fluorescent analogs of PS, PE, PC, and PA (Figure S5a). While controls corroborated that ALA6 was expressed at the yeast plasma membrane (Figure S5b), it remains unclear if ALA6 has a very different substrate specificity compared to other characterized flippases, or if the heterologous system was lacking a component that is uniquely required for ALA6 activity.

### Specialization of P4-ATPases

The P4-ATPase protein family in plants can be divided into five subfamilies (Baxter et al., 2003), each containing at least one pollen-expressed ALA isoform (Figure S1a). Of the seven pollen expressed ALA isoforms, three have been linked to pollen fertility: ALA3, subfamily 4 (Zhang and Oppenheimer, 2009; McDowell et al., 2013) and ALA6/7, subfamily 3 (this report). While decreased pollen fertility was observed for both *ala3* and *ala6/7* mutants, the underlying defects were distinct. For example, disorganized cytoplasmic streaming was observed in *ala3* pollen tubes (McDowell et al., 2013) but not *ala6/7* (Figure S6). Also, a change in lipid composition was observed in *ala6/7* pollen grains (Figure 6, File S1), but not *ala3* (McDowell et al., 2013). The differences between the *ala3* and *ala6/7* knockout phenotypes suggest that ALA3 and ALA6/7 have different cellular functions. Furthermore, the inability of the other four pollen-expressed ALA isoforms to compensate for the loss of ALA3 or ALA6/7 suggests that each ALA subfamily might have unique biological functions. The biological functions of the four other pollen-expressed ALA isoforms are currently unknown.

### Role of P4-ATPases in Temperature Stress Tolerance

One of the most dramatic defects associated with *ala6-1/7-2* was hypersensitivity to temperature stress. While seed set was reduced to ~55% of wild-type under unstressed conditions (Figure 2), *ala6-1/7-2* plants were completely sterile under hot-day/cold-night temperature stress (Figure 3). In comparison, the seed set reduction in wild type plants was ~2-fold. Interestingly, temperature hypersensitivity has also been reported for *ala3* knockouts (McDowell et al., 2013) and a knockdown of ALA1 (Gomès et al., 2000). In addition, the yeast P4-ATPase Drs2p is required for cell growth at or below 23°C (Ripmaster et al., 1993; Siegmund et al., 1998). It is not clear if these hypersensitivities are caused by defects in stress signaling, or biophysical differences resulting from changes in lipid composition or secretory pathway functions.

### Models for ALA6 and ALA7 in Pollen Tube Growth

From the evidence presented in this study, we propose that ALA6 and ALA7 function to directly or indirectly change the distributions and concentrations of PA and PI. PA and the phosphorylated derivatives of PI (phosphatidylinositol phosphates, PIPs), have well-established roles as signaling molecules in plants,

animals, and yeast (Wang, 2004; Stace and Ktistakis, 2006; Michell, 2008; Raghu et al., 2009; Ischebeck et al., 2010; Potocki et al., 2014). In plants, PA and PIPs are known to function as membrane-localization signals by binding specific protein targets, and can also regulate protein activity by inducing conformational changes (Wang et al., 2006; Munnik and Testerink, 2009; Xue et al., 2009; Munnik and Nielsen, 2011; Testerink and Munnik, 2011). Both PA and PIPs also have well-established roles in vesicular trafficking and pollen development (Thole and Nielsen, 2008; Fu, 2010; Ischebeck et al., 2010; Testerink and Munnik, 2011). For example, PA has been shown to affect the actin cytoskeleton of *Arabidopsis* pollen tubes via direct interaction with the actin capping protein AtCP (Huang et al., 2006). Altered PA levels result in disruptions to the pollen tube actin cytoskeleton and reduced tip growth (Potocký et al., 2003; Monteiro et al., 2005; Pleskot et al., 2013). Similarly, transient knock-down studies of NtPLD $\beta$ 1, a PA-producing enzyme in tobacco, revealed a reduction in pollen tube growth that could be rescued by the addition of exogenous PA (Pleskot et al., 2010).

While it is not yet clear how the loss of ALA6 and ALA7 results in pollen tube growth defects, two non-exclusive models warrant consideration. First, the loss of ALA6 and ALA7 might result in the defective regulation of plasma membrane lipid asymmetry, which could compromise membrane-associated functions such as protein recruitment, localized membrane curvature, signaling, or membrane fluidity. For example, a reduced ability to flip PA or PI to the inner leaflet of the pollen tube plasma membrane might alter the ability of the PM to recruit proteins involved in exocytosis and polar cell growth. In a second model, loss of ALA6 and ALA7 might impair membrane curvature during the formation of endomembrane vesicles, thereby causing a general disruption of the secretory and endocytosis pathways. These pathways are of general importance for all cells, especially pollen tubes, which display one of the most rapid polar growth rates of any plant cell.

Regardless of mechanism, we show here that the P4-ATPases ALA6 and ALA7 are critical for rapid, sustained pollen tube growth as well as tolerance to a temperature stress. Together, these results support a model in which ALA6 and ALA7 have distinct activities from the five other pollen-expressed ALA proteins and directly or indirectly change membrane features important for pollen fitness.

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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.2015.00197/abstract>

**Supplementary Movie S 1 | Movie of NaAz-treated pollen tube expressing GFP-ALA6.** Movie depicts the pollen tube shown in Figure 5c. See caption to Figure 5 for details. Images were taken at regular intervals of 1.25 s over a 2 m time period. Movie plays at 15x speed.

**Supplementary Movie S 2 | Movie of growing pollen tube expressing GFP-ALA6.** Movie depicts the pollen tube shown in Figure 5d. See caption to Figure 5 for details. Images were taken at regular intervals of 1.25 s over a 2 m time period. Movie plays at 15x speed.

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# Knockin' on pollen's door: live cell imaging of early polarization events in germinating *Arabidopsis* pollen

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Pollen tubes are an excellent system for studying the cellular dynamics and complex signaling pathways that coordinate polarized tip growth. Although several signaling mechanisms acting in the tip-growing pollen tube have been described, our knowledge on the subcellular and molecular events during pollen germination and growth site selection at the pollen plasma membrane is rather scarce. To simultaneously track germinating pollen from up to 12 genetically different plants we developed an inexpensive and easy mounting technique, suitable for every standard microscope setup. We performed high magnification live-cell imaging during *Arabidopsis* pollen activation, germination, and the establishment of pollen tube tip growth by using fluorescent marker lines labeling either the pollen cytoplasm, vesicles, the actin cytoskeleton or the sperm cell nuclei and membranes. Our studies revealed distinctive vesicle and F-actin polarization during pollen activation and characteristic growth kinetics during pollen germination and pollen tube formation. Initially, the germinating *Arabidopsis* pollen tube grows slowly and forms a uniform roundish bulge, followed by a transition phase with vesicles heavily accumulating at the growth site before switching to rapid tip growth. Furthermore, we found the two sperm cells to be transported into the pollen tube after the phase of rapid tip growth has been initiated. The method presented here is suitable to quantitatively study subcellular events during *Arabidopsis* pollen germination and growth, and for the detailed analysis of pollen mutants with respect to pollen polarization, bulging, or growth site selection at the pollen plasma membrane.

**Keywords:** pollen activation, cell polarization, bulging, vesicular trafficking, ARO1, actin cytoskeleton, sperm cells

## Introduction

The pollen tube (PT) of flowering plants is formed by the pollen grain vegetative cell and represents a cell of enormous specialization, responsible for the transport of the two male gametes through the female tissues of the pistil to the ovule. It is the fastest elongating plant cell (Sanati Nezhad et al., 2014) and can reach lengths of 30 cm, with growth rates up to 1 cm/h (Mascarenhas, 1993). PT growth is monotropic by expansion at an annular region

**Abbreviations:** ARO, Armadillo Repeat Only; epiBL, 24-epibrassinolide; F-actin, filamentous actin; GFP, green fluorescent protein; PGM, pollen germination medium; PT, pollen tube; RFP, red fluorescent protein; TIRF, total internal reflection fluorescence.

located at the tip in a process called polar tip growth (Taylor and Hepler, 1997; Geitmann, 2010).

Deeply embedded in the tissues of the pistil, *in vivo* PT growth is difficult to investigate with high temporal and spatial resolution and has been achieved so far only by using two-photon microscopy (Feijó and Moreno, 2004; Cheung et al., 2010). As an advantageous alternative, pollen can be germinated *in vitro* to study the cellular dynamics and complex signaling pathways that coordinate polar tip growth (Qin and Yang, 2011). From these studies we know that intensive exo- and endocytosis at the tip supported by regulated vesicle trafficking and cytoskeleton dynamics, as well as coordinated changes in cell wall properties are essential cellular activities of the growing PT (for review see Geitmann, 2010; Guan et al., 2013). Great advances have been made during the past years in identifying key signaling molecules for the proper elongation of the PT tip, such as Rho GTPases, calcium ions, and phosphoinositides (for review see Cheung and Wu, 2008; Qin and Yang, 2011; Steinhorst and Kudla, 2013). These key regulators are components of distinct signaling pathways forming a complex network that controls the cellular activities of tip-growing PTs (Guan et al., 2013). However, there are still significant gaps in our knowledge of PT growth regulation, especially with regard to the question when and how symmetry breaking in the apparently unpolar pollen vegetative cell occurs, and what the molecular mechanism for selecting the growth site is.

Polar tip growth of PTs is very similar to the polar elongation of root hairs on genetic and mechanistic levels (reviewed in Šamaj et al., 2006; Campanoni and Blatt, 2007; Kost, 2008; Lee and Yang, 2008). Root hair growth is known as a multi-phasic process, consisting of cell fate determination, the formation of a root hair bulge, and the initiation of tip growth in the root hair bulge, each of which is characterized by distinct physiological and mutant phenotypes in the model plant *Arabidopsis* (Schiefelbein and Somerville, 1990; Parker et al., 2000; Schiefelbein, 2000; Bibikova and Gilroy, 2003; Müller and Schmidt, 2004). Since pollen germination and the initiation of PT tip growth is rapid and much faster than root hair growth, it is technically more demanding to perform live cell imaging in order to study the cellular dynamics and the growth kinetics during pollen hydration, activation, germination and PT formation. Moreover, *in vitro* germination rates and growth dynamics of *Arabidopsis* pollen are known to be highly variable (Johnson-Brousseau and McCormick, 2004; Boavida and McCormick, 2007), complicating its use for cellular and molecular genetic studies of pollen germination and growth. However, methodological advances in germination techniques meanwhile facilitated the experimental use of *Arabidopsis* pollen (Bou Daher et al., 2009; Rodriguez-Enriquez et al., 2013; Vogler et al., 2014), offering possibilities to establish methods for larger-scale screening and quantitative phenotyping of wild type and mutant pollen.

To optimize high throughput time-lapse live imaging of germinating *Arabidopsis* pollen, we established an inexpensive and easy mounting technique suitable for every standard microscope, based on an improved pollen germination medium (Vogler et al., 2014). Using this setup for Spinning Disc confocal microscopy we investigated the growth kinetics and morphology changes of *Arabidopsis* PTs expressing GFP in the cytoplasm of the vegetative

pollen cell. We focused on early cell polarization events during pollen activation and germination by studying the spatiotemporal localization of GFP-labeled Armadillo Repeat Only 1 (ARO1), which is known to be essential for polar PT growth (Gebert et al., 2008). ARO1-GFP accumulates in the inverted cone-shaped region of growing PT tips in a brefeldin A and latrunculin B sensitive manner and TIRF microscopy, applied in this study, confirmed that ARO1-GFP localizes vesicle-associated at the PT tip.

We used *Arabidopsis* marker lines expressing ARO1-GFP and tagRFP-T-Lifeact in the pollen to study vesicle and filamentous actin (F-actin) dynamics before and during pollen germination. Furthermore, we used a pollen marker line with fluorescently labeled sperm cell nuclei and plasma membranes (Sprunck et al., 2012) to address the question when the two sperm cells, physically linked to the nucleus of the vegetative cell forming a male germ unit (MGU) (McCue et al., 2011; Zhou and Meier, 2014), are transported from the pollen grain into the germinated PT.

Our time-lapse live imaging of germinating *Arabidopsis* PTs revealed similarities between root hair formation and pollen germination as we observed successive phases of cell polarization, bulge formation, growth site selection, and the initiation of rapid tip growth. Prior to pollen germination, we observed a characteristic polarization of vesicle-associated ARO1-GFP and tagRFP-T-Lifeact labeled F-actin in the pollen grain. After bulging, a transition phase is observed where vesicle-associated ARO1-GFP heavily accumulates at the distal end of the bulge and adopts an inverted cone-like shape before the PT switches to rapid tip growth. At the same time, long F-actin cables appear, extending in parallel orientation from within the pollen grain into the PT, while the volume of the vacuole, arising opposite the germination site, increases. During the phase of rapid tip growth, F-actin bundles massively accumulate at the germination site and increasing vacuolization occurs, followed by sperm cell transport into the PT.

## Materials and Methods

### Plant Material

*Arabidopsis thaliana* (accession Col-0) plants were grown under long-day conditions (16 h light, 20°C, 70% humidity) in growth chambers after seeds were subjected to stratification 2 days at 4°C in the dark. Homozygous lines carrying the *P<sub>Lat52</sub>:GFP* transgene were used to express cytoplasmic GFP in the vegetative PT cell (Twell et al., 1990). A C-terminal GFP fusion of the ARO1 protein under control of its endogenous promoter (Gebert et al., 2008) was used to investigate its subcellular localization in pollen and PTs. A double homozygous marker for *P<sub>HTR10</sub>:HTR10-RFP* and *P<sub>HTR10</sub>:TET9-GFP* line (Sprunck et al., 2012) was used to visualize sperm cell nuclei and sperm cell plasma membranes.

### Molecular Cloning and Generation of Transgenic Lines

A double stranded DNA fragment encoding for a 17 aa actin binding domain termed Lifeact (Riedl et al., 2008) with additional 5' and 3' *Hind*III restriction sites was synthesized by proof-reading PCR on the partially overlapping template

oligonucleotides 5'-GGGGCCATGGAAGCTTGGGACCAGC CGTAGGAATGGGTGTTGCTGATCTTATTAAGAACGTCGA GTCTATTCTAAGGAGG-3' and 5'-GGGAAGCTTATGCC ATGGCTCCAGCTACAGGTGCTCCGCCCTCCTCCTCC TTAGAAATAGACTCGAACTTCTTAA-3' with the PCR primers Lifeact-fwd (5'-GGGGCCATGGAAGCTTGG-3') and Lifeact-rev (5'-GGGAAGCTTATGCCATGGC-3'). After *Hind*III digestion, the PCR product was ligated behind the fluorophore coding sequence into the modified Gateway destination vector pENTR-tagRFP-T (Denninger et al., 2014) to obtain pENTR-tagRFP-T-Lifeact. To achieve expression in pollen, 712 bp of the *ARO1* promoter with additional 5' *SacI* and 3' *SpeI* sites were amplified from the 95P-Nos-*ARO1p*:*ARO1-GFP* plasmid (Gebert et al., 2008) with the primers pARO1-II-for (5'-TCGGGTACCGAGCTCAGATCTAAGCTG-3') and pARO1-II-rev (5'-TGTGACCGCGGCCGACTAGtCAGATC-3'). The 35S promoter of the binary gateway expression vector pB2GW7 (Karimi et al., 2002) was replaced by the *ARO1* promoter via *SacI*/*SpeI* to obtain pB2GW7-*ARO1p*. Gateway LR reaction with pENTR-tagRFP-T-Lifeact and pB2GW7-*ARO1p* was performed according to the manufacturer's recommendations (Life Technologies) to obtain the expression vector pARO1:tagRFP-T-Lifeact that was used for *Agrobacterium*-mediated plant transformation by floral dip method (Clough and Bent, 1998).

## Pollen Mounting and Live Cell Imaging

Micro-germination slides were prepared in either a single-well or a multi-well setup (Figures S1,S2). To prepare a single-well micro-germination slide, a 1–2 mm high planar plasticine layer was added on the margin of the ring of a slide with an attached glass ring (L4246, PLANO, Wetzlar, Germany). The well was filled with molten pollen germination medium (PGM) according to Vogler et al. (2014), containing 10 μM 24-epibrassinolide (epiBL, Sigma-Aldrich E-1641) and solidified with 0.5% low melting point agarose. After solidification the center of the well was hand pollinated using single dehiscent anthers, manually removed from flowers at flower stage 13–14 (according to Smyth et al., 1990). The well was then sealed by gently pressing evenly a 24 × 24 mm No. 1.5 cover slip onto the plasticine until it slightly touched the PGM. The illustrated instruction on how to prepare a single-well micro-germination slide is shown in Figure S1. Multi-well micro-germination slides were prepared by attaching a 12 well silicon profile (flexiPERM® micro12, SARSTEDT, Germany) to a standard microscope slide (26 × 76 mm) and filling each well with 50–75 μL molten PGM. After solidification, the silicon profile was removed and another 25 μL of molten PGM were added on top of each agar pad to obtain convex shapes. After a frame of plasticine was modeled around the agar pads, they were hand pollinated and then sealed by gently pressing evenly a 24 × 60 mm No. 1.5 cover slip on the plasticine frame. The scheme on how to prepare a multi-well micro-germination slide is shown in Figure S2. Immediately after pollen application, micro-germination slides were used for live-cell imaging. No obvious differences in germination or PT growth were observed between single-well or multi-well micro-germination setups. By contrast, much lower and highly variable germination rates as

well as slower PT growth rates were observed when PGM without 10 μM epiBL was used to prepare the micro-germination slides, while the different phases of pollen germination and tube growth described in this work were unaffected.

Microscopy was performed on a ZEISS Cell Observer Spinning Disc confocal microscope (Yokogawa CSU-X1) equipped with a motorized stage using 20×/0.8 NA dry, 40×/1.30 NA DIC oil immersion or 63×/1.40 NA DIC oil immersion objectives. GFP fluorescence was excited with a 488 nm laser line and emission was detected from 505 to 545 nm. A 561 nm laser line was used to excite tagRFP-T and emission was detected from 570 to 640 nm. Free GFP in the pollen cytoplasm and *ARO1-GFP* fusion protein were imaged every 3 min, tagRFP-T-Lifeact every 10–15 min over 4–6 h in z-stacks of 11 optical slices at each of 10–20 positions representing individual pollen spots.

## Morphological Modeling of Pollen Germination

We assumed two extreme morphological models describing cellular geometries of germinating PTs and simulated these models graphically with Illustrator CS4 software (Adobe). In the “protrusion model” we proposed linear growth at the tip of a protuberance, generating a constantly elongating cylinder with a dome-shaped tip that emerges from the germination site. In the “bulging model” a first phase of isodiametric inflation was assumed for the germinating PT, followed by a second phase in which isodiametric growth switches to polar growth at a dome-shaped tip. Thus, in the “protrusion model,” a tubular object constantly emerges out of an ellipse, representing the pollen grain. To generate the “bulging model” a circle, representing the PT, was placed below the upper margin of an ellipse, representing the pollen grain. The diameter of the circle was frame-wise and constantly increased, while keeping its position constant at the lowermost point. After 20 frames, we changed the distal region of the circle into a dome-shaped tip, which then constantly elongates in form of a cylinder like in the “protrusion model.” In both morphological models, the width of the dome-shaped tip was set identical and did not change during elongation. Furthermore, the net increase in PT area was set identical for both models. Modeled PTs were measured in ImageJ like described for microscopic images (Image Processing and Quantitative Analysis).

## Pollen Staining and Microscopy

For membrane staining with FM4-64, pollen of *ARO1-GFP* expressing plants were germinated in 35 mm petri dishes on solidified PGM as described above. Three hours after pollination a small agar piece was excised and mounted upside down on a cover slip in a droplet of 8 μM FM4-64 (Life Technologies) dissolved in liquid PGM. Images were taken with an inverted SP8 Confocal Laser Scanning Microscope (Leica Microsystems) with a 40×/1.3NA oil immersion objective and 1 airy unit pin-hole opening. GFP and FM4-64 were excited simultaneously with a 488 nm laser line. GFP emission was detected from 495 to 550 nm and FM4-64 emission from 650 to 725 nm using HyD detectors. For DAPI staining, pollen of plants expressing *PARO1:tagRFP-T-Lifeact* were put in a droplet of DAPI staining solution (2.5 μg/ml 4',6-diamidino-2-phenylindole (DAPI), 0.01% Tween-20, 5% DMSO, 50 mM PBS, pH 7.2). Confocal

z-stacks were acquired at the Spinning Disc system described above using a  $100\times/1.40$  NA oil immersion objective. DAPI fluorescence was excited with a 365 nm LED illumination (COL-IBRI, ZEISS) and emission light was filtered by the microscope stand built-in filter cube (emission filter: 447–507 nm) and channeled through an empty Spinning Disc position to display DAPI fluorescence on the same camera as for tagRFP-T and DIC channels.

## TIRF Microscopy

For TIRF microscopy of PTs, a very planar gel pad was generated by laying two microscope slides orthogonal on the edges of three adjacent slides (Figure S3). 500  $\mu\text{l}$  of molten PGM containing 2% agarose was pipetted to the middle of the lower slides and immediately covered with another slide. After solidification, the uppermost slide and all flanking slides were removed and the PGM pad was hand pollinated as described above (Pollen Mounting and Live Cell Imaging). Pollinated slides were kept in a damp box for 3–5 h. Prior to microscopy, a droplet of double distilled water was pipetted onto the pad and a No. 1.5H cover slip was added. TIRF illumination was generated in a Delta Vision Elite (GE, Healthcare, Applied Precision) system with an Olympus IX-71 microscope, equipped with an Insight SSI(TM) solid state illumination system and an X4 laser module. Images were taken with an Olympus UAPON 100XOTIRF 1.49 NA oil immersion objective and recorded with a CoolSnap HQ2 CCD camera (Photometrics, Tucson, USA). GFP was excited with the 488 nm laser line and emission was detected between 501 and 549 nm. Image exposure time and TIRF angle were adjusted according to sample fluorescence intensity and specimen location.

## Image Processing and Quantitative Analysis

All images were processed in ImageJ (<http://rsbweb.nih.gov/ij>, version 1.45). In time-lapse experiments, the frame before a PT emerged from the germination site was set to zero. Z-stacks of time lapse images of pollen expressing *P<sub>Lat52</sub>:GFP* were subjected to projection algorithms. Bright field images were sum slice, GFP images maximum intensity projected. Afterwards, for the GFP channel a threshold was applied to obtain binary images. The implemented WAND tool was used to determine the pollen area, which was then subtracted from all images for a given PT and subsequently the PT area was measured for each frame. The analysis of PTs was only carried out with those PTs where growth was not disturbed by any other object and which could be observed for at least 1 h. Of all PTs the shape descriptor “roundness” was measured, given by  $4*\text{area}/(\pi*(\text{major axis})^2)$  of a respective PT. To compare the frame wise PT area increase shortly after germination and in later PT growth phases, the mean frame wise increase of the first 10 and the last 10 frames was calculated and compared in a Friedman’s 2-way variance analysis. Z-stacks of time lapse images of pollen expressing *P<sub>ARO1</sub>:ARO1-GFP* were also subjected to maximum intensity projections first. For those pollen that were monitored at least half an hour before and after the time point of germination, images were cropped in a rectangular selection containing only the pollen and emerging PT. All frames of a PT were included in a stack histogram that was used for subsequently computing gray values for setting a 70% signal

threshold to determine the PT shape (false colored in red) and a 0.5% signal threshold (false colored in yellow) to determine the maximum intensity peaks for ARO1-GFP. Frame-wise PT area increase was measured by overlaying unbiased PT shape outlines that were obtained using the WAND tool, which was also used to determine the size of individual PT areas. ARO1-GFP maximum intensity peaks were quantified using a variable ROI selection and measuring its mean gray value that was subsequently multiplied by the ROI size. To compare multiple PTs in a mean value computation, ARO1-GFP maximum intensity was normalized for each PT to its maximum signal value. Z-stacks of images of pollen expressing tagRFP-T-Lifeact were maximum intensity projected and to better visualize the maximum signal intensities false colored using the “spectrum” LUT. Calculations were performed with Excel2010 (Microsoft) and statistical analyses were computed with SPSS22 (IBM).

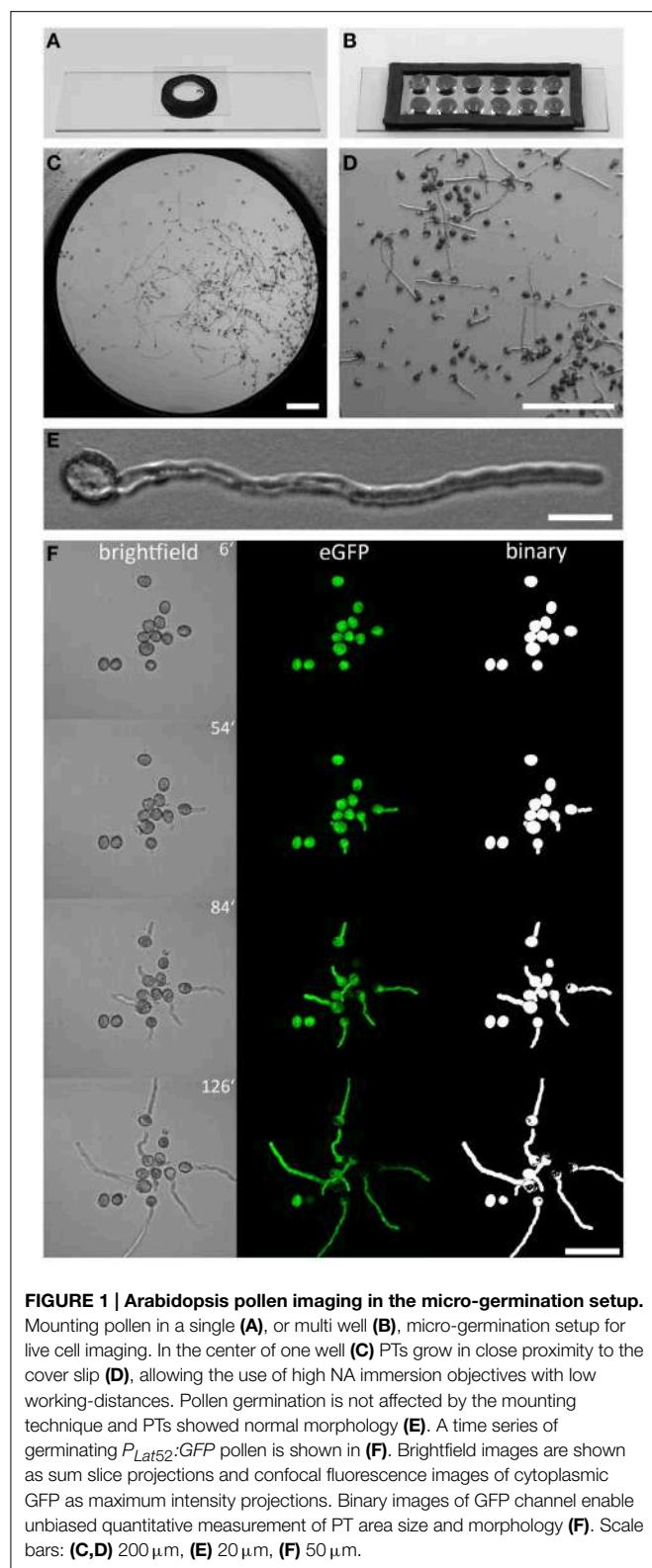
## Results

### Live Cell Imaging of Pollen Germination and PT Growth

To facilitate time lapse live cell imaging of *Arabidopsis* pollen germination and PT growth using high NA immersion objectives, we designed a single-well and a multi-well micro-germination setup as shown in **Figures 1A,B**. Both setups are fast and easy to prepare (see Figures S1,S2), based on inexpensive components. Pollen germination and PT growth of up to 12 genetically different plants can be simultaneously observed over many hours when using the 12-well micro-germination setup. Pollen germinates in the direct proximity to the cover slip in a film of PGM that is formed when the cover slip is gently pressed on the medium to seal the well (**Figure 1C**). Pollen germination rates within this film are very high (>80%) and homogeneous (**Figures 1D,F**), with normal PT morphology (**Figure 1E**). In an exemplary 10 well-setup, no temporal or morphological deviations in pollen germination or PT growth were observed (**Supplemental Movie 1**). This technique can be broadly used in every lab, adapted to many microscopic techniques and may be even up-scaled for the simultaneous imaging of pollen from more than 12 individuals.

### Pollen Tube Growth Kinetics

We evaluated a total of 66 PTs expressing cytoplasmic GFP in the PT vegetative cell that fulfilled our quality criteria for quantitative PT analyses, that is the absence of any obstacle during germination and growth and the complete recording of at least 1 h after germination. As PTs represent 3-dimensional cylindrical objects, we did not determine PT length in  $\mu\text{m}$  but measured the PT as area in  $\mu\text{m}^2$ . Automatic size measurements using the WAND tool (ImageJ) were performed with thresholded binary images of maximum intensity projections (**Figure 2A**). The growth kinetics of this PT is depicted as frame-wise increase in PT area and as cumulative increase in PT area over time, respectively (**Figure 2B**). After germination, no marked increase in PT area can be observed during the first 21 min of PT growth. Twenty four minutes after germination, PT growth strongly increases and



rises even more after 42 min. Comparing the growth rate determined by PT area measurements with PT length as a measure of growth revealed similar growth kinetics (Figure S4). When

we estimated the ratio of PT length to PT area over time, we calculated an approximated conversion factor of  $0.18 \mu\text{m}^{-1}$  for transferring PT area ( $\mu\text{m}^2$ ) in PT length ( $\mu\text{m}$ ).

When all 66 PTs were included into the quantification of growth kinetics, a high overall PT growth rate of  $400.2 \mu\text{m}^2/\text{h}$  (mean cumulative PT area) was observed (Figure 2C). We observed no or only very low increase in the PT area during the first 12 min of PT formation, resembling a lag phase. To statistically test this, we compared the mean PT area increase rate for the first and the last 10 frames of PT growth (inset in Figure 2C). In almost all cases (59 of 66), the mean PT area increase during the first 30 min was lower than for the last 30 min. Friedman's 2-way variance analysis revealed that PT growth during the first 30 min is highly significantly slower ( $p < 0.001$ ) than during the last 30 min.

From these results we conclude that PT growth in *Arabidopsis* starts with a first distinct phase of slow growth shortly after germination that is followed by a second phase of rapid PT growth. This is furthermore corroborated by the finding that in case of PT burst, also a short protuberance is first initiated but obviously does not pass over to the next phase of rapid elongation (Figure 2D). A later short phase of decelerated growth could be observed in about one third (21 of 66) of all PTs investigated (Figure 2B). This lag phase occurred when PTs reached a mean size of approximately  $200 \mu\text{m}^2$  and it was more variable and less pronounced (Figure 2B; Figure S5).

## Changes in PT Shape after Pollen Germination

We investigated the PT morphologies during germination and the transition to rapid tip growth in more detail and compared them with two extreme morphological models describing possible PT geometries. The “protrusion model” assumes that a PT would emerge from the pollen grain as an elongating cylinder with a dome-shaped tip that is maintained during germination and rapid tip growth (Figure 2E). As a result the diameter of the junction between the pollen grain and the PT, which is the site of germination, would remain rather constant in this model. By contrast the “bulging model” assumes that the PT initially exhibits isodiametric growth, leading to a round bulge emerging at the germination site (Figure 2F, red arrowhead). In a second phase, isodiametric growth would have to switch to polar tip growth by selecting a growth site and forming an elongating cylinder. A unique feature of the “bulging model” is that the isodiametric inflation of the bulge will increase the diameter of the germination site over time.

The differences between the two morphological models are highlighted in Figure 2G. For both models we determined a similar net increase in PT area and the same width of the dome-shaped tip. When we compared the increase in PT area over time it was indeed almost identical for both models (Figure 2H). We computed the course of “roundness” for both morphological models during germination and found the “roundness” to increase linearly to a sharp peak in the “protrusion model,” followed by a rapid decrease when the PT continues to elongate (Figure 2I). In the “bulging model,” by contrast, the course of roundness of a germinating PT forms a rather

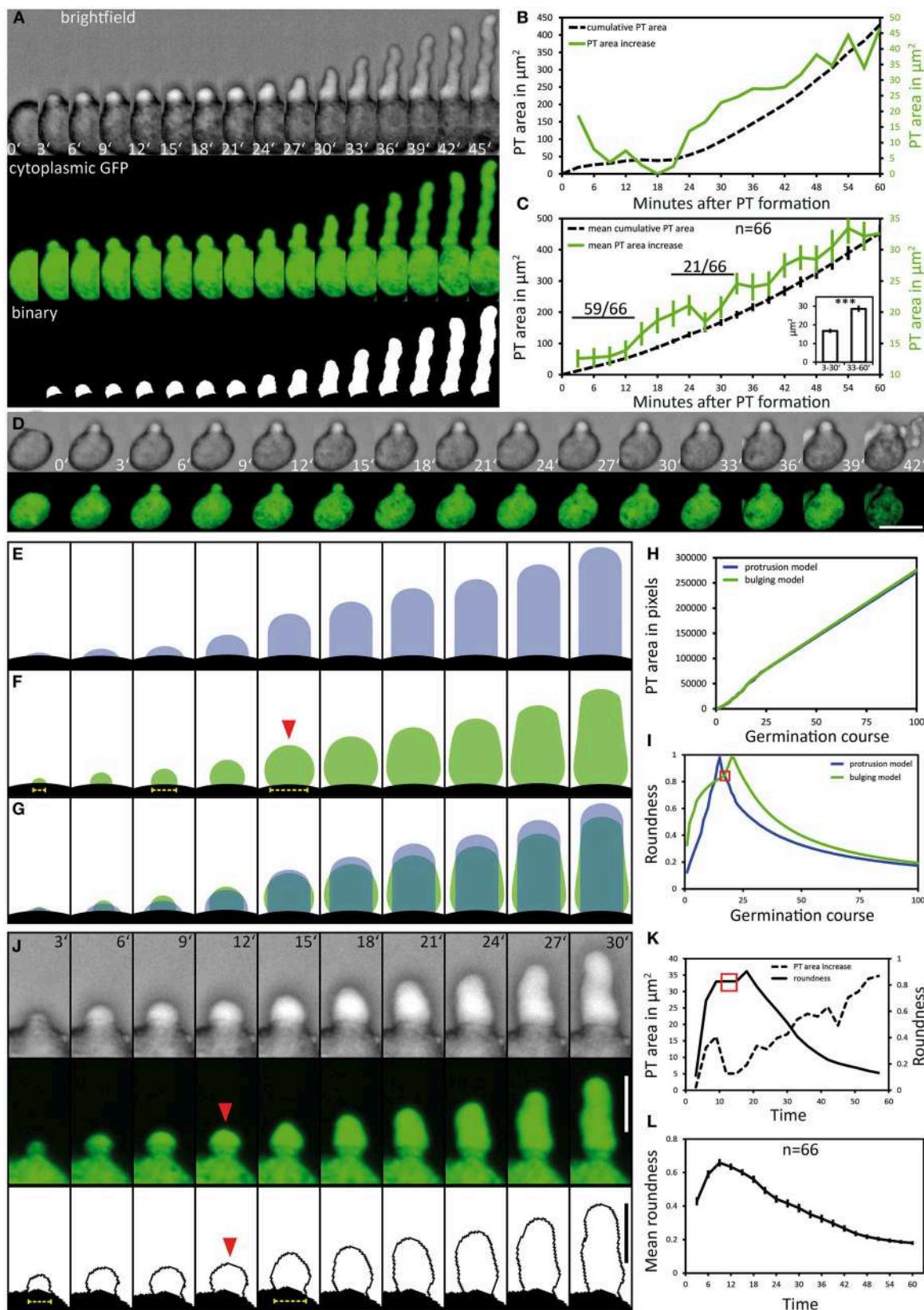


FIGURE 2 | Continued

**FIGURE 2 | Pollen tube growth kinetics and morphology changes.** Pollen expressing GFP in the cytoplasm of the vegetative cell ( $P_{Lat52}\text{-GFP}$ ) was used to quantitatively assess the kinetics of PT growth and PT morphology during germination. Brightfield and fluorescence channel, together with the binary image generated from the fluorescence channel, are shown in (A). Time point 0' indicates the last frame before germination. The quantification of the cumulative PT area and frame-wise increase in PT areas over time is given in (B). Mean values  $\pm$  1 SE of cumulative PT areas and the frame-wise increase in PT areas of 66 evaluated PTs are shown in (C). Numbers in (C) represent the frequency of an observed pattern. The mean frame-wise increase in PT areas for the first 10 frames of each pollen tube is significantly lower compared to the last 10 frames of each PT

[inset of (C); asterisks indicate statistically highly significant differences,  $p < 0.001$ ]. A pollen that germinates but fails to bursts after germination is shown in (D). “Protrusion” and “bulging” model for PT morphology changes during pollen germination and PT elongation are shown in (E,F). The overlay of both morphological models is shown in (G), their PT area increase over time is shown in (H). The course of “roundness” for both morphological models is shown in (I). Red solid arrowhead in (F) and red square in (I) highlights the transition from unpolar bulging to polar elongation in the “bulging model.” A representative germinating PT is shown in (J) and its course of PT area increase and “roundness” is shown in (K). Mean values  $\pm$  1 SE of the “roundness” of 66 PTs are given in (L). Scale bars: (A,B) 25  $\mu\text{m}$ , (J) 12.5  $\mu\text{m}$ .

hyperbolic increasing curve with a broader maximum leading to an accentuated peak (Figure 2I).

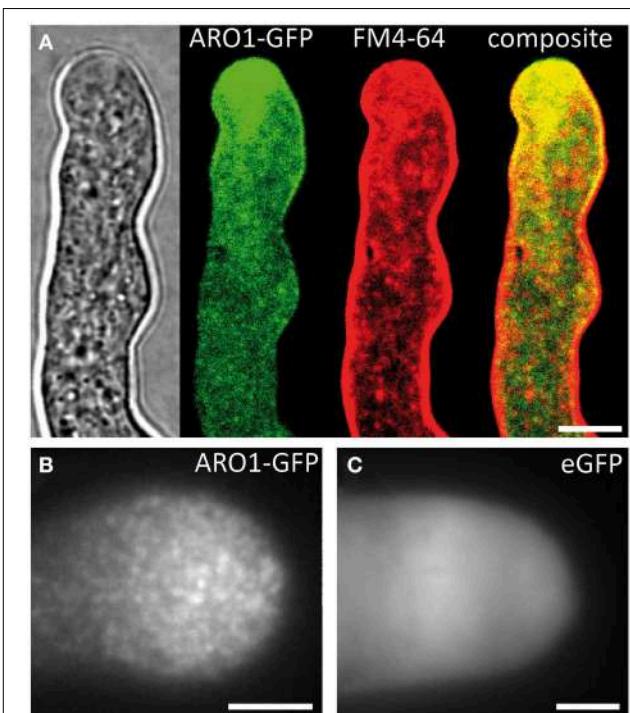
Notably, live imaging of germinating pollen revealed considerable similarities to the “bulging model” (Figures 2J,K). During the first 12 min of germination the pollen vegetative cell forms a round bulge at the germination site that exhibits isodiametric growth. Afterwards, the uniformly expanding bulge undergoes the transition into a polar growing PT (Figure 2J; 15' to 18'). The changes in its shape are reflected by the course of “roundness” plotted for this PT (Figure 2K). A hyperbolic increase with a broad maximum (red box in Figure 2K) is characteristic for the phase of bulging. The following peak defines the transition phase, when the bulge starts to form a dome-shaped tip, followed by a switch to rapid tip growth (Figure 2K).

We found the same tendency when we plotted the mean course of “roundness” for all 66 PTs (Figure 2L). Furthermore, in 37 of 66 examined pollen the increasing diameter of the germination site during bulging was clearly visible (Figure 2J, yellow dotted line), which is in line with the unique feature predicted by the “bulging model” (Figure 2F, yellow dotted line).

### ARO1-GFP is Associated to Vesicles

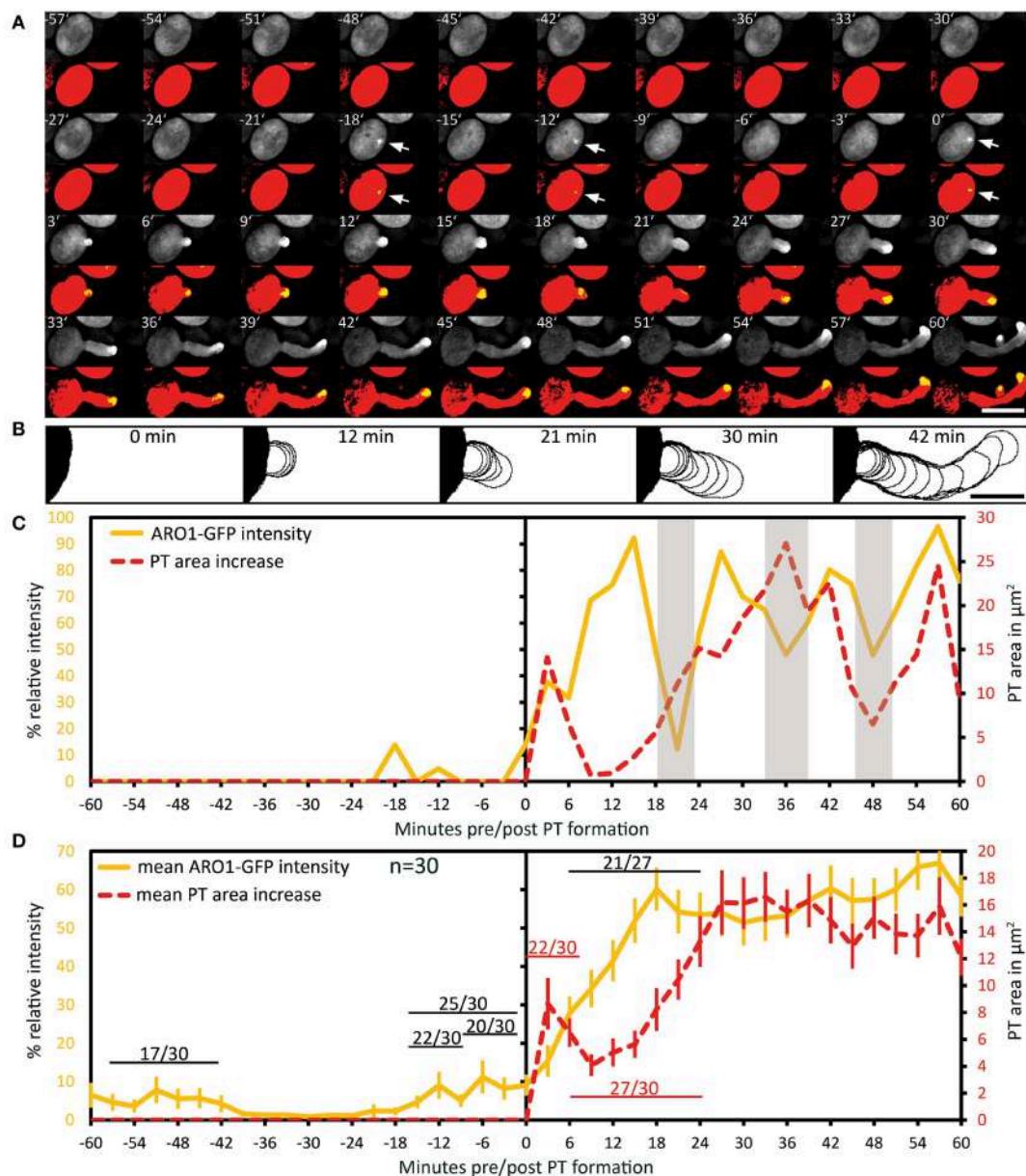
In the growing PT the GFP fusion of Armadillo Repeat Only 1 (ARO1) accumulates in the vesicle-rich “clear zone” (Figure 3A; Supplemental Movie 2). We observed partial co-localization of ARO1-GFP fluorescence with FM4-64 in the “clear zone,” but almost no co-localization in the subapical part of the PT (Figure 3A). Spinning Disc confocal time-lapse imaging of growing PTs furthermore revealed that ARO1-GFP streams in a reverse fountain pattern (Supplemental Movie 2). The fact that ARO1-GFP accumulates in the PT tip in a brefeldin A-sensitive manner (Gebert et al., 2008) suggested that ARO1-GFP is associated to vesicles in the PT tip.

We performed TIRF microscopy to confirm the proposed vesicle-association of ARO1-GFP. By illuminating only a thin region of the PT tip, including the cytoplasmic zone immediately beneath the PT plasma membrane, we compared the fluorescent signals of ARO1-GFP with that of free GFP. As shown in Figure 3B, ARO1-GFP signals appeared as numerous dot-like structures with a size of approximately 200 nm in the PT tip. In contrast PTs expressing cytoplasmic GFP showed a homogenous fluorescence (Figure 3C).



**FIGURE 3 | ARO1-GFP localizes to vesicles at the pollen tube tip, accumulating in the inverted cone-shaped region.** (A) At the PT tip, ARO1-GFP predominantly accumulates in the vesicle-rich inverted cone-shaped region and partially co-localizes with FM4-64. No co-localization of ARO1-GFP and FM4-64-stained membrane compartments is detected in the subapical region of the PT. (B) TIRF microscopy reveals that ARO1-GFP signals appear as discrete punctate structures of approximately 0.2  $\mu\text{m}$  in the PT tip. These punctate structures are not observed in PTs that express cytoplasmic GFP (C). Scale bars: 5  $\mu\text{m}$ .

**ARO1-GFP Decorated Vesicles Peak at the Future Germination Site During Pollen Activation**  
We then investigated the subcellular localization and signal intensity changes of vesicle-associated ARO1-GFP before and during pollen germination using Spinning Disc microscopy (Figure 4A; Supplemental Movie 3). The respective outlines of the frame wise increase in PT area are shown in Figure 4B. The quantification of ARO1-GFP signal intensity in relation to the increase in PT area is given in Figure 4C.



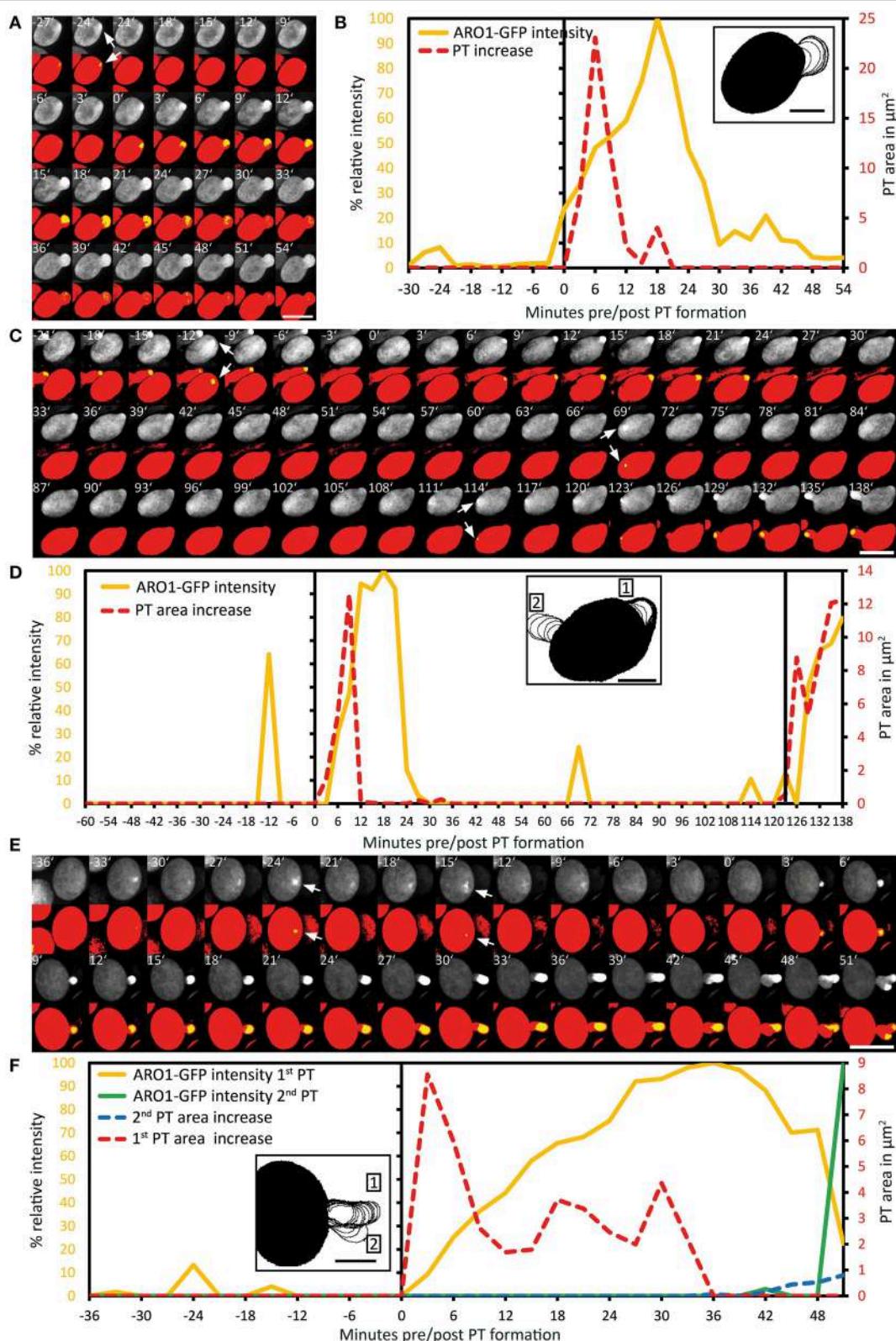
**FIGURE 4 | Polarization of vesicle trafficking in activated pollen predetermines the site of pollen tube emergence.** Time series of fluorescence signal in germinating pollen expressing *P<sub>ARO1</sub>·ARO1-GFP*. Maximum intensity projected fluorescence raw signal and thresholded signals of a representative PT are shown in (A). GFP fluorescence is shown in yellow (0.5% of highest intensities), and in red (70% of highest intensities) representing the ARO1-GFP maxima and the pollen cytoplasm. Outlines of PT shape for successive frames are drawn in (B). The

frame-wise increases in PT area over time and relative ARO1-GFP intensities for the PT in (A) are plotted in (C). Gray shaded areas indicate phases of PT growth reorientation. Mean value  $\pm 1$  SE of frame-wise PT area increase and normalized ARO1-GFP signal maxima for 30 PTs are given in (D). Numbers in (D) represent the frequency of an observed pattern. Time point 0' indicates the last frame before germination. Arrows point to ARO1-GFP intensity maxima before germination. Scale bars: (A) 25  $\mu$ m, (B) 10  $\mu$ m.

Notably, we observed 18 and 12 min before pollen germination high intensity peaks of ARO1-GFP subjacent to the future site of PT outgrowth (arrows in Figure 4A; peaks in Figure 4C), resembling two knocks on the door. During the following bulging phase (0–12 min), ARO1-GFP steadily accumulated at the distal pole of the bulge (Figures 4A,C). High fluorescence intensities at the PT tip, shaped as inverted cone, were observed when the

switched to rapid tip growth (24–60 min), with moderate down-turns during short phases of tube growth re-orientation (30–36 min; 45–48 min), which also took place during the transition to rapid tip growth (18–24 min).

The quantitative analysis of 30 PTs is shown in Figure 4D. Sixty to thirty six minutes before germination, intensity peaks of ARO1-GFP appeared in 17 out of 30 pollen grains



**FIGURE 5 |** Exceptional pollen germination events confirm the correlation between local vesicle accumulation and pollen tube emergence. The breakdown of vesicle accumulation in the pollen tube

bulge is accompanied with the failure to switch to rapid PT tip growth (**A,B**). After unsuccessful transition to rapid PT tip growth, the pollen cell may also (*Continued*)

**FIGURE 5 | Continued**

change the direction of polar vesicle trafficking, resulting in the establishment of a second germination site, as shown in **(C,D)**. A branching pollen tube is shown in **(E,F)**. After successful bulging the transition to rapid PT tip growth fails and a second growth site is selected, indicated by the accumulation of ARO1-GFP decorated vesicles in the tip of the PT branch. Maximum intensity projected fluorescence raw images and composite thresholded

images are shown in **(A,C,E)**. GFP fluorescence is shown in yellow (0.5% of highest intensities) and in red (70% of highest intensities), representing the ARO1-GFP maxima and the pollen cytoplasm. Frame-wise PT area increase and relative ARO1-GFP intensity are shown in **(B,D,F)**, where insets show frame-wise overlaid PT shape outlines. Time point 0' indicates the last frame before germination. Arrows point to ARO1-GFP intensity maxima before germination. Scale bars: **(A,C,E)** 25  $\mu$ m, insets in **(B,D,F)** 10  $\mu$ m.

**(Supplemental Movie 3)**. These intensity peaks, indicating rapid and local vesicle accumulation, were often but not always located near the future site of PT outgrowth. However, shortly before germination in 25 out of 30 pollen at least one high-intensity peak was detected subjacent to the future site of PT outgrowth (**Figure 4D**). 22 of 30 pollen grains showed one peak 12 to 9 min before germination at the future germination site, and in two thirds of observed pollen the ARO1-GFP high-intensity peak was recorded 6 to 3 min before germination. In 50% of the pollen two high-intensity peaks were visible (**Supplemental Movie 3**), while one third of pollen showed a single peak before germination. Frequencies and statistics of ARO1-GFP intensity peaks in pollen subjacent to the future site of PT outgrowth are shown in Figure S6.

Taken together, the temporary polar accumulation of ARO1-GFP decorated vesicles in activated pollen precedes pollen germination and marks the future site of PT outgrowth. Furthermore, we observed a strong increase of ARO1-GFP signal intensity after bulging, indicating the transition to rapid PT elongation (**Figure 4D**). Like observed for PTs expressing cytoplasmic GFP, almost all (27 of 30) ARO1-GFP expressing PTs showed an initial lag phase of growth after germination. During this lag phase, including bulging and transition phase, ARO1-GFP signal intensity strongly increased at the distal end of the bulge/dome-shaped tip in 21 of 27 PTs. Nine minutes after ARO1-GFP reached its maximum signal intensity at the tip of the tube, PT elongation rates reached their maxima, recognized by the rapid increase in PT area over time (**Figure 4D**). By contrast, during the first 9 min of the bulging phase 22 of 30 bulges substantially expanded while the accumulation of ARO1-GFP decorated vesicles at the distal end of the bulge was delayed, suggesting that bulging does not depend on pronounced vesicle trafficking to the very tip of the bulge.

### Patterns of Abnormal PT Growth Correlate with Deviating ARO1-GFP Signals

Using our multi-well micro-germination setup a high number of pollen germinated and thus we were able to observe very rare events (less than 3.3%) of abnormal PT growth, such as bulging without subsequent elongation (**Figures 5A,B**), the initiation of a second PT from one grain (**Figures 5C,D**) or the branching of a PT (**Figures 5E,F**).

In the first case of a PT that did not switch to the phase of rapid PT elongation, a high intensity peak of ARO1-GFP signals appeared 24 min before germination at the future site of PT outgrowth (**Figures 5A,B**). During germination a roundish PT bulge was formed showing isodiametric expansion and constant increase in ARO1-GFP fluorescence intensity with a maximum 18 min after germination. However, during the

following 12 min ARO1-GFP fluorescence rapidly decreased to only 10%, detected 30 min after germination. The decrease in ARO1-GFP signal intensity was accompanied with arrested PT growth (**Figures 5A,B**).

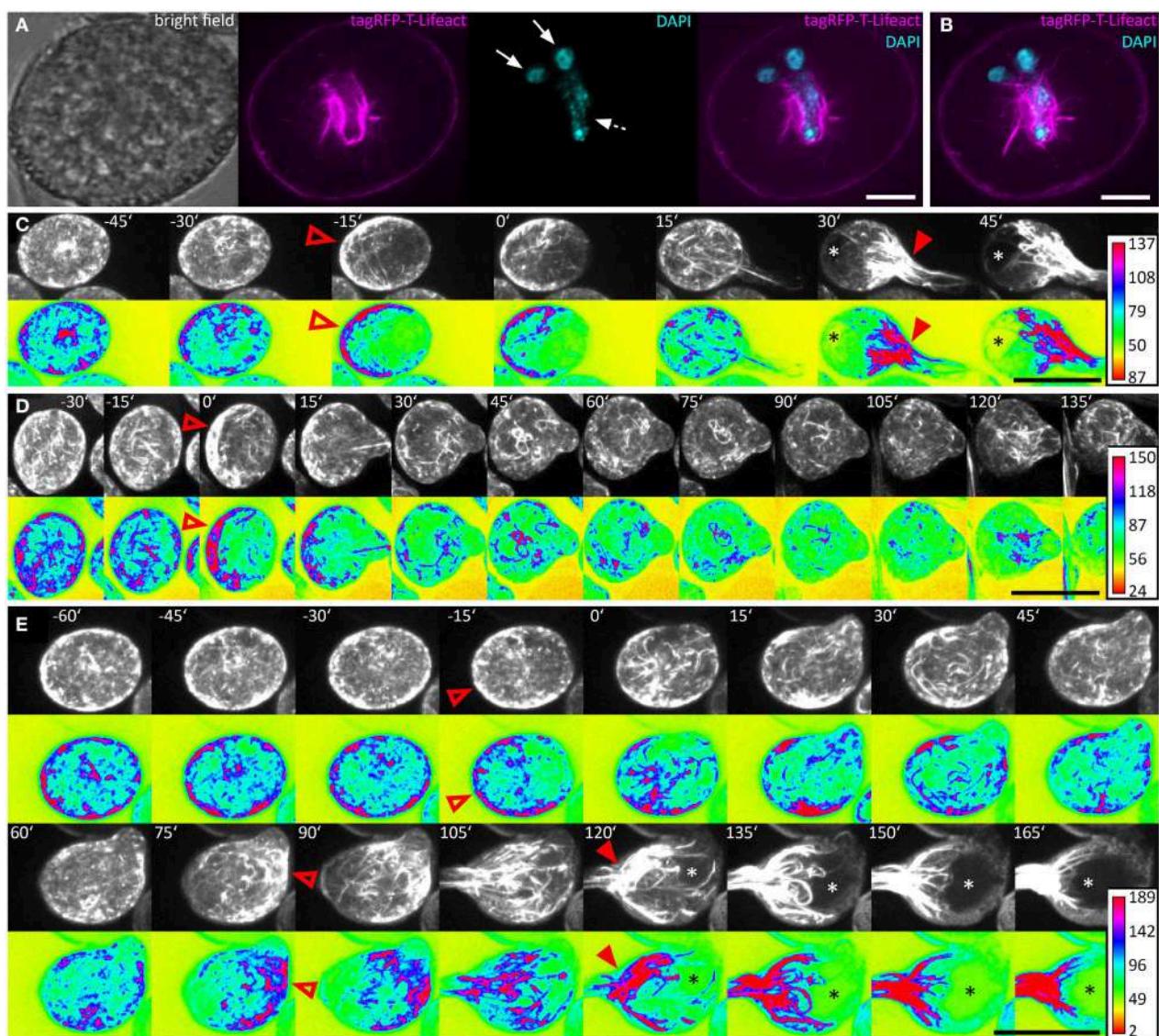
In one pollen grain a second tube was established during germination (**Figures 5C,D**). A sharp ARO1-GFP intensity maximum appeared subjacent to the future site of PT outgrowth, 12 min before pollen germination, the area of the bulge increased after pollen germination and ARO1-GFP accumulated in the bulge. However, 21 min after germination, ARO1-GFP fluorescence rapidly decreased and during the following 45 min neither the PT area increased, nor was any ARO1-GFP intensity maximum observed. Sixty nine minutes after first bulging, a new ARO1-GFP intensity maximum arose within the pollen grain, followed by another peak at the same site 45 min later. Nine minutes after the second ARO1-GFP fluorescence maximum the pollen grain started to germinate at this site. After bulging, ARO1-GFP steadily accumulated at the distal end of the second bulge and rapid PT elongation was successfully initiated (**Figures 5C,D**).

In the case of a branching PT (**Figures 5E,F**), two maxima of ARO1-GFP intensity occurred 24 and 15 min before pollen germination. During the following phase of bulging, ARO1-GFP signal intensity at the distal end of the bulge steadily increased and reached a maximum in the remarkable long transition phase, 36 min after germination. However, the switch to rapid PT growth did not occur at this site but a second growth site was selected, marked by ARO1-GFP signals appearing at the tip of the branching PT (**Figure 5E**; 42 min). During the following 6 min ARO1-GFP signal intensity at the first tube tip rapidly decreased while the PT branch expanded. Nine minutes after the PT initiated branching, another ARO1-GFP intensity maximum was detected in the new tip of the PT, while the signal in the old tip diminished (**Figures 5E,F**; 51 min).

### The Actin Cytoskeleton Polarizes Prior to Germination and Undergoes Characteristic Changes during PT Growth

We used the *ARO1* promoter to drive moderate expression of the tagRFP-T-Lifeact fusion protein in pollen. DAPI staining was performed to visualize the nuclei of the vegetative cell and the sperm cells in pollen grains. Immediately after pollen mounting, the pollen actin cytoskeleton was not distributed with any polarity and showed homogenous accumulation in the cell periphery and pronounced fluorescent signals around the vegetative nucleus (**Figures 6A,B**), which has also been reported for mature *Brassica napus* pollen (Hause et al., 1992; Gervais et al., 1994).

We germinated tagRFP-T-Lifeact expressing pollen in our micro-germination setup and observed that within half an hour



**FIGURE 6 |** The actin cytoskeleton undergoes characteristic changes during pollen germination. Pollen expressing tagRFP-T-Lifeact was used for live-cell imaging of F-actin architecture and dynamics during pollen germination and growth. A single optical slice through a representative DAPI stained pollen grain is shown in (A). Solid arrows mark the sperm cells and dashed arrows the vegetative nucleus. A composite maximum intensity projection of 19 optical slices is shown in (B), the typical pattern of actin dynamics during pollen germinating is presented in (C). A pronounced F-actin network accumulates at the periphery of the pollen vegetative cell, opposite to the future site of PT outgrowth (open arrowhead). Rapid PT tip growth is associated with

vacuole formation (asterisk) and the massive appearance of parallel F-actin bundles extending from within the pollen grain, into the pollen tube (solid arrowheads). In (D) a PT that failed to switch to rapid PT tip growth after bulging is shown. The germination of a second PT after first unsuccessful bulging is shown in (E). Maximum intensity projections of raw images and intensity based false-colored images are shown in (C–E) with respective calibration bars. Open red arrowheads indicate polar F-actin accumulation at the periphery of the pollen vegetative cell. Solid red arrowheads point at massively accumulating F-actin bundles that extend into the PT. Asterisks mark vacuoles. Time point 0' indicates the last frame before germination. Scale bars: (A) 10  $\mu$ m, (B) 5  $\mu$ m (C–E) 25  $\mu$ m.

before germination F-actin accumulated at the periphery of the pollen vegetative cell, opposite to the future site of PT outgrowth (**Figures 6C–E**). Almost all (35 of 38) pollen grains showed this pattern of F-actin polarization before germination.

Within 15 min after pollen germination, we observed an increase in longitudinal actin cable formation pointing toward the PT axis and partially reaching into the tube (**Figure 6C**).

Very articulate actin reorganization appeared in all investigated PTs around 30 min after germination, when the PTs reached a mean size of  $300 \pm 17 \mu\text{m}^2$ . The actin cytoskeleton assembled at the site of PT outgrowth forming prominent longitudinal F-actin bundles that reached from the pollen grain into the PT. The formation of a large vacuole opposite to the germination site was observed simultaneously with the prominent F-actin assembly

near the site of PT outgrowth (**Figure 6C**). In later stages of PT growth, this dense assembly of F-actin cables was shifted into the PT (**Supplemental Movie 4**).

Again, we looked for exceptional germination scenarios and identified a PT that stopped growth after bulging (**Figure 6D**) and a PT that initiated a second tube from one pollen grain (**Figure 6E**). In the case of PT growth arrest after bulging, the F-actin polarized prior to germination at the pole opposite to the germination site and longitudinal actin cables reaching from the pollen grain into the PT bulge were present 15 min after germination. However after 30 min, the actin cytoskeleton started to depolarize and transition to rapid tip growth was not initiated (**Figure 6D**).

In the case of additional tube formation from one pollen grain, 15 min before germination the actin cytoskeleton accumulated at the periphery, opposite to the future site of PT outgrowth but after bulge formation, the F-actin almost completely depolarized until 60 min after the first germination (**Figure 6E**). F-actin repolarization was observed 75 min after the first germination event, opposing to the site where the second tube bulged later on. Fifteen to thirty minutes after the second F-actin polarization was observed at the periphery of the pollen vegetative cell, a second bulge was formed and underwent transition to rapid tip growth, showing all F-actin features of a normal growing PT.

### Sperm Cell Transport Starts When the Switch to Rapid Tip Growth Has Taken Place

We used a marker line showing RFP fluorescence in the sperm cell nuclei and GFP fluorescence in the sperm cell plasma membranes to investigate whether the two sperm cells are relocated into the PT at a distinct growth phase (**Supplemental Movie 5**). The GFP-labeled sperm cell membranes show that the two sperm cells are closely interlinked and that one long membrane extension connects one of the sperm cells to the nucleus of the vegetative cell (**Figure 7**), thereby forming a transport unit known as the male germ unit (MGU). In *Arabidopsis*, the entrance of the three MGU components into the PT follows a regular order, both *in planta* (Lalanne and Twell, 2002) and *in vitro* germinated

pollen (Zhou and Meier, 2014): the vegetative nucleus always precedes the sperm cells during entrance into the PT (Lalanne and Twell, 2002; own observations).

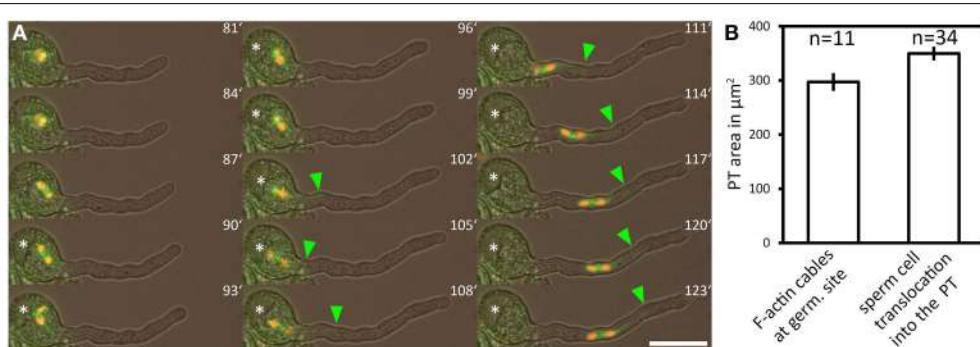
The tip of the GFP labeled long membrane extension of the leading sperm was used as a tracer for the position of the vegetative nucleus as it is hooked up to the vegetative nucleus. We defined the time point of MGU relocation into the PT when the tip of the sperm membrane extension became permanently visible outside the pollen grain (**Figure 7A**, green arrowhead). From 34 PTs we calculated a mean PT size of  $350 \pm 13 \mu\text{m}^2$  at the time point of MGU translocation into the PT (**Figure 7B**), indicating that the sperm cell transport into the PT does not occur at random but when the PT has reached a certain length and growth phase. Two processes associated with rapid PT tip growth, the formation of a large vacuole within the pollen grain and the accumulation of prominent F-actin cables at the base of the growing PT (**Figure 7B**), have already taken place when we detected the sperm membrane extension in the PT.

## Discussion

The PT is an attractive model for the analysis of tip growth mechanisms on the molecular and cellular level, especially in plant species amenable to forward genetic screens and with excellent genomic and bioinformatic resources such as *Arabidopsis thaliana*. Nevertheless, quantitative imaging of growing *Arabidopsis* PTs remained challenging, due to highly variable pollen germination rates in different experiments.

Here, we describe an inexpensive and easy mounting technique to simultaneously track germinating pollen from up to 12 genetically different plants. Our multi-well germination-slide with modified pollen germination medium yields high germination percentages and allows live cell imaging and subsequent quantitative image analysis of the whole process of *Arabidopsis* pollen activation, germination, and the establishment of polar tip growth.

By using our setup and pollen from different fluorescent marker lines we were able to precisely describe the kinetics of



**FIGURE 7 |** The male germ unit is transported into the PT after the transition to rapid tip growth. A marker line labeling both sperm nuclei (red fluorescence) and the sperm plasma membrane (green fluorescence) was used for time lapse imaging of pollen germination. **(A)** Green arrowhead points to the tip of the long sperm cell membrane extension physically

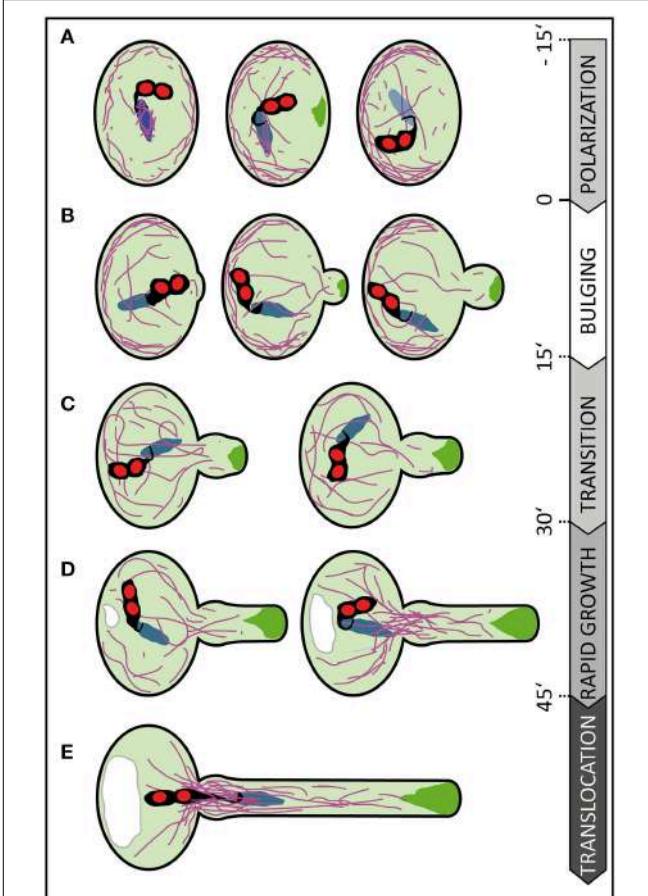
associated to the vegetative cell nucleus, which has been transported into the pollen tube. Bar plots in **(B)** show average PT areas when F-actin bundles massively accumulate at the germination site (indication for rapid tip growth), compared to the mean PT area when the male germ unit is relocated into the PT. Scale bar in **(A)**:  $25 \mu\text{m}$ .

Arabidopsis pollen germination *in vitro*. We expressed tagRFP-T-Lifeact in pollen to investigate F-actin dynamics during pollen activation, germination and tube growth, as Lifeact has become the actin marker of choice in the PT (Qu et al., 2015). Vesicles within the pollen grain and the germinating PT were visualized by ARO1-GFP (Gebert et al., 2008). The accumulation of ARO1-GFP in the apical region of growing PTs and the rapid dissipation of this tip localization by brefeldin A treatment is reminiscent of YFP-RabA4d, an exocytotic vesicle marker of PTs (Lee et al., 2008; Szumlanski and Nielsen, 2009), and of GFP-Rab11b-tagged vesicles in tobacco PTs (de Graaf et al., 2005; Cheung and Wu, 2008). Transport vesicles in the tip of angiosperm PTs are known to follow a reverse fountain-streaming pattern (for review see Bove et al., 2008; Cheung and Wu, 2008; Chebli et al., 2013), as is the case for ARO1-GFP (**Supplemental Movie 2**). By TIRF microscopy, a method that has been successfully used to image secretory vesicles in *Picea meyeri* PTs (Wang et al., 2006), we were able to show that ARO1-GFP is associated to vesicles in the PT tip. The size of the punctate ARO1-GFP signals was approximately 200 nm in diameter, which is very close to the calculated size of 182 nm described for vesicles in Arabidopsis PTs (Ketelaar et al., 2008). Based on the vesicle-like appearance of ARO1-GFP in TIRF microscopy, its reverse fountain-streaming pattern and the BFA sensitive tip localization we conclude that ARO1-GFP shows a bona-fide vesicle association in the tip of growing PTs.

### Germinating Arabidopsis Pollen Reveal Characteristic Tube Morphologies and Growth Kinetics, Accompanied with F-actin and Vesicle Polarization

Tip growing cells confine cellular expansion to a small area. The occurrence of a single growth site includes at least two distinct phases: the initiation of growth and the elongation phase (Geitmann, 2010). Our quantitative imaging of tube morphologies and growth kinetics enabled us to dissect the early events of germination and to define characteristic features associated with distinct phases (**Figure 8**). We observed successive phases of cell polarization before germination, bulge formation at the beginning of PT germination, the transition to polar growth and subsequent initiation of rapid tip growth.

The germination phase is characterized by an emerging PT that shows isodiametric expansion at the germination site. Some longer F-actin bundles become visible during that phase, extending from within the pollen grain into the bulge (**Figure 8B**). The growth rate of the expanding bulge, measured as increase in area over time, is rather slow during the first 15 min ( $16.7 \pm 1.1 \mu\text{m}^2$ ), compared with later tube growth of  $57.0 \pm 4.0 \mu\text{m}^2$  at 45–60 min after germination. In our experimental setup this slower growth phase persists on average 30 min and includes the transition phase (**Figure 8C**) in which the bulge slightly elongates and adopts a dome-shaped form before switching to the phase of rapid tip growth. Notably, we observed that the bulge expands while the accumulation of ARO1-GFP associated vesicles at the distal end of the bulge is slightly delayed. The fact that the Arabidopsis PT starts forming a uniformly expanding bulge before vesicles massively accumulate at the future site of polar



**FIGURE 8 | Scheme summarizing subcellular changes observed during different phases of pollen germination and tube growth.** At least five distinct phases were recognized in our live cell imaging studies on *in vitro* germinating Arabidopsis pollen. **(A)** In early rehydrating pollen F-actin is uniformly distributed at the pollen cell cortex and forms prominent bundles around the vegetative nucleus. Polarization of the pollen grain is indicated by ARO1-GFP decorated vesicles, transiently accumulating subjacent to the future germination site approximately 3–20 min prior germination, and by F-actin accumulating at the cell periphery, in the half of the pollen vegetative cell opposite to the later germination site. **(B)** During the following bulging phase a local protuberance becomes visible, showing isodiametric expansion. ARO1-GFP decorated vesicles start to accumulate in the bulge and first longitudinal F-actin bundles extend from the grain into the bulge. **(C)** The transition phase is indicated when the bulge becomes slightly tubular-shaped. Transition to tip growth is accompanied by a strong accumulation of ARO1-GFP decorated vesicles in the shape of an inverted cone and by the reorganization of the actin cytoskeleton. The polar dense F-actin at the cell periphery of the pollen grain dissipates and long actin bundles, often oriented toward the emerging PT, arise. **(D)** During the subsequent phase of rapid tip growth the PT area increases significantly. A vacuole is formed in the pollen grain, across from the germination site and F-actin bundles start to extend from within the pollen grain into the pollen tube. The accumulation of ARO1-GFP-decorated vesicles in the very tip of the growing pollen tube is most pronounced. **(E)** The translocation phase is initiated when the MGU becomes transported into the growing pollen tube. Sperm cell translocation is preceded by the formation of massive parallel F-actin bundles at the germination site. The vacuole in the pollen grain rapidly enlarges. Objects are not to scale. Color code: purple lines, F-actin; green areas, ARO1-GFP; red areas, sperm cell nuclei; black areas surrounding sperm cell nuclei, sperm cell membranes and cytoplasm; blue area, vegetative cell nucleus. Numbers indicate approximate time points for each phase before or after germination in minutes.

growth suggest that the bulging phase represents a rather turgor-driven deformation process, like assumed by Geitmann (2010). It furthermore indicates that the transition phase was preceded by the selection of a defined plasma membrane region for local exocytosis within the bulge. Thus, the burst of PTs soon after germination, especially observed in a number of pollen mutants such as *aro1-3* and *seth4* (Lalanne et al., 2004; Gebert et al., 2008), may be a turgor-driven event when the establishment of a local growth site was unsuccessful.

ARO1-GFP labeled vesicles heavily accumulate at the distal end of the bulge and finally adopt an inverted cone-like shape (**Supplemental Movie 3**). In elongating PTs the inverted cone-shaped zone at their apex is referred to as the “clear zone,” because this region is almost exclusively occupied by vesicles but lacks refracting starch containing amyloplasts (Hepler and Winship, 2015). The establishment of the vesicle-rich “clear zone” depends on acto-myosin-dependent long distance transport of vesicles toward the tip of the PT. This transport is mediated by F-actin cables, which are oriented parallel to the longitudinal axis of the PT and a cortical network of fine filaments located in the subapical region of the cell (Cai and Cresti, 2009; Chebli et al., 2013). Cortical actin filaments in the shank of angiosperm PTs are believed to be oriented with their barbed ends toward the apex, while the central actin bundles are thought to comprise filaments with the barbed ends pointing backwards (Chebli et al., 2013). The resulting reverse fountain-like cytoplasmic streaming observed in angiosperm PTs is likely to be involved in maintaining the “clear zone” by producing a constant shear between the anterograde and retrograde transport lanes, by which many of the vesicles, especially those near the surface of the inverted cone, will re-enter the tipward lanes and flow back to the apex (Hepler and Winship, 2015). Thus, the local accumulation and inverted cone-shaped appearance of ARO1-GFP labeled vesicles at the end of the bulging phase indicate that a distinct growth site has been selected, which also becomes apparent by the change in PT morphology and the increase in the median growth rate in the following transition phase (**Figure 8C**).

When rapid tip growth is initiated (**Figure 8D**), longitudinal actin cables extend from the pollen grain toward the apex of the PT and the volume of the vacuole opposite the germination site continuously increases. We observed the appearance of massive F-actin bundles near the germination site, extending from the pollen grain into the PT, when the PT area reached the average size of  $300 \mu\text{m}^2$ . Likewise, Rhodamine-phalloidin staining of *Pyrus communis* PTs showed articulate staining of actin at the PT base and of cables ranging into the tube (Tiwari and Polito, 1988). Notably, the male germ unit, comprising the two sperm cells associated to the vegetative cell nucleus, is transported from the pollen grain into the PT only when the PT completed its transition to rapid tip growth. In our experimental setup we detected the long membrane extension connecting the leading sperm cell to the vegetative nucleus in the PT when its area is  $350 \pm 13 \mu\text{m}^2$  (**Figure 8E**), which equals to a PT length of  $63 \pm 2.3 \mu\text{m}$ . Zhou and Meier (2014) determined a PT length of approximately  $35 \pm 10 \mu\text{m}$  when the vegetative cell nucleus permanently enters the PT. This difference may

be attributed to a different experimental setup but also to the fact that Zhou and Meier (2014) used a marker line with a mCherry-labeled vegetative nucleus rather than labeled sperm nuclei and membranes as we did. While sperm cell nuclei are spheres, the vegetative nucleus is elongated and irregularly shaped and can reach a remarkable length ( $>20 \mu\text{m}$ ) in the growing PT.

## New Insights into *Arabidopsis* Pollen Activation, Provided by Live Imaging of Vesicle Dynamics and F-actin

Most pollen grains are metabolically quiescent and highly desiccated (Edlund et al., 2004). They need to attain a certain degree of hydration before they germinate, which will increase the turgor and transform the unpolar pollen grain to a highly polarized cell, a process termed pollen activation. In the past many studies were performed on morphological and ultrastructural changes in activated pollen revealing that, inter alia, the grain starts to organize its cytoskeleton and endoplasmic reticulum, and forms secretory vesicles (Raghavan, 1997 and references cited therein). Depending on the species examined, PTs either grow out of preformed germinal pores (apertures) or break directly through the exine wall, as is the case with *Arabidopsis* pollen grains. The presence of cytoplasmic vesicles subjacent to the aperture was detected by ultrastructural studies on pollen from *Lycopersicum peruvianum*, *Nicotiana alata* and *Narcissus pseudonarcissus* L. (Cresti et al., 1977, 1985; Heslop-Harrison and Heslop-Harrison, 1992). However, it is not yet clear how the pollen perceives external polarization signals and how they are transduced to select the site for tube emergence.

It was reported that the cytoplasmic  $\text{Ca}^{2+}$  concentration in *Arabidopsis* pollen increases at the potential germination site soon after hydration (Iwano et al., 2004) and that *in vitro* germination involves the formation of a “germination plaque” at the future site of tube emergence, containing cellulose, callose, pectin, and at least partly de-esterified pectin (Hoedemaekers et al., 2015). When we performed our live cell imaging on the dynamics of ARO-GFP1 labeled vesicles in hydrating pollen grains we observed the initial appearance of weak transient ARO1-GFP signals, arising at various areas of the pollen cell periphery. However, approximately 3–20 min before germination either one or two very strong fluorescent peaks of ARO1-GFP labeled vesicles appeared in the region where the PT protoplast will break through the exine, suggesting targeted vesicle secretion and probably local softening of the cell wall at this site. This would be in line with previous assumptions that vesicles filled with cell wall material and cell wall-modifying enzymes are directed toward the future emergence site to produce a local weak point at which the turgor-driven bulge formation is initiated afterwards (Krichevsky et al., 2007; Geitmann and Ortega, 2009; Cai et al., 2011).

The polarization of the actin cytoskeleton toward the site of tube emergence has been reported in activated *Pyrus communis* pollen (Tiwari and Polito, 1988) by using rhodamine-phalloidin labeling. Similar observations were made by TRITC-phalloidin staining for actin in hydrated *Narcissus pseudonarcissus* pollen (Heslop-Harrison and Heslop-Harrison, 1992). Notably, we

did not observe a similar pattern of polarization in our live imaging setup with hydrating *Arabidopsis* pollen grains expressing tagRFP-T-Lifeact: F-actin mainly accumulated at the cell periphery opposite to the future germination site and no conspicuous polarization toward the site of tube emergence was observed before PT bulging. We assume that the spatial configuration of actin arrays at the periphery of the other half of the activated *Arabidopsis* pollen grain may form a mechanical counter-bearing for the turgor-driven PT bulging.

Species-dependent variations in F-actin polarization during pollen grain activation would be conceivable, on the other hand previous reports using actin-binding proteins or their actin-binding domains have shown that each F-actin marker produces a different labeling pattern (Thomas et al., 2006; Wilsen et al., 2006; Cheung et al., 2008). However, the distribution of the actin cytoskeleton in hydrating *Arabidopsis* pollen grains by fluorescent phalloidin has, to our knowledge, not been investigated in detail and will be difficult to interpret without having any information about the cellular dynamics before and after the moment of fixation.

## Conclusions

Our live imaging studies on germinating *Arabidopsis* PTs using the described mounting technique revealed characteristic growth phases and kinetics, together with specific spatiotemporal changes in vesicle transport and actin cytoskeletal organization. The method presented here allows the phenotypic assessment of larger numbers of *in vitro* germinating *Arabidopsis* pollen from wild type and mutant plants by live imaging. It facilitates the analyses of morphological alterations and growth kinetics, and the identification and subcellular localization of players contributing to cell polarity formation and growth site selection in germinating pollen.

## Author Contributions

FV and SS designed the experiments, SS directed the project. Generation of constructs and transgenic plants, single- and multi-well micro-germination setup establishment, Spinning Disc microscopy, quantitative image analysis and statistics was carried out by FV. TIRF microscopy was performed by SK. FV wrote the manuscript with input from SK and SS.

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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.2015.00246/abstract>

**Supplemental Movie 1 | Time lapse imaging of pollen germinating in the multi-well micro-germination slide.** Imaging of ARO1-GFP expressing pollen in an exemplary 10 well micro-germination setup was performed over 6 h. Note that position B1 was readjusted after 1 h PT growth. Time stamper shows hours and minutes. Scale bar: 50 μm.

**Supplemental Movie 2 | Vesicle-associated ARO1-GFP is enriched in the inverted cone-shaped “clear zone” of the pollen tube tip and shows reverse fountain streaming pattern.** Time lapse Spinning Disc confocal imaging of ARO1-GFP at the PT tip. Royal LUT was used to highlight increasing signal intensities (low to high: blue to yellow to red to white). Time stamper shows minutes and seconds. Scale bar: 5 μm.

**Supplemental Movie 3 | Polar vesicle accumulation during pollen germination and PT tip growth, indicated by ARO1-GFP intensity changes.** Time lapse imaging of a representative pollen expressing ARO1-GFP before germination, during bulging and PT growth. PT outlines are drawn in cyan, ARO1-GFP low-intensity threshold in red (70% highest intensities) and signal maxima in yellow (0.5% highest intensities). White arrowheads point to ARO1-GFP maxima in the ungerminated pollen grain, green arrows indicate ARO1-GFP maxima after germination. Time stamper shows minutes pre/post germination. Scale bar: 10 μm.

**Supplemental Movie 4 | F-actin dynamics during pollen germination and PT tip growth.** Time lapse imaging of tagRFP-T-Lifeact expressing pollen before germination and during PT growth. Solid arrowheads highlight F-actin assembly at the periphery of the pollen cell, opposite to the later germination site. Empty arrowheads point at the massive accumulation of F-actin bundles at the PT base, during rapid PT growth. Time stamper shows minutes pre/post germination. Scale bar: 15 μm.

**Supplemental Movie 5 | Sperm cell translocation into the growing PT.** Time lapse imaging of germinating pollen with fluorescently labeled sperm cell nuclei and plasma membranes. Solid arrowheads highlight the sperm plasma membrane protrusion that connects one sperm cell to the vegetative cell nucleus. Time stamper shows minutes pre/post germination. Scale bar: 15 μm.

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# Epigenetic landscape of germline specific genes in the sporophyte cells of *Arabidopsis thaliana*

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In plants, the germline lineages arise in later stages of life cycle as opposed to animals where both male and female germlines are set aside early in development. This developmental divergence is associated with germline specific or preferential expression of a subset of genes that are normally repressed for the rest of plant life cycle. The gene regulatory mechanisms involved in such long-term suppression and short-term activation in plant germline remain vague. Thus, we explored the nature of epigenetic marks that are likely associated with long-term gene repression in the non-germline cells. We accessed available *Arabidopsis* genome-wide DNA methylation and histone modification data and queried it for epigenetic marks associated with germline genes: genes preferentially expressed in sperm cells, egg cells, synergid cells, central cells, antipodal cells or embryo sac or genes that are with enriched expression in two or more of female germline tissues. The vast majority of germline genes are associated with repression-related epigenetic histone modifications in one or more non-germline tissues, among which H3K9me2 and H3K27me3 are the most widespread repression-related marks. Interestingly, we show here that the repressive epigenetic mechanisms differ between male and female germline genes. We also highlight the diverse states of epigenetic marks in different non-germline tissues. Some germline genes also have activation-related marks in non-germline tissues, and the proportion of such genes is higher for female germline genes. Germline genes include 30 transposable element (TE) loci, to which a large number of 24-nt long small interfering RNAs were mapped, suggesting that these small RNAs take a role in suppressing them in non-germline tissues. The data presented here suggest that the majority of *Arabidopsis* gamete-preferentially/-enriched genes bear repressive epigenetic modifications or regulated by small RNAs.

**Keywords:** epigenetic, germline, gene repression, histones, gametophyte, sporophyte

## Introduction

In land plants, the sexual structures are developed late in their life-cycle since the gamete-holding organs are initiated on the fully developed mature sporophyte. In contrast, metazoans separate their germline cell lineage very early following gametic fusion. For example, in humans the primordial germ cells developmental fate is established less than a week after fertilization (Richardson and Lehmann, 2010). Flowering plants maintain a population of stem cells that differentiate into

various vegetative tissues for the most part of their life but also into the reproductive organs after alteration to the gametophyte phase (reviewed in Berger and Twell, 2011). The specification of maleness and femaleness involves an orchestration of various regulatory mechanisms, and our understanding of such complex gene regulation has improved in recent years (reviewed in Armenta-Medina et al., 2011; Twell, 2011 and Berger and Twell, 2011). Following fusion of gametes through fertilization, plants return to the sporophytic life phase. The alternation between sporophyte and gametophyte generation in the plant's life-cycle implies that genes that are specifically down-regulated or up-regulated in gametes have to be inversely activated or silenced within the non-germline tissues of the dominant phase sporophyte. This silencing and activation require complex and fine-tuned regulatory mechanisms.

Gene regulation contributing to tissue specificity can occur directly through transcription or indirectly by post-translation modification on histones and DNA methylation. Haerizadeh et al. (2006) identified the germline-restrictive silencing factor (GRSF) that specifically represses a sperm cell gene in non-germline cells of lily. However, gene regulation by transcription factors is one type of regulatory mechanism. Gene expression modulation at the transcription level also encompasses epigenetic regulation that leads to changes on the DNA or histone status to block or guide the expression of target genes in the locus vicinity of the changes. Epigenetics is the study of traits, which are defined as "stably heritable phenotypes resulting from changes in a chromosome without alterations to its DNA sequences" (Berger et al., 2009). DNA 5-methyl-cytosine modification (DNA methylation; 5mC) has a repressive nature while histone moieties can have either a positive or a negative effect on gene regulation at the locus where the histone modification occurred. The types of covalent modification on histones vary. Specific acetylated forms, such as H3K9ac, H3K18ac, and H3K27ac or the ubiquitination of H2Bub are epigenetic marks that lead to gene activation, while histone methylation has a less define response on gene expression. As such, the specific methylated amino acid, its hypermethylated state or the surrounding epigenetic context can all lead to a different gene activation/repression status (Liu et al., 2010). In some instance, histone methylation can acts as a repressive mark as it is the case for H3K9me2, H3K27me1, and H3K27me3, or it can be interpreted as a activator mark when found on H3K4me3, H3K9me3, H3K36me2, and H3K36me3. The gene regulation of some histone methylation marks, like H3K4me1 and H3K4me2, is modulated by the other epigenetic marks at that same locus. As such, H3K4me1 and H3K4me2 can act as either a repressive or an activating mark depending on the neighboring epigenetic context. Another type of epigenetic control is emphasized through the use of histone variants, as shown by promoters with a H3.3 variant enrichment that are transcriptionally more active (Shu et al., 2014).

Advances in microarray and DNA-sequencing technologies have allowed the recent expansion of epigenetic modification analyses on many model organisms. Whole-genome tiling array technologies were utilized for not only gene expression profiling including alternative splicing but also the investigation of DNA-methylation (Mockler et al., 2005; Gregory et al., 2008), which

have a conserved role in silencing gene expression (Martienssen and Colot, 2001). Microarray technology was also applied to the post-translational modifications of histones (Zhu et al., 2001; Dindot et al., 2009; Moghaddam et al., 2011). Next-generation ultrahigh-throughput sequencing is also actively utilized for exploring the epigenetic modifications of DNA and histone status (Cokus et al., 2008; Ma et al., 2011; Marques et al., 2011). Both methods have investigated the epigenetic characteristics of various tissues of *Arabidopsis thaliana*, making *Arabidopsis* one of the most extensively studied model plant for epigenetic studies.

Recent studies have identified a subset of flowering plant genes that show preferential or enriched expressions in germline cells (Steffen et al., 2007; Borges et al., 2008; Wuest et al., 2010; Drews et al., 2011). These genes are, by definition, suppressed or down regulated in the non-germline tissues. Although many studies have shown the relationships between the tissue-specific gene expressions and the status of epigenetic traits, the mechanisms of epigenetic suppression or down-regulation of germline genes in non-germline tissues are poorly understood. Hence, in this study, we explored the nature of epigenetic marks that are likely associated with long-term gene repression of germline genes in non-germline cells.

## Materials and Methods

### Genes with Epigenetic Marks

Genes with H3K4me1, H3K4me2, and H3K4me3 in 3-week old seedlings were identified from the genomic coordinates of these histone modification marks provided by Zhang et al. (2009). Genes with H3K9me2 (i.e., target genes of H3K9me2) in 3wk-old shoots were selected using the pre-processed sequencing data deposited in Gene Expression Omnibus (GEO) repository in NCBI, GSE12383 (Bernatavichute et al., 2008). Each sequence used in the original paper by Bernatavichute and colleagues was tagged with Z-score of log-ratio between Cy5 (H3K9me2 signal) and Cy3 (H3 signal), and those with Z-score higher than 0.2 were extracted and mapped onto TAIR9 *Arabidopsis* genome sequence using Bowtie (Langmead et al., 2009). Genes overlapping the coordinates of selected probe sequences were regarded as targets of H3K9me2. All the remaining epigenetic marks in a given tissue were extracted directly from the data of corresponding papers listed in Table 1; their respective role is indicated at the end of each epigenetic mark name: "(r)" repression-related; "(a)" activation-related; and "(a/r)" activation or repression depending on other accompanying epigenetic marks.

### *Arabidopsis* Germline Genes

Borges et al. (2008) reported 81 *Arabidopsis* genes that are preferentially expressed in sperm cells. For female germline genes, we combined the results from Wuest et al. (2010), Drews et al. (2011), and Steffen et al. (2007) and extracted 855 genes in total that exhibit preferential expression in female gamete tissues. These female genes were further classified into six groups: 165, 157, 125, 16, and 11 genes that are specifically up-regulated in egg cell, synergid cells, central cell, antipodal cells, and embryo sac, respectively, and 381 genes enriched in female gamete tissues as a whole but not in a particular tissue

**TABLE 1 | Epigenetic studies using somatic tissues of *Arabidopsis thaliana*.**

| Tissue                             | Marks   | References   |
|------------------------------------|---|--|
| Seedlings (10 days old)            | 5mC(r), H2Bub(a), H3K27me1(r), H3K27me3(r), H3K36me3(a), H3K4me2(a/r), H3K4me3(a), H3K9me2(r), H3K9me3(a)               | Turck et al., 2007; Zhang et al., 2007; Roudier et al., 2011 |
| Seedlings (3 weeks old)            | H3K4me1(a/r), H3K4me2(a/r), H3K4me3(a)  | Zhang et al., 2009   |
| Seedlings (5 days old, dark-grown) | H3K27ac(a), H3K27me3(r), H3K9ac(a), H3K9me3(a)  | Charron et al., 2009   |
| Leaves                             | H3K27me3(r), H3.3.TTS(a)*, H3.3.TTS.Promoter(a)*, H3.3.Promoter(a/r)*   | Lafos et al., 2011; Shu et al., 2014                         |
| Shoot apical meristem              | H3K27me3(r)   | Lafos et al., 2011   |
| Shoots (3 weeks old)               | H3K9me2(r)  | Bernatavichute et al., 2008                                  |
| Roots (10 days old)                | H3K27me3(r), H3K4me3(a)   | Roudier et al., 2011   |
| Aerial tissue (2 weeks old)        | H3K4me2(a/r), H3K4me3(a), H3K9Ac(a), H3K9me2(r), H3K18Ac(a), H3K27me1(r), H3K27me3(r), H3K36me2(a), H3K36me3(a), 5mC(r) | Luo et al., 2012   |

\*Genes with H3.3 mark were grouped by the position of H3.3: H3.3.TTS, H3.3 near transcription termination sites (TTS); H3.3.Promoter, H3.3 in promoter; H3.3.TTS.Promoter, H3.3 near TTS and in promoter.

(**Table 2, Supplementary Data 1**). Proportion of genes carrying specific epigenetic modification was calculated by dividing the number of cell-type genes with an epigenetic modification by the total number of genes of that cell-type. For the calculation of proportion of germline genes with epigenetic marks, germline genes in each group were considered separately (1 group for male and 7 groups for female) or combined by sex (81 male germline genes and 855 female germline genes). Statistically significance of the data was determined by using Pearson coefficient with *t*-test *p*-values.

### Small RNAs from TE Genes

Small RNA sequencing data were collected from various studies using *Arabidopsis* (Axtell et al., 2006; Kasschau et al., 2007; Montgomery et al., 2008; Fahlgren et al., 2009; Moldovan et al., 2010) and mapped to the TAIR9 *Arabidopsis* genome sequence using Bowtie (Langmead et al., 2009). Sequences that were uniquely aligned within the 30 *Arabidopsis* germline specific transposable elements (TEs) were retained and scored by their length (**Supplementary Data 2**).

### Gene Ontology Term Enrichment Test

Gene ontology (GO) term enrichment analysis was performed by goEAST with default parameters, which included multi-test adjustment using Yekutieli method (Zheng and Wang, 2008).

## Results

### Epigenetic Marks on *Arabidopsis* Germline Genes in Different Non-germline Tissues

#### Epigenetic Marks of Germline Genes in Seedlings

Seedlings are the most extensively examined material for investigating genome-wide epigenetic modifications in *Arabidopsis* (**Table 1**, epigenetic marks and references therein). Ten days old seedlings (10d-old) were examined for 5mC, H2Bub, H3K27me1, H3K27me3, H3K36me3, H3K4me2, H3K4me3, H3K9me2, and H3K9me3 (Turck et al., 2007; Zhang et al., 2007; Roudier et al., 2011), and seedlings grown under dark condition for 5 days were studied for H3K9me3, H3K27me3, H3K9ac, and H3K27ac (**Supplementary Data 3, 4**, respectively) (Charron et al., 2009). A complementary study examined H3K4me1, H3K4me2, and H3K4me3 marks in 3 weeks old (3wk-old) seedlings (**Supplementary Data 5**) (Zhang et al., 2009). Among the repression-related marks found in the non-germline tissues of the 10d-old seedling (grown under normal condition), H3K27me3 was shown to be more abundant at sperm cell-specific genes and those with enriched expression in antipodal cell-specific and central cell-specific genes (**Figure 1A**). For genes over-expressed in female germlines other than antipodal genes, the proportion of germline genes with DNA methylation (5mC) is comparable between the different female cell type genes (**Figure 1A**). In contrast, H3K9me2, another repression-related mark, is nearly absent for germline genes, while H3K27me1 (also repression-related) occupies ~20% of germline genes regardless of germline tissues in which their expression is enriched (**Figure 1A**). Among all epigenetic marks, H3K4me2, which can be related to either activation or repression depending on other accompanying epigenetic marks, is the most common epigenetic mark in 10d-old seedlings for female germline genes. However, H3K27me3 remains as the most common mark for male germline genes (i.e., those that are preferentially expressed in sperm cells) in the same tissues (**Figure 1A**). When germline genes are grouped by sex-type and compared to activity state, the majority of germline genes have one or more types of repression-related marks in 10d-old seedlings grown under normal conditions, although the fraction of female germline genes with activation-related marks is also high compared to male germline genes (**Figure 1B**).

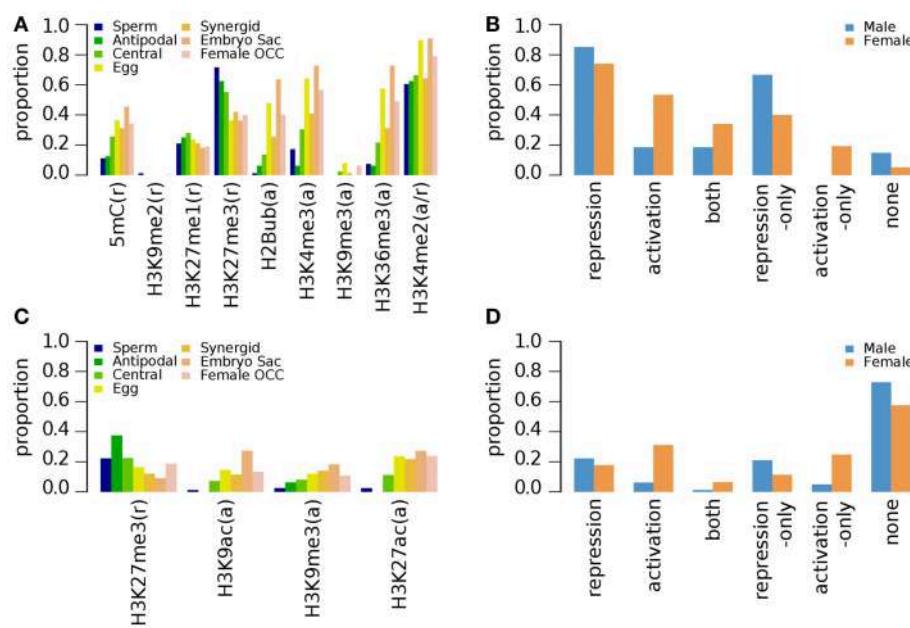
In 5 days old (5d-old) dark-grown seedlings, H3K27me3 (*t*-test *p*-value of 0.001) and H3K9me3 (*t*-test *p*-value 0.008) mark substantially less male and female germline genes (**Figure 1C**) compared to 10d-old seedlings grown in normal condition (**Figure 1A**). The fractions of germline genes marked by other epigenetic marks are also low in general in 5d-old dark-grown seedlings (**Figure 1C**), suggesting that the environmental conditions might have influenced the epigenetic modification status. Nevertheless, the fraction of germline genes having activation-related marks is still higher for female germline genes than for male germline genes in 5d-old dark-grown seedling (**Figure 1D**). Furthermore, in the 5d-old dark-grown seedling, most germline-specific genes have lost most of their regulatory histone marks as shown in **Figure 1D**.

**TABLE 2 | Germline- and gamete-specific genes of *Arabidopsis thaliana*.**

|       | Male  |     | Female        |              |                |            |             |
|-------|-------|-----|---------------|--------------|----------------|------------|-------------|
|       | Sperm | Egg | Synergid Cell | Central Cell | Antipodal Cell | Embryo Sac | Female OCC* |
| Genes | 81    | 165 | 157           | 125          | 16             | 11         | 381         |

\*Female OCC, Female Other Cell-type Combination.

Male specific genes from Borges et al. (2008); Female specific genes from Wuest et al. (2010); Drews et al. (2011); Steffen et al. (2007).

**FIGURE 1 | Distribution of DNA methylation (5mC) and specific histone modifications on *Arabidopsis* germline genes in seedlings.**

Genes were selected for their specific expression in one of six different reproductive cell type classes. **(A,B)** Epigenetic marks on germline genes in 10 day-old seedlings grown under normal light conditions. **(C,D)** Epigenetic marks on germline genes in 10 day-old seedlings grown under

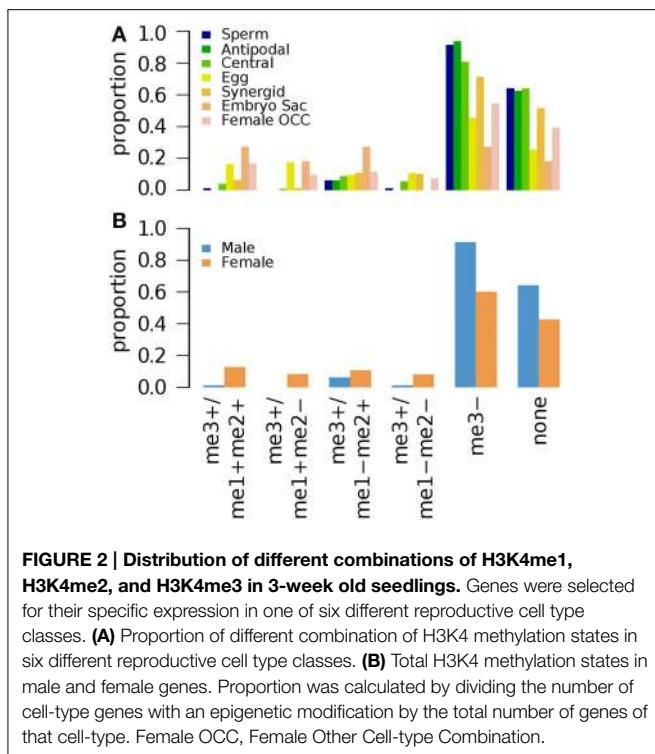
dark conditions. For **(B,D)**, epigenetic marks are grouped by their repressive or activating nature and the genes are grouped by male and female germline specific expression. Proportion was calculated by dividing the number of cell-type genes with an epigenetic modification by the total number of genes of that cell-type. Female OCC, Female Other Cell-type Combination.

*Arabidopsis* seedlings grown for 3 weeks (3wk) under normal conditions were examined for different combinations of epigenetic marks H3K4me1, H3K4me2, and H3K4me3 (Zhang et al., 2009). A small fraction of germline genes has these marks regardless of the combinations (Figure 2). Zhang et al. (2009) showed that the presence of H3K4me3 for a gene is associated with medium to high level of expression regardless of the type of other accompanying marks (none, either or both of H3K4me1 and H3K4me2). Thus, it is expected to have a small fraction of germline genes to be marked by H3K4me3 with or without H3K4me1 and/or H3K4me2 (Figure 2). We aforementioned that activation-related marks are more common for female germline genes in 5d/10d-old seedlings regardless of growth condition (Figure 1). Likewise, a larger fraction of female germline genes are marked by H3K4me3 with either or both of H3K4me1 and H3K4me2 (me3+/me1+me2+, me3+/me1+me2-, and me3+/me1-me2+) or H3K4me3 alone (me3+/me1-me2-) compared to male germline genes (Figure 2), although these proportions are generally small. Sperm genes show a depletion

of the H3K4me3 marks, where around 90% of sperm genes show an absence of this specific activator mark and around 60% of sperm genes are without any H3K4 methylation moieties (Figure 2). This was also observed at antipodal cell and central cell genes, where those female cell type genes follow sperm cell genes regulation between 10d-old seedlings and 3wk-old seedlings (Figures 1A,B, 2, respectively).

### Epigenetic Marks on Germline Genes in Aerial Tissue

A recent investigation of nine histone modification marks and DNA methylation on 2 week old (2wk-old) aerial tissue was conducted by Luo et al. (2012) (Supplementary Data 6). H3K27me3 is the most common mark among the repression-related marks for germline genes except for those with enriched expression in the female gamete as a whole (Figure 3A). Large proportion of female germline genes also has activation-related marks, some of which are marked by only activation-related marks, whereas only a small fraction of male germline genes, have activation-related marks (Figure 3B), which results in the

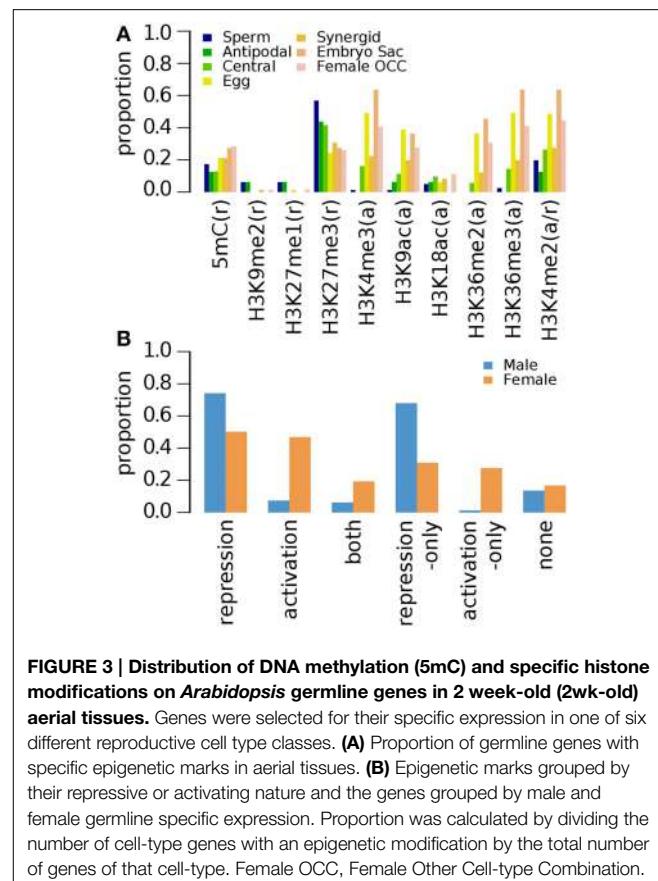


similar overall distribution of epigenetic marks as in that of 10d-old seedlings when marks are grouped by the regulatory effects and the genes were grouped by sex (**Figure 1B** compare to **Figure 3B**): Pearson's correlation coefficients: 0.98 for male (*p*-value 0.0006) and 0.89 for female (*p*-value 0.0168). The 2wk-old seedling dataset shows a relatively weak abundance of activating acetylated histone marks in all genes, but again, the depletion is more pronounced in sperm cell specific genes (**Figure 3**).

### Epigenetic Marks on Germline Genes in Roots, Shoots, Leaves and Shoot Apical Meristem

Epigenetic studies on other tissues are limited to a few marks, such as H3K27me3, H3K9me2, and H3K4me3 (**Table 1**) (**Supplementary Data 7–10**). In roots, H3K27me3 (repression-related) is associated with ~50% of sperm-preferential genes and below ~40% of female germline genes regardless of their preferred female germline tissue (**Figure 4A**). The occupancy of H3K27me3 in female germline genes in roots further decreases when all female germline genes are combined (**Figure 4B**). The activation-related mark H3K4me3 occupies less than 20% of germline genes specifically over-expressed in sperm, antipodal, central and synergid cells. However, a relatively high proportion of female germline genes specific to other germline tissues appear to have elevated the overall occupancy of H3K4me3 in female germline genes (**Figure 4**).

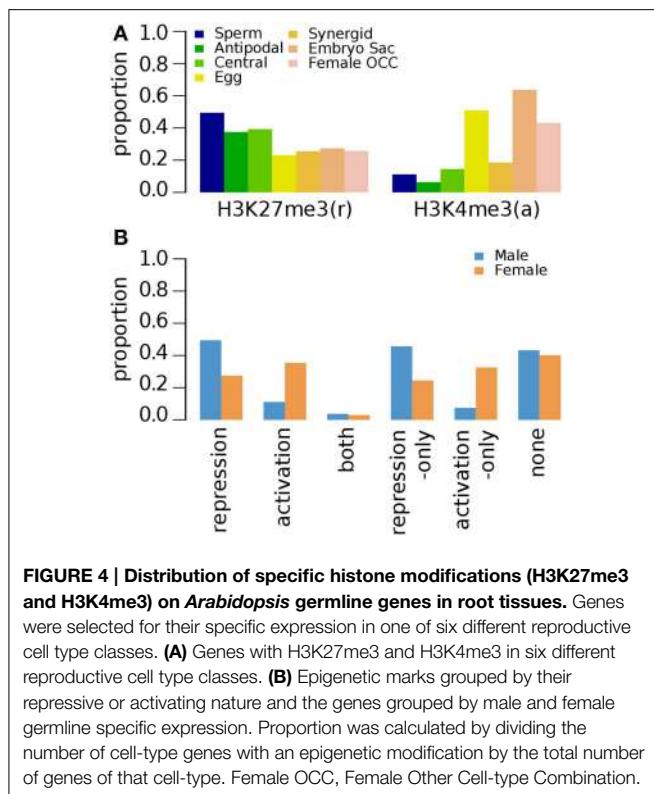
H3K27me3 modification (**Supplementary Data 11**) was similarly distributed in seedlings (10d-old), roots, shoot apical meristem and leaves, with 10d-old seedlings having the most abundant with nearly 80% occupancy (**Figure 5**). However, light-deprived seedlings have fewer germline genes marked by H3K27me3 (**Figure 5**) as previously depicted in **Figures 1C,D**.



Three week old (3wk-old) shoots were examined for H3K9me2, a repression-related mark (**Table 1**) (Bernatavichute et al., 2008). In contrast to 10d-old and 2wk-old aerial tissues where H3K9me2 is nearly absent for germline genes, majority of germline genes are marked by H3K9me2 in 3wk-old shoots, suggesting that H3K9me2 plays an important role for the down-regulation of germline genes in shoots (**Figure 6**).

### Epigenetic Marks on Germline Genes in Non-germline Tissues as a Whole

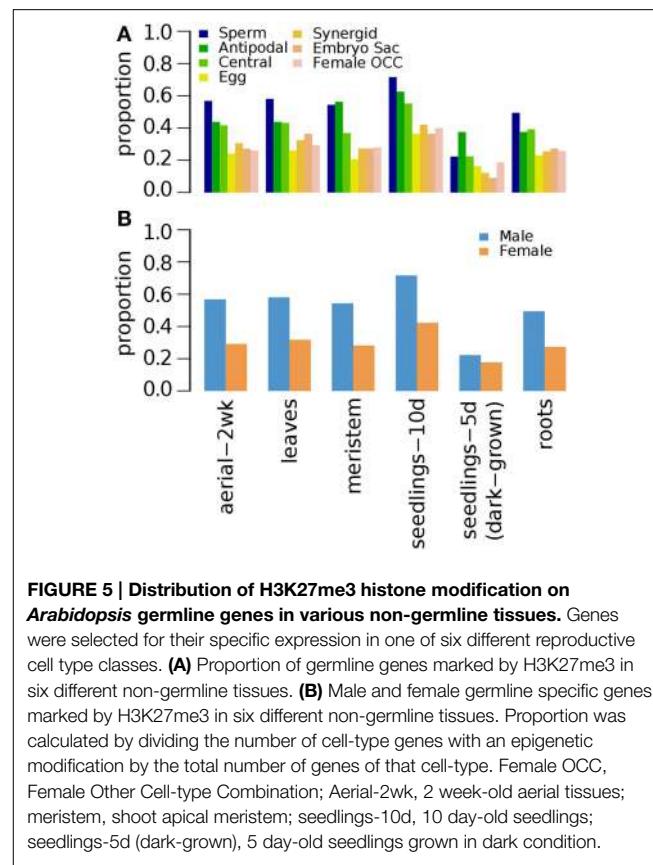
We combined the epigenetic mark information for germline genes in all examined non-germline tissues (**Supplementary Data 12**). The combined analysis of epigenetic mark data in various non-germline tissues shows that different epigenetic marks are found in different fraction of male and female germline genes in non-germline tissues as a whole but the overall trends are similar: Pearson's correlation coefficient 0.80 (*p*-value: 9.7e-5) (**Figure 7A**). As expected, all male germline genes and almost all female germline genes (97%, i.e., 830 genes) have one or more repression-related epigenetic marks in non-germline tissues (**Figure 7B**). Among the repression-related marks, H3K9me2 marks the majority of all female germline genes in one or more of non-germline tissues (**Figure 7A** and **Supplementary Figure S1**). For male germline genes, H3K27me3 is the most common repression-related mark in non-germline tissues (75%) and H3K9me2 also marks the majority



of sperm-preferential genes (73%) (**Figure 7A**). Similarly, H3K27me3 is the second most common repression-related mark on female germline genes in non-germline tissues (51%) (**Figure 7A**). Interestingly, a substantial fraction of female germline genes (74%) have one or more types of activation-related marks in non-germline tissues, and 21 female germline genes have only activation-related marks in non-germline tissues (**Figure 7B**). In comparison, the fraction of male germline genes with activation-related marks in non-germline tissue is much lower than that of female germline genes, whereas no male germline genes are marked only by activation-related marks (**Figure 7B**). The variant H3.3, known to be linked to up-regulated promoters (Shu et al., 2014), was found to have very low abundance (less than 10%) of all germline-specific genes in non-germline tissues (**Figure 7A**).

### Germline Genes with Only Repression-related Marks in Non-germline Tissues

As germline genes are found to be up-regulated only in germline tissues, a substantial fraction of the germline genes are associated with only repression-related marks: 50 out of 81 male germline genes (62%) and 222 out of 855 female germline genes (26%). GO term enrichment analysis performed by goEAST (Zheng and Wang, 2008) reveals that nine GO terms are enriched in 50 male germline genes that have only repression-related marks in non-germline tissues, which are biological processes related to gametophyte development or reproduction (**Table 3**). However, no particular GO term is enriched in 222 female germline genes that have only repression-related marks.



Among the male and female germline genes, 30 are annotated as TEs, 29 of which have one or more types of repression-related epigenetic marks (**Supplementary Data 13**). While DNA methylation is associated with less than 40% of all germline genes (**Figure 7A**), 27 germline TEs (90%) are methylated in non-germline tissues. This is consistent with the previous studies that showed the silencing of retrotransposon DNA in plants through DNA methylation (Hirochika et al., 2000; Miura et al., 2001). Along with DNA methylation, we also observed small RNAs being derived from these germline TEs in non-germline tissues. The most abundant class of small RNAs is 24-nt long small interfering RNAs (siRNAs), which is known to be over-expressed in and around transposons and retroelements in *Arabidopsis* along with 23-nt long siRNAs (Kasschau et al., 2007) (**Figure 8**). The observation of germline TEs having both DNA methylation and 24-nt long siRNAs in non-germline tissues is in agreement with the reported involvement of siRNAs in the gene silencing pathway via RNA-directed DNA methylation (Hamilton et al., 2002).

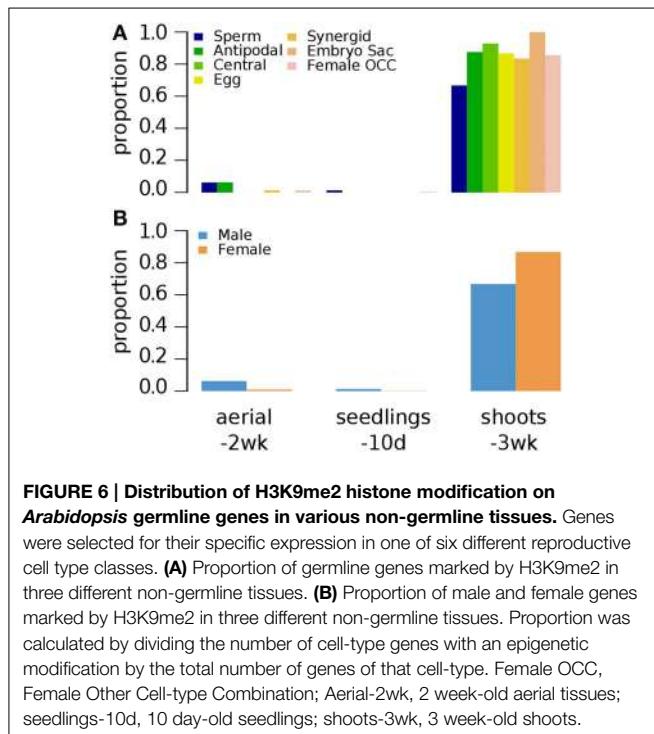
### Germline Genes with Only Activation-related Marks in Non-germline Tissues

While the repression-related epigenetic marks and small ncRNAs are likely involved in down-regulation of germline genes in non-germline tissues in an epigenetic manner, 21 germline genes are associated with only activation-related marks (**Table 4**). The most common activation-related mark is H3K4me3 (16 genes).

Interestingly, in 10d-old seedlings these 16 genes are co-marked by H3K4me2, which acts as either activation- or repression-related mark- depending on accompanied marks. Similarly, among 11 genes that have H3K4me3 in 2wk-old aerial tissue, 9 also have H3K4me2 marks.

## Discussion

Current published plant genomic studies cover only a fraction of the total known epigenetic marks (Tessarz and



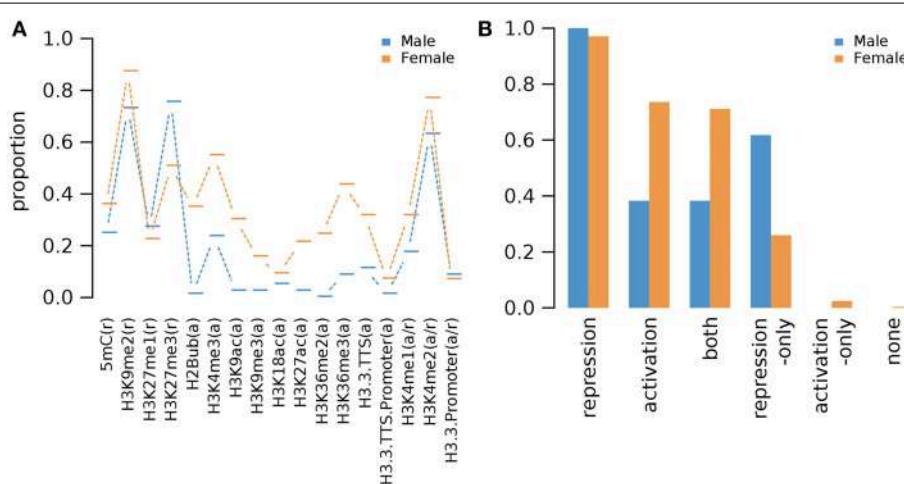
**FIGURE 6 | Distribution of H3K9me2 histone modification on *Arabidopsis* germline genes in various non-germline tissues.** Genes were selected for their specific expression in one of six different reproductive cell type classes. **(A)** Proportion of germline genes marked by H3K9me2 in three different non-germline tissues. **(B)** Proportion of male and female genes marked by H3K9me2 in three different non-germline tissues. Proportion was calculated by dividing the number of cell-type genes with an epigenetic modification by the total number of genes of that cell-type. Female OCC, Female Other Cell-type Combination; Aerial-2wk, 2 week-old aerial tissues; seedlings-10d, 10 day-old seedlings; shoots-3wk, 3 week-old shoots.

Kouzarides, 2014) while some epigenetic marks with known regulatory function have not been examined at a genome-wide level yet. Here we report the current state of knowledge regarding epigenetic regulation of germ-line specific genes. By analyzing a broad range of whole-genome studies,

**TABLE 3 | GO term enrichment analysis of male germline genes with repression-only marks in non-germline tissues.**

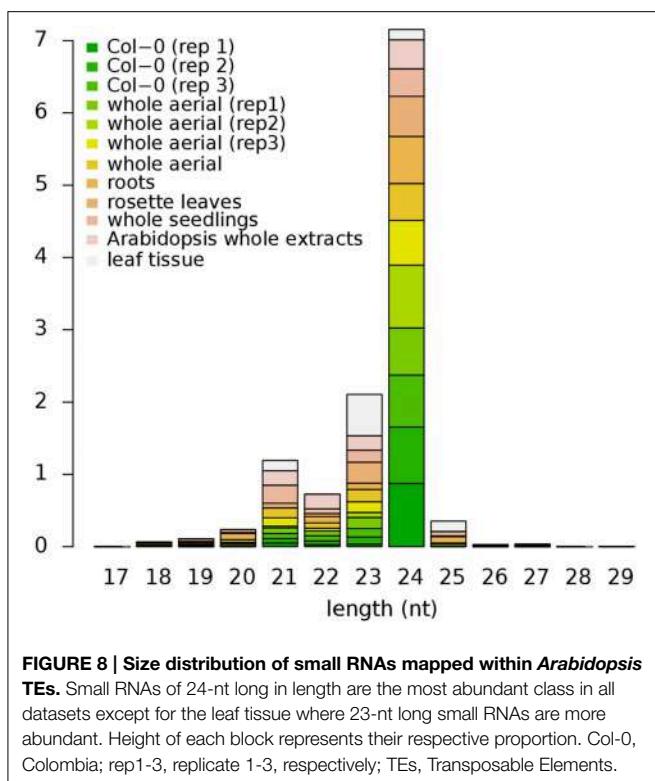
| GOID       | Term  | Gene symbols                | FDR*     |
|------------|---|-----------------------------|----------|
| GO:0048235 | Pollen sperm cell differentiation                                   | MGH3; DAZ1; HAP2; DAZ3      | 3.94E-05 |
| GO:0048232 | Male gamete generation  | MGH3; DAZ1; HAP2; DAZ3      | 6.52E-05 |
| GO:0055046 | Microgametogenesis  | MGH3; DAZ1; HAP2; DAZ3      | 7.07E-05 |
| GO:0019953 | Sexual reproduction   | MGH3; DAZ1; HAP2; DAZ3; KPL | 0.00165  |
| GO:0009555 | Pollen development  | MGH3; DAZ1; HAP2; DAZ3; KPL | 0.00613  |
| GO:0022412 | Cellular process involved in reproduction in multicellular organism | MGH3; DAZ1; HAP2; DAZ3      | 0.007707 |
| GO:0007276 | Gamete generation   | MGH3; DAZ1; HAP2; DAZ3      | 0.010165 |
| GO:0044702 | Single organism reproductive process                                | MGH3; DAZ1; HAP2; DAZ3; KPL | 0.010412 |
| GO:0048229 | Gametophyte development   | MGH3; DAZ1; HAP2; DAZ3; KPL | 0.036192 |

\*False Discovery Rate after multiple-test adjustment by Yekutieli method (Zheng and Wang, 2008).



**FIGURE 7 | Proportions of *Arabidopsis* germline genes having epigenetic marks in one or more of non-germline tissues.** **(A)** Individual epigenetic marks. **(B)** Epigenetic marks grouped by their repressive or activating nature and the genes grouped by male and

female germline specific expression. Proportion was calculated by dividing the number of cell-type genes with an epigenetic modification by the total number of genes of that cell-type. Female OCC, Female Other Cell-type Combination.



**FIGURE 8 | Size distribution of small RNAs mapped within *Arabidopsis* TEs.** Small RNAs of 24-nt long in length are the most abundant class in all datasets except for the leaf tissue where 23-nt long small RNAs are more abundant. Height of each block represents their respective proportion. Col-0, Colombia; rep1-3, replicate 1-3, respectively; TEs, Transposable Elements.

we uncover a spatial and temporal understanding of the epigenetic repression of these genes in the somatic tissues (Table 2).

### Disparity and Similarity between Male and Female Regulation

We show that the epigenetic regulation differs between the two types of gamete-specific genes. There is a preponderance of H3K27me3 deposition in male germline-genes while there is a combination of different marks for the female counterpart with a preference for H3K9me2 (Supplementary Figure S1). The molecular machinery involved in the pre- and post-deposition of epigenetic marks could differ between the male- and female-specific expressed genes. As such, different SET domain-containing proteins, methyltransferases, are likely to be involved as well as the recognition machinery that delivers the specific SET proteins to the to-be-repressed loci. The methylation is then followed by recognition from different proteins acting as reader and /or effectors. Different protein recognizes different histone marks leading to a set downstream effect (reviewed in Liu et al., 2010). Although the resulting function of those marks are to shut down specific germline expression in non-germline tissue, the mechanisms used between male and female genes will most likely differ, as different epigenetic marks are accumulated at their respective loci. Another striking difference between the two types of germline gene regulation is the use of the activator marks for the female gametophyte gene regulation (Figures 1–4, 7). Although H3K4me1/2 marks are context dependent, H3K4me3 is a well-established activator mark. As one of the most important lineage cell, the egg cell shows the most of this specific mark

(Supplementary Figure S1). Wuest et al. (2010) described a very specific and tight regulation of the *Arabidopsis* egg cell specific genes. As such it is surprising to see an abundance of H3K4me3 at these loci in tissue where their expression is repressed. Although H3K4me3 was rarely seen alone at these loci (Figure 2), hinting to the possibility that H3K4me3 might act in similar ways as its contextual undermethylated counterparts. In fact, we found that the variant H3.3 was absent on these germline genes (Figure 7A) indicating that H3K4 might act as repressor mark on those loci. The higher ratio of the variant H3.3 to H3 is an indicative of gene activity (Shu et al., 2014). This trend is supported by our current findings. We also highlight a similarity in epigenetic control between the sperm cell-specific genes and some of the female gametophyte cell specific genes. The antipodal cells, the central cell, and the sperm cell gene loci behave in a related pattern (Supplementary Figure S1). This could be the ancestral germline-specific repression mechanism and where the egg cell would have evolved additional regulation mechanisms over time. It would be interesting to test this hypothesis if epigenetic whole-genome analysis were available in the lower plant species like *Marchantia polymorpha* and *Physcomitrella patens*. However, we show here that the repression of gamete-specific genes in the somatic tissue could be encompassed by epigenetic regulation, which could restrict these genes to their expression zones. The mechanism involved to achieve this seems to have recruited different repressive strategies between the two types of germline-specific genes; a predominant H3K27me3 pathway for sperm cell-specific genes and a combination of both activating and repressive marks for the egg cell-specific genes.

### H3K27me3 as a Sperm Cell-specific Gene Repression Mechanism

In *Arabidopsis*, the mature pollen grain is composed of three cells: a vegetative cell that produces the pollen tube and two sperm cells that are transported down the pollen tube to participate in the double fertilization in the female gametophyte. In this instance, both sperm cells are considered the male germline cells. In somatic tissues, our analysis demonstrated that repressive epigenetic marks were found at the 81 sperm cells specific loci (Figures 1, 3–7). Detection of methylation on lysine K4 and K36 of histone H3 was minimal at those loci in somatic tissues, although the contextual epigenetic mark H3K4me2 was found at a higher level in some tissues (Figures 1, 3). As a context dependent epigenetic mark, H3K4me2 could either act as an activating or a repressive mark. At the vicinity of the 81 sperm cell specific loci, we are tempted to conclude that H3K4me2 could depict a repressive nature only. This is supported in Figure 2, where the diverse composition of methylated H3K4 showed that the presence of the dimethylated status was always low in combination of the other two methylation states known as activator marks. Sperm cell-specific repressor inside the somatic cells is most likely the trimethylated form on H3K27. This histone modification was the most abundant repressive mark found at male germline genes in 10d-old seedlings, 2wk-old seedlings and root tissues (Figures 1, 3, 4, respectively). This finding is agreement with Hoffmann and Palmgren (2013) study using whole male gametophyte.

**TABLE 4 | Description of protein function of germline-specific genes with activation epigenetic marks only.**

| Activation-related marks  | Description  |
|---|--|
| AT2G42930 H2Bub; H3K36me3   | Glycosyl hydrolase family protein 17                                       |
| AT1G10330 H3K36me3; H3K4me3; H3K27ac  | Pentatricopeptide (PPR) repeat-containing protein                          |
| AT2G35730 H3.3.TTS  | Heavy-metal-associated domain-containing protein                           |
| AT3G58100 H3K36me3; H3K4me3; H3K9ac; H3K27ac  | PDCB5 (PLASMODESMATA CALLOSE-BINDING PROTEIN 5); Nucleic acid binding      |
| AT3G62320 H2Bub   | Ferrodoxin hydrogenase   |
| AT4G16440 H3K36me2; H3K36me3; H3K4me3; H3K9ac; H3.3.TTS; H2Bub; H3K9me3; H3K27ac          | Transcriptional coactivator p15 (PC4) family protein                       |
| AT5G09240 H3K9ac; H3.3.TTS; H3K36me3; H3K4me3; H3K27ac                                    | Lectin protein kinase family protein                                       |
| AT5G60270 H3K36me3; H3K4me3; H3.3.TTS; H2Bub  | Transcription factor   |
| AT3G04410 H3.3.TTS.Promoter; H3K4me3; H3K27ac   | Unknown protein  |
| AT3G09310 H3K4me3; H3K9ac; H3.3.TTS; H2Bub; H3K36me3                                      | LDL2 (LSD1-LIKE2); amine oxidase/electron carrier/oxidoreductase           |
| AT3G13682 H3K18ac; H3K36me2; H3K36me3; H3K4me3; H3K9ac; H3.3.TTS; H2Bub; H3K27ac; H3K9me3 | Arabidopsis homolog of ATP58IPK  |
| AT5G03160 H3K36me2; H3K36me3; H3K4me3; H3.3.TTS; H2Bub; H3K27ac                           | Unknown protein  |
| AT1G48780 H3K36me2; H3.3.TTS; H2Bub; H3K36me3; H3K4me3                                    | Unknown protein  |
| AT1G49150 H3K4me3   | Unknown protein  |
| AT1G61450 H3K36me3  | Unknown protein  |
| AT2G18650 H3K4me3; H3K9ac; H3K36me3   | MEE16 (maternal effect embryo arrest 16); protein binding/zinc ion binding |
| AT3G18120 H3K4me3   | F-box family protein-related   |
| AT3G22670 H3K36me3; H3K4me3; H3K9ac; H2Bub; H3K27ac                                       | Pentatricopeptide (PPR) repeat-containing protein                          |
| AT4G25560 H3.3.TTS; H2Bub   | AtMYB18 (myb domain protein 18); DNA-binding/transcription factor          |
| AT5G06410 H3K18ac; H3K36me3; H3K4me3; H3K9ac; H2Bub                                       | DNAJ heat shock N-terminal domain-containing protein                       |
| AT5G15760 H3K4me3; H3K9ac; H2Bub; H3K36me3  | Plastid-specific 30S ribosomal protein 3, putative/PSRP-3, putative        |

In their study, both K27me3 and K4me2 were detected at high level in pollen-specific loci of the non-pollen tissues. It would be interesting to see if there is a distinction between the nature of the repressive marks between sperm cell specific loci and vegetative cell specific loci found in the somatic cells. Immunofluorescence analyses of global histone methylation marks showed differential methylation states between the generative and the vegetative cell nuclei of the mature bicellular pollen in *Lilium longiflorum* (Okada et al., 2006; Sano and Tanaka, 2010). Trimethylation at K27 was shown to be abundant in most mature non-germline cells of the anthers (O'Brien et al., 2014) including the vegetative cell of the pollen (Sano and Tanaka, 2010; O'Brien et al., 2014) while HK4me2 was abundant only in the vegetative cell (Okada et al., 2006). As such, sperm cell-specific genes in non-germline cells could see their expression repressed through H3K27me3-mediated recruitment of repressor complexes.

### Repression through Both Activating and Repressing Marks in Female Germline-specific Genes

The embryo sac of *Arabidopsis* is composed of 7 cells and 8 nuclei: the egg cell and the bi-nucleate central cells that give rise to the embryo and the endosperm respectively, while antipodal cells and synergids do not contribute to the genetic lineage of the offspring but are still components of the female gametophyte. Although H3K9me2 was the most abundant repressive mark, we found a combination of both repressive marks as well as activation marks at the loci of the different female gametophyte

specific genes (**Supplementary Figure S1**). In somatic tissues of male germline-specific genes, repressive marks are dominant (our study and Hoffmann and Palmgren, 2013). It is quite intriguing that female germline specific genes have a different regulation mechanism where activating marks are present as well as repressive marks. Overall, there is a large variation in epigenetic marks found at the different tissue specific loci of the egg cell, the central cell, and the synergid cell, where repressor marks are more abundant in the central cell followed by synergid cells and in less frequent in the egg cell, while activator marks show an opposite pattern (**Figure 1**). Regulation of female gametophyte-specific gene expression seems to follow divergent epigenetic pathway depending on the cell type inside the embryo sac. What keeps the egg cell-specific genes repressed in the sporophytic tissue while a large proportion of these genes are host to activator marks is not clear? It is quite possible that the overall repertoire of repressive epigenetic marks is not fully revealed for the egg cell-specific genes and additional coverage with other epigenetic marks are needed. As such, a variety of known repressive marks have not been investigated at the whole genome level to date. Histone methylation on arginine is one of them. Alternatively, recent whole-genome studies of repressive context like the interactive heterochromatic islands (Feng et al., 2014) and the heterochromatic histone variant H2A.W (Yelagandula et al., 2014) have not been integrated in the current studies. Data from these two studies could possibly contribute toward explaining why egg cell-specific genes harbor a large proportion of activator epigenetic marks as compared to repressive marks. Are uncharacterized epigenetic marks able

to influence negatively the expression of genes even in the presence of activator marks at their loci? Or alternatively, can activator marks expression potential be made ineffective by either a large abundance of different repressive marks? Can some specific epigenetic mark readers have a higher affinity toward some repressive marks than some activator marks readers toward activator marks? At the molecular level, a mechanism must be in place to shut down the gene expression of female-specific expressed genes in the somatic tissues in spite of the presence of activator marks found at those loci in non-germline tissues.

### Differences in Spatial and Temporal Regulation of the Same Germline Genes

Interestingly, while H3K9me2 is associated with the majority of germline genes in 3wk-old shoots (**Figure 6**), it is nearly absent from gamete-expressed genes in 10d-old seedlings (**Figures 1, 6**) and of 2wk-old shoots (**Figure 3**). This is also the case for the H3K4me2 mark that show dynamic changes, where 10d-old seedlings and 2wk-old shoots have abundance of the mark while 3wk-old shoots show a lack of H3K4 marks altogether (**Figure 2**). This is not surprising as 10d-old shoot and 2wk-old shoots are developmentally similar. This finding implies that different tissues as well as broader developmental stages use different types of epigenetic marks to repress the same set of genes implying that epigenetic marks are dynamic, versatile, and the type of mark as such is not as important as its inherent property to keep germline genes repressed in the non-gametophytic tissues. The inflorescence and the cauline leaves tissue could contribute toward differences seen between the two tissues in 1 week time frame (2wk-old vs. 3wk-old), pointing toward an intriguing regulatory system involving two different gene repression mechanisms.

### Germline-specific TEs Regulation in Non-germline Tissues

In this study we report that 93% of germline specifically expressed TEs are methylated at their respective loci in the non-germline tissues. Germline-specifically expressed TEs raise the question of why such elements are active in these cells. TE transcripts have also been previously reported in rice pollen and germ cells (Russell et al., 2012, 2014). TE genomic DNA methylation occurs through RNA-directed DNA methylation (RdDM) where small interfering RNA (siRNA) directs *de novo* DNA methylation to its cognate homologous DNA region. The DNA-dependent RNA polymerase (RNAP) enzymes IV and V are involved in two different pathways for DNA methylation. The RNAP IV and V complexes are highly similar except for their corresponding largest subunits NRPD1 (At1g63020) and NRPE1 (At2g40030) respectively. RNAP IV acts upstream of RdDM and in conjunction with other protein partners, generates 24 nucleotide long siRNAs, while RNAP V acts downstream of RNAP IV and facilitates *de novo* DNA methylation through siRNA-charged ARGONAUTE 4 (AGO4) at specific targeted loci (reviewed in Haag and Pikaard, 2011). From the 29 germline-expressed TEs, we could identify an enrichment of corresponding 24 nucleotide long siRNAs in somatic cells (**Figure 8**), revealing

that RdDM pathways are involved in TEs repression in non-germline cells. ATGene Express reports that RNA expression of both NRPD1 and NRPE1 are at its lowest in the pollen, which could explain the activation of TEs expression in germline cells while the siRNA would keep the germline-expressed TEs in check in somatic tissue. These results indicate that plants might have evolved a mechanism to specifically and voluntarily regulate TEs in their germline. This could in turn allow for random selective opportunities through genomic shuffling of the gamete genetic material, a mean to adaptive selection.

### Concluding Remarks

We used whole genome analysis to show that a large proportion of germline-specific genes show repressive epigenetic marks at their respective loci in somatic tissues. Repressive marks, H3K9me2 and H3K27me3 could be used at these loci to maintain the status of the germline-specific genes in a repressed state outside the germline cells. These two marks (H3K9me2 and H3K27me3) were found to be abundant at sperm cell-specific genes making H3K9me2 and H4K27me3 the key epigenetic modifications behind the repressed states of the genes in the non-germline cells. A similar situation was also observed in the case of egg cell-specific genes. However, a larger abundance of activator marks were also present at female germ-line cells gene loci. Thus, our study shows that epigenetic control of gene expression is likely to be a dominant mechanism for repressing germline genes in somatic tissues, paving the way for discovering additional marks in future large-scale genomic studies.

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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.2015.00328/abstract>

**Supplementary Data 1 | List of genes specifically expressed in different germline cells.**

**Supplementary Data 2 | Normalized read-counts of small RNA sequences (count-per-million) mapped within germline transposable elements.**

**Supplementary Data 3 | Presence of various epigenetic marks on germline genes in 10 day-old seedlings.**

**Supplementary Data 4 | Presence of various epigenetic marks on germline genes in 5 day-old dark-grown seedlings.**

**Supplementary Data 5 | Presence of various epigenetic marks on germline genes in 3 week-old seedlings.**

**Supplementary Data 6 | Presence of various epigenetic marks on germline genes in 2 week-old aerial tissues.**

**Supplementary Data 7 | Presence of various epigenetic marks on germline genes in roots.**

**Supplementary Data 8 | Presence of various epigenetic marks on germline genes in 3 week-old shoots.**

**Supplementary Data 9 | Presence of various epigenetic marks on germline genes in leaves.**

**Supplementary Data 10 | Presence of various epigenetic marks on germline genes in shoot apical meristem.**

**Supplementary Data 11 | Presence of the epigenetic mark, H3K27me3 on germline genes in 2 week-old aerial tissues, leaves, shoot apical meristem, 10 day-old seedlings, 5 day-old dark-grown seedlings and roots.**

**Supplementary Data 12 | Combined counts and proportions of germline genes with various epigenetic marks in non-germline tissues.**

**Supplementary Data 13 | Presence of various epigenetic marks on germline TEs in non-germline tissues.**

**Supplementary Figure S1 | Proportions of germline genes with various epigenetic marks in non-germline tissues.** Related germline tissues are in X-axis, and the epigenetic marks are in Y-axis. Proportions are indicated in cells and are color-coded as shown in the legend at top-right side of the figure. Proportion: Number of genes carrying specific epigenetic modification was calculated by dividing the amount of cell-type genes with an epigenetic modification by the total amount of genes of that cell-type.

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# Large genetic screens for gynogenesis and androgenesis haploid inducers in *Arabidopsis thaliana* failed to identify mutants

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Gynogenesis is a process in which the embryo genome originates exclusively from female origin, following embryogenesis stimulation by a male gamete. In contrast, androgenesis is the development of embryos that contain only the male nuclear genetic background. Both phenomena are of great interest in plant breeding as haploidization is an efficient tool to reduce the length of breeding schemes to create varieties. Although few inducer lines have been described, the genetic control of these phenomena is poorly understood. We developed genetic screens to identify mutations that would induce gynogenesis or androgenesis in *Arabidopsis thaliana*. The ability of mutant pollen to induce either gynogenesis or androgenesis was tested by crossing mutagenized plants as males. Seedlings from these crosses were screened with recessive phenotypic markers, one genetically controlled by the female genome and another by the male genome. Positive and negative controls confirmed the unambiguous detection of both gynogenesis and androgenesis events. This strategy was applied to 1,666 EMS-mutagenised lines and 47 distant *Arabidopsis* strains. While an internal control suggested that the mutagenesis reached saturation, no gynogenesis or androgenesis inducer was found. However, spontaneous gynogenesis was observed at a frequency of 1/10,800. Altogether, these results suggest that no simple EMS-induced mutation in the male genome is able to induce gynogenesis or androgenesis in *Arabidopsis*.

**Keywords:** gynogenesis, androgenesis, *Arabidopsis thaliana*, haploid, EMS mutagenesis, genetic screen

## Introduction

In sexual reproduction, the fusion of the male and female haploid gametes leads to the formation of a diploid embryo. Both parents contribute equally to the nuclear genome of the embryo. In contrast, the cytoplasmic genome (mitochondrial and chloroplastic), is solely from female origin (Berger et al., 2008). *In situ* gynogenesis and androgenesis are two deviations of sexual reproduction. Gynogenesis leads to embryos that exclusively originate from the female genetic background, with no contribution of the male in the embryo's genome, even if a male gamete is required to stimulate embryogenesis. This differs from another mode of reproduction, parthenogenesis, where embryogenesis occurs spontaneously, in absence of a male gamete. Conversely, *in situ* androgenesis leads to the development of embryos that contain only the nuclear male genetic background, with

no contribution of the female to the nuclear genome of the embryo. In both *in situ* gynogenesis and androgenesis, the cytoplasmic genome is of female origin.

Obligate gynogenesis is the natural mode of reproduction for some vertebrate species, such as some salamanders or fishes. In these unisexual organisms, males do not exist. Females produce diploid eggs and the male stimulation is performed by the sperm of related species (Neaves and Baumann, 2011). *In situ* androgenesis seems to be rare in nature (Seguí-Simarro, 2010), used for example by some clam families, the Mexican axolotl Siredon, and the Cyprus *C. serpervirens* for which another Cyprus species acts as a surrogate mother (Pichot et al., 2008).

Gynogenesis and androgenesis are of great interest for plant breeders because genome-wide homozygosity can be achieved in a single generation, reducing the time requirements of breeding programs (Dunwell, 2010; Germanà, 2011). The elimination of one genome parent leads to haploids which can then be diploidized by a step of chromosome doubling. Moreover, androgenesis can be useful to improve the cytoplasmic male sterility (CMS) system (Budar et al., 2001). The main issue of this system is the introgression of a selected nuclear genome into a male sterile line that is under cytoplasmic control. A current method is carried out by several backcrosses that are time consuming. Efficiently using androgenesis, as the cytoplasmic genome remains from female origin, only one cross is necessary to transmit CMS. In addition *in situ* androgenesis or gynogenesis have been used to create a method of clonal reproduction through seed (Marimuthu et al., 2011).

For decades various methods to induce artificial gynogenesis and androgenesis in many crop species have been exploited. Biotechnological *in vitro* approaches such as anther cultures and isolated microspore cultures are widely used to produce doubled haploids in many species. It should be noted that in the case of *in vitro* androgenesis, the mitochondrial, and plastidial genome has a male origin, in contrast to *in situ* androgenesis. For gynogenesis, ovule, ovary, and flower culture, with or without the use of mentor or irradiated pollen, is used to produce gynogenic doubled haploids in some species (Bohanec, 2009; Seguí-Simarro, 2010; Germanà, 2011). Another method is to induce *in situ* gynogenesis/androgenesis through interspecific crosses. The most documented examples are *Triticum aestivum* × *Zea mays* (Laurie and Bennett, 1988) and *Hordeum vulgare* × *Hordeum bulbosum* crosses (Kasha and Kao, 1970). Although androgenesis has been reported in a few cases in barley (Kasha and Kao, 1970; Lange, 1971; Finch, 1983), gynogenesis is more common (Houben et al., 2010). Irradiated pollen can also be used to induce *in situ* gynogenesis (Chat et al., 2003; Froelicher et al., 2007). Finally, specific lines that induce *in situ* gynogenesis or androgenesis following intraspecific crosses have been also reported in the literature, notably in maize (Kermicle, 1969; Eder and Chalyk, 2002; Zhang et al., 2008). This trait appears to be under genetic control. For example, the *gynogenesis inducer 1* (*ggi1*) locus has been shown to control gynogenesis induction, and is widely used in maize breeding, but the underlying gene(s) has not been identified yet (Barret et al., 2008). Another haploid inducer in maize is the *indeterminate gametophyte* (*ig*)

mutant (Huang and Sheridan, 1996; Evans, 2007). *ig* can induce both androgenesis and gynogenesis (Kermicle, 1969). This process has been used to produce plants with a male nuclear genome and a female cytoplasm genome (Kindiger and Hamann, 1993). In barley, a haploid initiator mutant (*hap*) prevents fertilization of the egg cell but not the central cell. For this reason, endosperm can be formed normally and haploid embryos containing only the female genome can be developed (Mogensen, 1982). In *Arabidopsis*, the centromeric histone *CENH3* was manipulated leading to the TailSwap (TS) and Genome Elimination (GEM) line able to stimulate both gynogenesis and androgenesis (Ravi and Chan, 2010; Marimuthu et al., 2011; Ravi et al., 2014). These lines carry a null mutation in the native *CENH3* which is rescued with one or two transgenes, respectively. The *CENH3* variant(s) are required for viability because the null mutant is lethal. The transfer of this method to crops might be difficult due to this genetic complexity. The GEM line has been used for the creation of synthetic apomixis, developed by Marimuthu et al. (2011). This method combined GEM and *MiMe* in which meiosis is turned into mitosis, to induce the production of clonal seeds. However, only ~30% of the seeds are clones because the penetrance of the GEM line is incomplete.

Hence the improvement of *in situ* haploid induction method, both in terms of frequency and availability in more species, would be of great interest. Here, with the aim of obtaining better knowledge of the genetic control of *in situ* androgenesis and gynogenesis, we ran a large scale genetic screen for mutations inducing these events in the model plant *Arabidopsis thaliana*.

## Materials and Methods

### Plant Material and Growth Conditions

The *A. thaliana* accession used for mutagenesis was Columbia (Col-0). The other 47 accessions (non-mutagenized) were Ms-0, Rubezhnoe-1, Kz-9, Kly-1, N7, N14, Leb-3, Altai-2, Sij-1, Shahdara, Bik-1, Ita-0, Cvi-0, Sei-0, Sah-0, Sakata, Ty-0, Ost-0, Lov-1, Yo-0, Pyl, Bur-0, Rld-2, Jea, Bla-1, Ran, Lod-2, Bozen-1a, Toufl-1, Cha-0, Are-0, Esc-0, Etna-1, Had-1b, Chab-1, Dja-1, Sorbo, Kondara, Kar-1, Bas-1, Nov-01, Rak-1, Chi-0, Bij, Keu-1, Shigu-2, and Stepn-1. Mutants for *APT* were used as female plants. These mutants are deficient for the enzyme *APT* [adenine phosphoribosyl transferase (EC2.4.2.7)] which confers 2FA (2-Fluoroadenine) resistance (Gaillard et al., 1998). The *apt* mutant is also male sterile, facilitating the crosses. Plants with a T-DNA insertion in *GLABRA1* (*gl1*) were used as male plants. *gl1* mutants do not have trichomes. Plants were cultivated in greenhouses with a 16 h/day and 8 h/night photoperiod, at 20°C and 70% humidity.

### EMS Mutagenesis

Seeds were incubated for 17 h at room temperature in 5 mL of 0.3% (v/v) EMS. Neutralization was performed by adding 5 mL of sodium thiosulfate 1 M for 5 min. Three milliliter of water was added to make the seeds sink. The supernatant was removed and

the seeds were washed three times for 20 min with 15 mL of water. The seeds were immediately sown in soil. EMS induce mutations by nucleotide substitutions which causes primarily G:C to A:T transitions.

## Oligonucleotides for PCR Genotyping and Sequencing

*APT* was amplified using primers with RT1 (5'-tccagaatc-cgctaaggattgc-3') and RT21 (5'-CTCAATTACGCAAGCAC-3'). Polymorphism between wild type and mutant alleles was revealed with Mva1 (Fermentas, Stockholm, Sweden) digestion at 37°C for 1 h. *GL1* gene was amplified using primers with GL135SF (5'-TTCAAAGACAAATTCAAAACA-3'), and GL135SR (5'-GATTGGCCGTTAAGTTGAT-3'), and mutant allele using GL135SR, and PKYPM1 (5'-CGCAATGTGTT ATTAAGTTGTCTAACGCG-3'). The DNA sequence of the coding region of the *APT* gene was amplified by PCR as 3 fragments from 500 to 1,200 bp which overlap. Differences between mutants and wild-type sequences were viewed using the Multalin program (<http://multalin.toulouse.inra.fr/multalin/>).

## Ploidy Determination

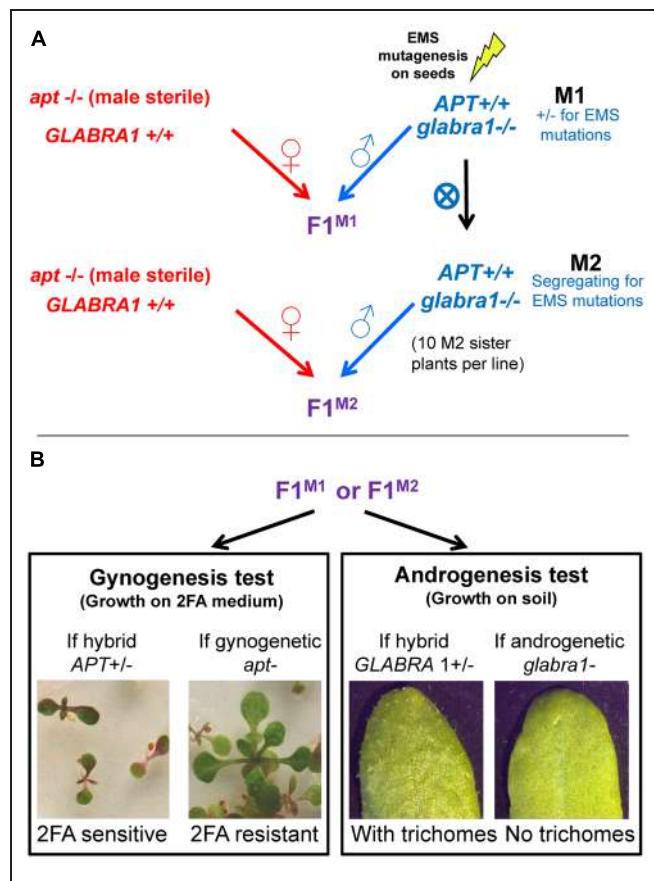
Chromosome spreads were prepared and stained with DAPI as described in Ross et al. (1996). Observations were made with a Leica DM RXA2 epifluorescence microscope using an oil PL APO 100X/1.40 objective (Leica). Chromosomes were counted on cells at mitotic metaphase.

## Experimental Design to Detect *in situ* Gynogenesis and Androgenesis Inducers

In this study, we developed a screen to detect gynogenesis and androgenesis events (Figure 1). The obtained plants would be haploid and would contain nuclear genetic information exclusively from either the female or the male genome, respectively. To identify such events we used two recessive phenotypic markers: 2FA resistance and absence of trichomes. The gynogenesis screen exploits the *apt* mutant which is 2FA resistant whereas the androgenesis screen is based on *gl1* mutant in which trichomes are absent on leaves. The ability of mutant pollen to induce either gynogenesis or androgenesis was tested by crossing female plants homozygous for the *apt* mutation and male plants homozygous for the *gl1* mutation.

EMS mutagenesis was applied on *gl1* seeds to produce the M1 generation (seven independent mutagenesis, Table 1). The screen was performed both at the M1 and M2 generations. At the M1 generation (one plant being one line), only dominant or gametophytic mutations can be detected. At the M2 generation (crossing ~10 M2 segregated plants per line) recessive mutations can also be detected.

To screen for gynogenesis events, about 100 seeds ( $F1^{M1}$  or  $F1^{M2}$ ) were grown on 2FA medium.  $F1$  seeds produced through regular fertilization would carry a functional *APT* allele (from the male) and thus would die on 2FA medium. In contrast, gynogenetic plants (haploid) would survive on 2FA medium because they would lack the *APT* wild type allele (Figure 1B, left). In parallel, to screen for androgenetic events, about 100  $F1$  seeds were grown on non-selective media and observed for



**FIGURE 1 | Design of the haploid inducer screen.** Seeds of Col-0 strains containing the *glab1* mutation are mutagenized to obtain M1 plants which are self-fertilized to produce M2 plants. These plants, either in M1 or M2, are crossed with an *apt* mutant (A). The two mutations, *apt* and *glab1* are recessive and confer resistance to 2FA and absence of trichomes, respectively (B). In F1, if gynogenesis or androgenesis haploids appear they would lack *APT* or *GLABRA* dominant allele, respectively, and be detected because of the corresponding phenotype.

**TABLE 1 | Number of tested plants and number of *apt* mutants detected.**

| Mutagenesis number | Number of mutagenized plant | Number of tested plant |            | Number of <i>apt</i> mutant found |
|--------------------|-----------------------------|------------------------|------------|-----------------------------------|
|                    |                             | in M1                  | in M2      |                                   |
| 1                  | 70                          | 70                     | 69         | 1                                 |
| 2                  | 94                          | 94                     | 83         | 1                                 |
| 3                  | 66                          | 66                     | 0          | 1                                 |
| 4                  | 172                         | 172                    | 0          | 1                                 |
| 5                  | 339                         | 339                    | 0          | 1                                 |
| 6                  | 79                          | 79                     | 0          | 2                                 |
| 7                  | 846                         | 44                     | 846        | 6                                 |
| <b>Total</b>       | <b>1666</b>                 | <b>864</b>             | <b>998</b> | <b>13</b>                         |

the presence/absence of trichomes.  $F1$ s would carry a functional *GL1* allele (from the female) and would have trichomes. Androgenetic haploid plants would show an absence of trichomes

because they would lack the *GL1* wild type allele (**Figure 1B**, right).

Novel *apt* mutations generated by the EMS treatment would be expected to be identified which also serves as an internal control. In that case, F1s would carry two deficient alleles for *APT* coming from both parents and 2FA resistant plants can be found in the F1s. These *de novo* *apt* mutations can be distinguished from gynogenetic events because (i) F1 plants are diploid, (ii) 2FA resistant plants would also been found in the self-fertilization progeny of the male parent, (iii) an additional mutation would be found in the *APT* gene. The number of *apt* mutations found in the screen can be used as a marker of the saturation level reached in the screen.

## Results

### Positive and Negative Controls

We first tested if the detection system described above is efficient to detect androgenesis and gynogenesis events, by using negative and positive controls. The negative controls were performed by crossing a Col-0 wild type plant with the two recessive markers chosen (crosses between *apt*−/− × wt [Col-0] or wt [Col-0] × *gl1*−/−). In that case, the F1 of these crosses should be hybrids. The phenotype conferred by recessive markers should not be seen because both would be heterozygous. Indeed, neither 2FA resistant ( $n = 108$ ) nor plant lacking trichomes were detected ( $n = 100$ ), in the F1 of these crosses. As a positive control, we used the GEM haploid inductor line (Marimuthu et al., 2011). We produced F1 seeds by crossing *apt*−/− × GEM and GEM × *gl1*−/−. If a gyno/androgenesis event takes place, it should be detected via the expression of recessive markers, as the dominant *GL1* and *APT* allele originating from the gem line would be eliminated. In these two positive controls, the two phenotypes were found: 16% of 2FA resistant plants ( $n = 158$ ) and 58% of plants lacking trichomes ( $n = 53$ ). These controls indicating that this screen allows the unambiguous detection of gynogenesis or androgenesis events.

### Spontaneous Gynogenetic Haploid Plants

Interestingly, 97 2FA resistant and haploid plants (determined by chromosome counting), thus from female origin, have been indeed recovered in the screen. These cases are interpreted as gynogenesis events. However, in all cases, only one such plant appeared per cross (around 100 seeds tested for any given cross in the screen). When a haploid plant was found in a cross, we further tested the corresponding line but never showed a heritable capacity to induce gynogenesis above background levels. Thus, this corresponds likely to the spontaneous apparition of haploids of female origin in *Arabidopsis*. As these 97 events were detected among ~1,047,700 seeds (10,477 crosses × ~100 seeds analyzed per cross), we estimate a spontaneous gynogenesis to occur at a frequency of ~1/10,800. In contrast, no androgenetic haploid were found among ~846,000 seeds tested.

### Number of Lines Tested and Mutation Saturation of the Genome

For the gynogenesis screen, 864 M1 lines were tested (**Table 1**). Each M1 was used to pollinate *apt* mutants, and the resulting F1s were grown on 2FA medium (~100 plants per cross). 2FA resistant plants were found in a total of 8 independent crosses among 864. The frequency of 2FA resistant plants in each F1 ranged from 3 to 36%. For each of these 8 cases, all F1 2FA resistant plants were diploid; 2FA resistant plants were found in the selfing progeny of the male plant at a proportion ranging from 0.5 to 21%, (likely reflecting the chimeric nature of M1 EMS mutants; Koornneef, 2002); and *de novo* point mutation were found in *APT* in each of the lines (**Table 2**). Thus, we conclude that these were not gynogenesis events but EMS-induced *apt* mutations. Having found 8 *apt* mutants for 864 M1 tested suggests that the screen reached a reasonable level of saturation, and that dominant/gametophytic mutations that would confer gynogenesis are unlikely to exist. We thus stopped the M1 screen and started the M2 screen. In the M2 gynogenesis screen, 998 families were tested. Ten M2 sister plants per family were used to pollinate *apt* mutants and each resulting F1 was individually grown on 2FA medium. These 998 M2 families include 152 families used in the M1 screen and 846 families that were not screened at the M1 generation. For 8 of 998 families, ~50% diploid 2FA resistant plants were found in at least one of the 10 F1s tested. Like in the M1 screen, these events are explained by EMS-induced *apt* mutations: 2FA resistant plants were also found in the selfing of the corresponding M2 plant (at a proportion of ~25%), and the presence of a novel *apt* mutation was confirmed by sequencing in all cases (**Table 2**).

For the androgenesis screen 44 M1 plants were tested, by pollinating them with *gl1* pollen, and observing the presence/absence of trichomes on leaves of ~100 plants of the resulting F1s. No potential androgenetic events were found among these 44 populations. We then tested 846 families at the M2 generation: 10 M2 plants per family were pollinated by *gl1* pollen and the resulting F1s were examined for the presence of trichomes (~100 plants

**TABLE 2 | New *apt* mutants found in the screen.**

| Mutant name | Fertile or sterile | Position and nucleotide change | Amino acid changes of the AT1G27450.2 |
|-------------|--------------------|--------------------------------|---------------------------------------|
| gl187.9     | Fertile            | CHr1:9532494 C > T             | P25 > S                               |
| gl23        | Sterile            | CHr1:9532503 G > A             | G28 > R                               |
| s3pl5       | Sterile            | CHr1:9532608 G > A             | D33 > N                               |
| gl129.11    | Sterile            | CHr1:9532887 G > A             | G70 > D                               |
| gl865.6     | Sterile            | CHr1:9532887 G > A             | G70 > D                               |
| s6pl30      | Sterile            | CHr1:9532899 G > A             | G74 > D                               |
| s11pl13     | Sterile            | CHr1:9533395 G > A             | E106 > K                              |
| gl172.10    | Sterile            | CHr1:9533483 G > A             | G135 > D                              |
| s11pl33     | Fertile            | CHr1:9533485 G > A             | G136 > R                              |
| s2pl6       | Fertile            | CHr1:9533486 G > A             | G136 > E                              |
| gl392.8     | Sterile            | CHr1:9533599 G > A             | splicing site of the 4th intron       |
| s1pl41      | Sterile            | CHr1:9533621 G > A             | C155 > Y                              |
| s10pl47     | Sterile            | CHr1:9533751 G > A             | splicing site of the 5th intron       |

per F1). However, no potential androgenetic events were detected in this screen. As the same families were used for the gynogenesis screen, we know that one among the 44 M1 plant tested and six among the 846 M2 families tested contained an *apt* mutation induced by the EMS treatment, suggesting that a certain level of saturation was reached.

Finally, we tested 47 different accessions genetically distant from Col-0 to explore the possibility that natural variation could be able to induce gynogenesis. For these crosses, each accession (Ms-0, Rubezhnoe-1, Kz-9, Kly-1, N7, N14, Leb-3, Altai-2, Sij-1, Shahdara, Bik-1, Ita-0, Cvi-0, Sei-0, Sah-0, Sakata, Ty-0, Ost-0, Lov-1, Yo-0, Pyl, Bur-0, Rld-2, Jea, Bla-1, Ran, Lod-2, Bozen-1a, Toufl-1, Cha-0, Are-0, Esc-0, Etna-1, Had-1b, Chab-1, Dja-1, Sorbo, Kondara, Kar-1, Bas-1, Nov-01, Rak-1, Chi-0, Bij, Keu-1, Shigu-2, and Stepn-1) was crossed as male with an *apt* mutant as female. For each cross, about 500 F1 seeds were grown on 2FA-containing medium, but no resistant plants were found.

## Discussion

In this study, we showed that spontaneous gynogenesis occurs at a frequency of ~1/10,800 in *Arabidopsis* crosses. In contrast, no androgenetic haploids were found among ~846,000 seeds tested, suggesting that spontaneous androgenesis occurs, if at all, at a much lower frequency than gynogenesis. It should be noted, that these figures were obtained after manual crosses, and that we cannot exclude that they could differ in a population obtained by selfing. It is not unusual to find spontaneous haploid in different plant species like in *Brassica napus* (Olsson and Hagberg, 1955) or in maize (Chase, 1963). Spontaneous haploids of female origin were detected in maize at a proportion of 1/1,000 whereas, haploid androgenesis were found on average at 1/80,000 (Chase, 1963). In maize, spontaneous haploid seems to appear more often than in *A. thaliana*.

The screens performed here were designed to identify male genetic factors influencing the occurrence of *in situ* gynogenesis or androgenesis. However, these screens failed to identify mutations that increase the occurrence of these events to detectable levels. The controls using a known inducer line confirmed that the experimental design was able to unambiguously detect gynogenetic and androgenetic events. However, it should be noted that, as ~100 seeds were tested per line, the screens could have missed mutations that would induce gyno/androgenetic events at frequencies lower than 5%. The mutagenized populations used in this study contained 13 independent, phenotypically detectable, mutations in *APT*. Thus *apt* mutations induced by the EMS treatment can be used as an internal measure of the mutagenesis saturation. It should be noted that not all gene are equally sensitive to mutagenesis because of their length, number of introns,

or number of codons that are essential for the function of the protein. Nevertheless, it is reasonable to assume that these populations contained at least one efficient mutation in most of the *Arabidopsis* genes. However, no mutation able to induce gynogenesis and androgenesis were recovered. This may suggest that such a mutation does not exist in *A. thaliana*. However such mutations may be very specific (e.g., gain of function, separation of function, or specific levels of residual activity) and then much less frequent than simple knock out/knock down. For example, it is possible that a single mutation in the centromeric histone CENH3 gene could recapitulate the subtle equilibrium observed in the TS/GEM lines. Indeed, a null mutation in CENH3 histone is lethal, while the TS/GEM lines contain modified versions of CENH3 (with GFP fusion and/or replacement of the CENH3 tail by the H3 tail). It appears that chromosomes loaded with these versions of CENH3 are able to segregate properly at mitosis, ensuring plant viability, but that they are eliminated when put in competition with chromosomes loaded with wild type CENH3, leading to genome elimination (Ravi and Chan, 2010; Marimuthu et al., 2011; Ravi et al., 2014). We can speculate that a single mutation in CENH3 could recapitulate the required level of CENH3 functionality. However, the screen we used here, that cannot be increased in size indefinitely as it relies on manual crosses, may very well have missed such subtle mutations.

In addition, our screen was designed to identify the ability of pollen grains to induce gynogenesis and androgenesis. Indeed, as EMS mutagenized lines were used only as male in the crosses, only mutations carried by the male genome were tested. As female inducers are known like *ig* in maize (Huang and Sheridan, 1996; Evans, 2007) or the *haploid initiator* in barley (Mogensen, 1982), it could be interesting to explore the female genetic control of androgenesis and gynogenesis in *Arabidopsis* by running screens with the appropriate design.

This study suggests that the rate of mutations that can induce androgenesis/gynogenesis in *Arabidopsis* is very low. This should stimulate alternative approaches to obtain better knowledge of the genetic control of androgenesis and gynogenesis. It could be relevant to run such screens in another species, such as maize in which spontaneous mutants have been found. Further, this underlines the need of the identification and characterization of the few known loci that control these processes, such as *ggi1* in maize.

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# Correlation between a loss of auxin signaling and a loss of proliferation in maize antipodal cells

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The plant life cycle alternates between two genetically active generations: the diploid sporophyte and the haploid gametophyte. In angiosperms the gametophytes are sexually dimorphic and consist of only a few cells. The female gametophyte, or embryo sac, is comprised of four cell types: two synergids, an egg cell, a central cell, and a variable number of antipodal cells. In some species the antipodal cells are indistinct and fail to proliferate, so many aspects of antipodal cell function and development have been unclear. In maize and many other grasses, the antipodal cells proliferate to produce a highly distinct cluster at the chalazal end of the embryo sac that persists at the apex of the endosperm after fertilization. The antipodal cells are a site of auxin accumulation in the maize embryo sac. Analysis of different families of genes involved in auxin biosynthesis, distribution, and signaling for expression in the embryo sac demonstrates that all steps are expressed within the embryo sac. In contrast to auxin signaling, cytokinin signaling is absent in the embryo sac and instead occurs adjacent to but outside of the antipodal cells. Mutant analysis shows a correlation between a loss of auxin signaling and a loss of proliferation of the antipodal cells. The leaf polarity mutant Laxmidrib1 causes a lack of antipodal cell proliferation coupled with a loss of DR5 and PIN1a expression in the antipodal cells.

**Keywords:** antipodal cells, embryo sac, maize, auxin, gametophyte, *Zea mays*

## Introduction

The plant life cycle has genetically active diploid and haploid phases, called the sporophyte and gametophyte (Walbot and Evans, 2003). The female gametophyte of angiosperms, called the embryo sac, has four cell types: the two synergids, the egg cell, the central cell, and the antipodal cells (Drews and Yadegari, 2002). The Polygonum type of embryo sac development is the most common. First, one megasporangium undergoes three rounds of free nuclear divisions to produce an eight-nucleate syncytium. After the first division the two nuclei migrate to opposite poles of the embryo sac and are separated by a central vacuole. The nuclei then undergo two rounds of synchronous divisions to produce an 8-nucleate syncytium with micropylar and chalazal clusters of nuclei. The migration and position of these nuclei are highly regular. The embryo sac then cellularizes to produce seven cells. One nucleus from each pole migrates to the center of the future central cell, followed by fusion of the two nuclei in some species, and migration to the micropylar end of the central cell.

The embryo sac is polarized along the micropylar-chalazal (M-C) axis with the egg cell and synergids at the micropylar end and the antipodal cells at the chalazal end of the embryo sac.

The polarization along the M-C axis is present at all stages of megagametogenesis and is present in the ovule and megasporocyte mother cell, thus anticipating polarity in the embryo sac. Polar distribution of cytoplasmic components along the M-C axis of the megasporocyte, a single cell, is present even before meiosis (Russell, 1979). After meiosis, the meiotic products located at the chalazal and micropylar ends can be distinguished by callose deposition and concentration of mitochondria and plastids. Only the chalazal-most of these products survives to become the functional megasporocyte. In the functional megasporocyte, a central vacuole forms during the one-nucleate stage and separates the chalazal and micropylar clusters of nuclei after the first free nuclear division. M-C polarization is apparent throughout the syncytial stages of megagametogenesis. The micropylar and chalazal poles have different concentrations of plastids and different patterns of divisions of the nuclei at each pole. Then following cellularization the cells differentiate into the cell types characteristic of their position along the M-C axis.

The antipodal cells lie at the chalazal pole of the embryo sac. Maize (*Zea mays L.*) antipodal cells are densely cytoplasmic compared to the neighboring nucellus and central cell. Maize antipodal cells can be multi- or uni-nucleate with incomplete cytokinesis so that they are only partially separated by cell walls (Diboll and Larson, 1966). The size of the antipodal cell vacuoles is also variable (Diboll, 1968). The microtubules of the antipodal cells are randomly oriented (Huang and Sheridan, 1994). They are hypothesized to function as transfer cells for the embryo sac in maize. This hypothesis for antipodal cell function is primarily based on studies of their morphology. The cell walls of maize antipodal cells adjacent to the nucellus are papillate, supporting a role for the antipodal cells as transfer cells for the embryo sac (Diboll, 1968). In maize, the antipodal cells continue to divide during embryo sac maturation reaching a final number of 20–100 cells with one to four nuclei each. Maize antipodal cells can persist and even continue dividing after fertilization during kernel development (Randolph, 1936). The antipodal cells of another cereal, barley, have similar cell wall invaginations juxtaposed to the surrounding nucellus and also persist beyond fertilization (Engell, 1994). Antipodal cells in maize have high sucrose synthase activity compared to the surrounding cells of the ovule, suggesting a high metabolic activity and nutritive function (Wittich and Vreugdenhil, 1998). However, the function of the antipodal cells has not been experimentally determined. Suppression of central cell identity in the antipodal cells requires the egg-cell secreted peptide ZmEAL1, indicating that egg cell signaling is critical for antipodal cell development (Krohn et al., 2012). Other factors required for antipodal cell growth and development have not yet been identified in maize.

In *Arabidopsis*, the antipodal cells do not proliferate and reportedly degenerate during embryo sac maturation, at least in starchless mutant line TL255 (Murgia et al., 1993). However, recent studies indicate that the antipodal cells of *Arabidopsis* persist after fertilization like those of maize, although they do not proliferate (Song et al., 2014). Interestingly, the antipodal cells of wheat also degenerate, although they proliferate first (An and You, 2004). While mutant studies have not revealed a definitive role for antipodal cells in *Arabidopsis*, the genetic and genomic analysis

of gametophyte biology has revealed some insights into regulation of antipodal cell development. Enhancer trap and other transcriptional reporter lines have revealed that the antipodal cells, not surprisingly, define a unique transcriptional domain (Yu et al., 2005; Steffen et al., 2007; Bemer et al., 2010; Wang et al., 2010a; Drews et al., 2011). The neighboring central cell seems to exert influence on the development of the antipodal cells in *Arabidopsis*. In embryo sacs mutant for the central cell expressed *FIONA* gene, antipodal cell lifespan is increased, suggesting that a normal central cell is required to prevent persistence of the antipodal cells (Kagi et al., 2010). Loss of function of the chromatin cohesion factor *CTF7* also results in delayed antipodal cell death (Jiang et al., 2010). Antipodal cell specific transcripts are also actively suppressed in central cells as can be seen by the ectopic expression of antipodal cell reporters in the central cells of *agl80* and *agl61/diana* mutants (Portereiko et al., 2006; Bemer et al., 2008, 2010; Steffen et al., 2008).

Auxin is involved in many developmental processes including lateral organ development, shoot branching, and root architecture, and auxin-mediated responses depend both on patterns of auxin biosynthesis and auxin transport (reviewed in (Leyser, 2006; Zhao, 2010; Sauer et al., 2013)). The main source of developmentally important auxin is a two-step tryptophan-dependent pathway (Mashiguchi et al., 2011; Phillips et al., 2011; Won et al., 2011). L-tryptophan is converted to indole-3-pyruvic acid (IPA) by *TAA1* aminotransferases (Stepanova et al., 2008; Tao et al., 2008) followed by the conversion of IPA to indole-acetic acid (IAA) by *YUCCA* (YUC) flavin monooxygenases (Dai et al., 2013). Control of auxin biosynthesis has been shown to be important for many environmental responses and developmental processes (reviewed in Sauer et al., 2013). Analysis of the dominant mutant *yuc1D* demonstrated that *YUCCA* flavin monooxygenases perform a rate-limiting step in auxin biosynthesis (Zhao et al., 2001). Auxin efflux under control of the PIN class of proteins is essential to achieve appropriate auxin maxima and for normal auxin signaling in a wide range of developmental contexts in *Arabidopsis* and maize (McSteen and Hake, 2001; Carraro et al., 2006; Gallavotti et al., 2008; Krecek et al., 2009; Forestan et al., 2012). Polar subcellular localization of PIN protein depends on the PINOID (PID) protein kinase and is required for normal root and shoot development (Christensen et al., 2000; Benjamins et al., 2001; Friml et al., 2004; Cheng et al., 2008). Auxin transport also depends on the ABC transporters, BRACHYTIC2 (BR2) in maize and PGP1/ABCB1 and PGP19/ABCB19 in *Arabidopsis* (Noh et al., 2001; Multani et al., 2003; Geisler et al., 2005) which have partially overlapping roles with PIN-dependent auxin transport (Bandyopadhyay et al., 2007; Blakeslee et al., 2007; Mravec et al., 2008). Additionally, auxin distribution is influenced by influx through AUX1 auxin influx carriers (Bennett et al., 1996; Yang et al., 2006). Auxin is perceived by the TIR1 auxin receptor, a component of an SCF-type ubiquitin protein ligase (Dharmasiri et al., 2005). Auxin binding by TIR1 leads to degradation of the AUX/IAA class of proteins; this in turn frees the AUXIN RESPONSE FACTOR (ARF) transcription factor proteins to bind DNA and modulate transcription in response to high auxin levels (for a review see, Leyser, 2006). Auxin contributes to the control of leaf polarity through *MONOPTEROS* and interactions

of ASYMMETRIC LEAVES1 (*AS1*) and *AS2* with tasiRNAs and *ETTIN/ARF3* and *ARF4* (Garcia et al., 2006; Qi et al., 2014). The maize ortholog of *AS2*, *indeterminate gametophyte 1* (*ig1*), controls both leaf polarity and embryo sac development (Evans, 2007), and the dominant mutation, *Laxmidrib1-O*, has the opposite effect on leaf polarity as the recessive *ig1* mutant (Schichnes et al., 1997; Schichnes and Freeling, 1998).

Arabidopsis plants expressing GFP under the control of a *DR5* promoter reveal an auxin maximum in the micropylar nucellus during the earliest stages of embryo sac development (Pagnussat et al., 2009). Increasing auxin levels by overexpressing *YUCCA1* under control of the embryo sac promoter *pES1* disrupts embryo sac patterning with expansion of micropylar fates. Conversely, down-regulating auxin responses by expressing an artificial microRNA targeting *ARF5*, *ARF7*, *ARF2*, *ARF19* (and to a lesser extent *ARF8*, *ARF6*, *ARF3*, *ARF4*, and *ARF1*) blocks expression of synergid-specific (i.e., micropylar) markers (Pagnussat et al., 2009). Additional studies did not find an auxin gradient in either the Arabidopsis or maize syncytial embryo sac, and no *DR5* expression was detected in any Arabidopsis embryo sac cells (Ceccato et al., 2013; Litviev et al., 2013). Instead auxin signaling is present in the micropylar nucellus of both species and in the antipodal cells of maize (Litviev et al., 2013). The nucellar expression of *PIN1* is required for embryo sac development in Arabidopsis (Ceccato et al., 2013). Other aspects of auxin signaling, namely *AUX1* and *PGP1* are localized to the plasma membrane of the female gametophyte in Arabidopsis. Here a role for auxin in the maize embryo sac is examined, including analysis of multiple gene families involved in auxin signaling and biosynthesis. Auxin signaling in maize is localized within the antipodal cell cluster, and loss of proliferation of antipodal cells is correlated with a loss of auxin signaling in the antipodal cells.

## Material and Methods

### Analysis of Maize Gene Families Involved in Auxin Biosynthesis, Distribution, and Signaling

To identify maize *YUCCA*, *TAA*, *AUX1*, *brachytic2-like* ABC transporters, *PID*, and *TIR1* genes present in the maize working gene set (ZmB73 v.5a.59), the Working Gene Set Peptide database was queried using BLAST at <http://maizedb.org> starting with the published Arabidopsis *YUCCA1*, *TAA1*, *AUX1*, *PID* (and maize *bif2*) and *TIR1* genes and their close homologs. To ensure that related *bona fide* maize orthologs of *YUCCA1* could be distinguished from other classes of monooxygenases, *FMO1* and related flavin monooxygenases of Arabidopsis (Bartsch et al., 2006) were included in the phylogenetic analysis of the *YUCCA1* family. Similarly, the Jasmonate receptor, *COI1* (Thines et al., 2007), was used as an outgroup for the *TIR1* auxin receptor family, and the amino acid transporter AT5G41800 as an outgroup for the *AUX1* family of auxin influx carriers. Maize *ARF* transcription factor nomenclature is based on published results (Xing et al., 2011) and the Grass Transcription Factor Database ([http://grassius.org/tf\\_browsefamily.html?species=Maize](http://grassius.org/tf_browsefamily.html?species=Maize)) (Yilmaz et al., 2009). Only *ARF* genes with a full-length B3 domain were included in the analysis. The list of maize *IAA* genes was taken

from the annotated gene set at [maizesequencing.org](http://www.maizesequencing.org) (<http://www.maizesequencing.org>), which includes the published *IAA* gene list (Wang et al., 2010b) with some modifications to the family members caused by the update of the ZmB73 genome from version 4a.53 to 5a.59. The maize *PIN* gene family nomenclature was taken from published analysis (Forestan et al., 2012) with one additional gene identified by BLAST query of the maize genome (ZmB73 v.5a.59). For the maize *PID/BIF2* protein kinase family, analysis focused on genes in the *PID/PID2/WAG1/WAG2* clade because these genes are functionally redundant for auxin control of cotyledon development (Cheng et al., 2008), although phylogenetic analysis included a larger group of serine-threonine kinases. For all phylogenetic analyses, alignments were made using the ClustalW algorithm in MegAlign (DNASTAR). Phylogenies were produced from these alignments using MrBayes v3.2.0 using default settings for amino acid analysis (Huelsenbeck and Ronquist, 2001). Each MrBayes analysis was performed for 100,000 generations or until the standard deviation of the split frequencies dropped below 0.05. The *PIN1* family analysis was run for 200,000 generations. The *ARF* family was run for 820,000 generations. The *AUX1*, *PID/WAG*, *YUCCA*, *TAA1*, *TIR1*, and ABC transporter family analyses were run for 100,000 generations each. Phylogenetic trees were drawn from the MrBayes files using FigTree v1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>). Gene expression values were taken from RNA-Seq data from Illumina sequencing of B73 mature, freshly shed pollen and B73 5-day old seedling shoot, and combined RNA-Seq data from Illumina sequencing of B73 embryo-sac-enriched samples and ovules with the embryo sacs removed and from SOLiD sequencing of W23 embryo-sac-enriched samples and ovules with the embryo sacs removed (Chettoor et al., 2014). This data was mined for expression levels based on Fragments per Kilobase per Million reads (FPKM) for the genes in the gene families above. Genes were considered up-regulated in the embryo-sac-enriched samples if they were 2-fold higher than the surrounding ovule tissue with an expression threshold above 0.1 FPKM.

### Microscopy of Embryo Sacs

Analysis of fixed embryo sacs by confocal microscopy was performed without additional staining after FAA fixation according to Phillips and Evans (2011) or alternatively after staining with Acriflavine alone or with both Acriflavine and Propidium Iodide. Tissues were stained with Acriflavine as a Schiff reagent as published previously (Vollbrecht and Hake, 1995) and some samples, after Acriflavine staining and before dehydration in ethanol and clearing in Methyl Salicylate, were stained with Propidium Iodide (Running et al., 1995). Acriflavine/Propidium Iodide stained samples were visualized on a Leica Sp5 point-scanning confocal microscope using excitations of 436 and 536 nm and emissions of 540 ± 20 and 640 ± 20 nm. For live cell imaging of fluorescent reporters in maize ovules, dissection of the ovules was performed similarly but without fixation from plants carrying one copy of either the *pHISTONE H1B(GRMZM2G164020)::HISTONE H1B-YFP*, *pDR5::RFP*, *pPIN1(GRMZM2G098643)::PIN1-YFP*, or *pTCS::TCSv2::NLS-tdTTomato* transgene in a B73 inbred background. All transgenic

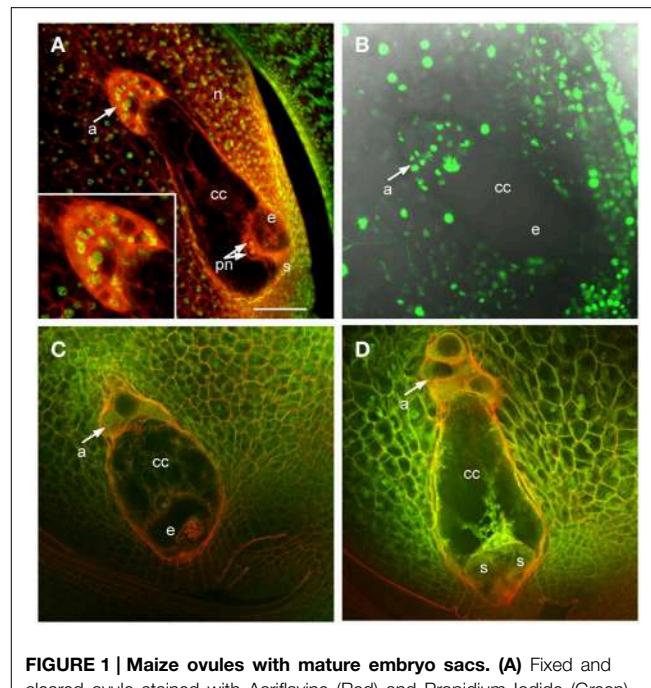
lines were generously supplied by the Maize Cell Genomics Project (<http://maize.jcvi.org/cellgenomics/index.php>) (Mohanty et al., 2009). To dissect out ovules for fluorescence microscopy, freshly harvested ears were kept in a humid environment with the husks only partially removed and only a few ovules dissected at a time. The silk was removed to the base of the silk to expose the ovule. The ovule was then bisected along the longitudinal axis of the ear to produce a cut surface within a few cell layers of the embryo sac. The cut surfaces were then placed against a cover slip in water for observation on an inverted microscope with a Leica SP5 point-scanning confocal microscope using an excitation of 514 nm and an emission of  $550 \pm 20$  nm for YFP, an excitation of 563 nm and an emission of  $600 \pm 20$  nm for RFP, and an excitation of 554 nm and an emission of  $600 \pm 20$  nm for tdTomato. For analysis of effects of *Lxm1-O* on expression of fluorescent reporters *Lxm1-O/+* plants were crossed as males to transgenic hemizygotes. Live cell imaging of ovules of plants hemizygous for the transgene and heterozygous for *Lxm1-O* were examined in the same way as wild type. Ovule/embryo sac staging was performed using silk length as a proxy for ovule age similarly to Huang and Sheridan (1994). Ovules of florets with silks over 15 cm in length were taken as mature stage, with shorter silks an estimate of progressively younger ovules.

## Results

### Maize Antipodal Cells

In maize and other grasses the antipodal cells are unique among embryo sac cells in proliferating after cellularization. This is one of the distinguishing features of grass embryo sacs compared to Arabidopsis, in which the antipodal cells do not proliferate. Analysis of mature embryo sacs reveals that the mature antipodal cells also have fundamental differences from other embryo sac cells. Nuclei of the antipodal cells are very distinct from those of the central cell or egg cell (the synergids have typically degenerated by maturity) and more closely resemble the surrounding nucellar cell nuclei (Figure 1). Egg cell nuclei and particularly polar nuclei have large prominent nucleoli, which stain with Acriflavine as a Periodic Acid Schiff reagent, and the nuclei overall stain faintly with Propidium Iodide. The antipodal cell nuclei, in contrast, lack prominent nucleoli and have an intense speckled staining pattern with Propidium Iodide. Additionally, the Histone H1B gene, GRMZM2G164020, is expressed in the antipodal cells and the nucellus, but not in mature central cells or egg cells (Figure 1B). Based on nuclear staining properties, nucleoli appearance, and Histone H1B expression, the antipodal cell nuclei are much more similar to the nucellar nuclei than they are to the other embryo sac nuclei.

Early after cellularization, most or all of the antipodal cells are cytoplasmically dense and fluoresce intensely with FAA fixation (Figures 1C,D). The size and fluorescent properties of the antipodal cells vary with inbred background (data not shown). The boundary between the antipodal cell cluster and the nucellus stains intensely with Acriflavine as a Schiff reagent. The most chalazal of the antipodal cells is often more vacuolated than the rest in early stages of antipodal cell cluster development; as the



**FIGURE 1 | Maize ovules with mature embryo sacs. (A)** Fixed and cleared ovule stained with Acriflavine (Red) and Propidium Iodide (Green). Inset shows higher magnification of antipodal cell region. **(B)** Live cell imaging of an ovule expressing *pHistoneH1B::HISTONEH1B-YFP*. **(C,D)** Fixed and cleared ovules stained with Acriflavine (Red) and fluorescence from formaldehyde fixation and autofluorescence (Green). a, antipodal cells; cc, central cell; e, egg cell; n, nucellus; pn, polar nuclei; s, synergid. Scale bar = 100  $\mu$ m.

antipodal cell cluster grows and matures more of them become vacuolated.

### Auxin Signaling in Maize Antipodal Cells

To analyze the pattern of auxin signaling in the maize embryo sac, the expression pattern of two fluorescent reporters in maize were studied: a transcriptional reporter of auxin levels, *DR5::RFP*, and a fluorescent protein fusion for a auxin efflux carrier (GRMZM2G098643\_ZmPIN1a) expressed from its native promoter, *pPIN1a::PIN1a-YFP* (Gallavotti et al., 2008). Maize whole embryo sac RNA-Seq data was mined to determine whether this *PIN* gene is likely to be expressed in the embryo sac. RNA-Seq of embryo-sac-enriched samples (with some attached nucellus) was compared to the remainder of the ovule lacking the embryo sac (Chettoor et al., 2014). *ZmPIN1a*, along with three other maize *PIN* genes, is up-regulated in the embryo sac (defined as having 2-fold higher expression in the embryo sac enriched sample compared to the surrounding ovule tissue and expression above 0.1 FPKM) (Table 1 and Table S1). These four maize *PIN* genes fall into three different groups, 1, 10, and 8 (using the nomenclature of Forestan et al., 2012) (Figure 2). Two additional genes are two-fold higher in the embryo sac compared to the surrounding ovule but have expression below the 0.1 FPKM threshold. One gene, GRMZM2G074267 in the clade with *ZmPIN1a* and *AtPIN1*, has the reverse expression pattern with higher expression in the surrounding ovule than the embryo sac.

**TABLE 1 | Expression of gene families related to auxin movement, signaling, and biosynthesis in maize embryo sacs.**

| Gene Family                | Genes up-regulated in the embryo sac compared to surrounding ovule tissue |  |
|----------------------------|---|--|
|                            | 2-fold Higher in ES vs. surrounding<br>ovule and above 0.1 FPKM           | 1.5-fold higher in ES vs. surrounding<br>ovule and above 0.05 FPKM |
|                            |   |  |
| PIN                        | 4 of 10   | 5 of 10  |
| AUX1                       | 1 of 5  | 3 of 5   |
| BR2-like                   | 3 of 8  | 4 of 8   |
| PID                        | 2 of 5  | 2 of 5   |
| ARF                        | 9 of 35   | 11 of 35   |
| AUX/IAA                    | 16 of 37 <sup>†</sup>   | 23 of 37 <sup>†</sup>  |
| TIR1-like                  | 0 of 8  | 2 of 8   |
| YUCCA                      | 5 of 11   | 5 of 11  |
| TAA                        | 4 of 6*   | 5 of 6 <sup>†</sup>  |
| All auxin-related families | 44 of 125 <sup>†</sup>  | 60 of 125 <sup>†</sup>   |
| Whole genome               | 9618 of 39,635 (3414 in surrounding ovule)                                | 13,579 of 39,635 (6354 in surrounding ovule)                       |

<sup>†</sup>Higher than expected based on whole genome frequency of ES up-regulated genes,  $p < 0.01$ .

\*Higher than expected based on whole genome frequency of ES up-regulated genes,  $p < 0.05$ .

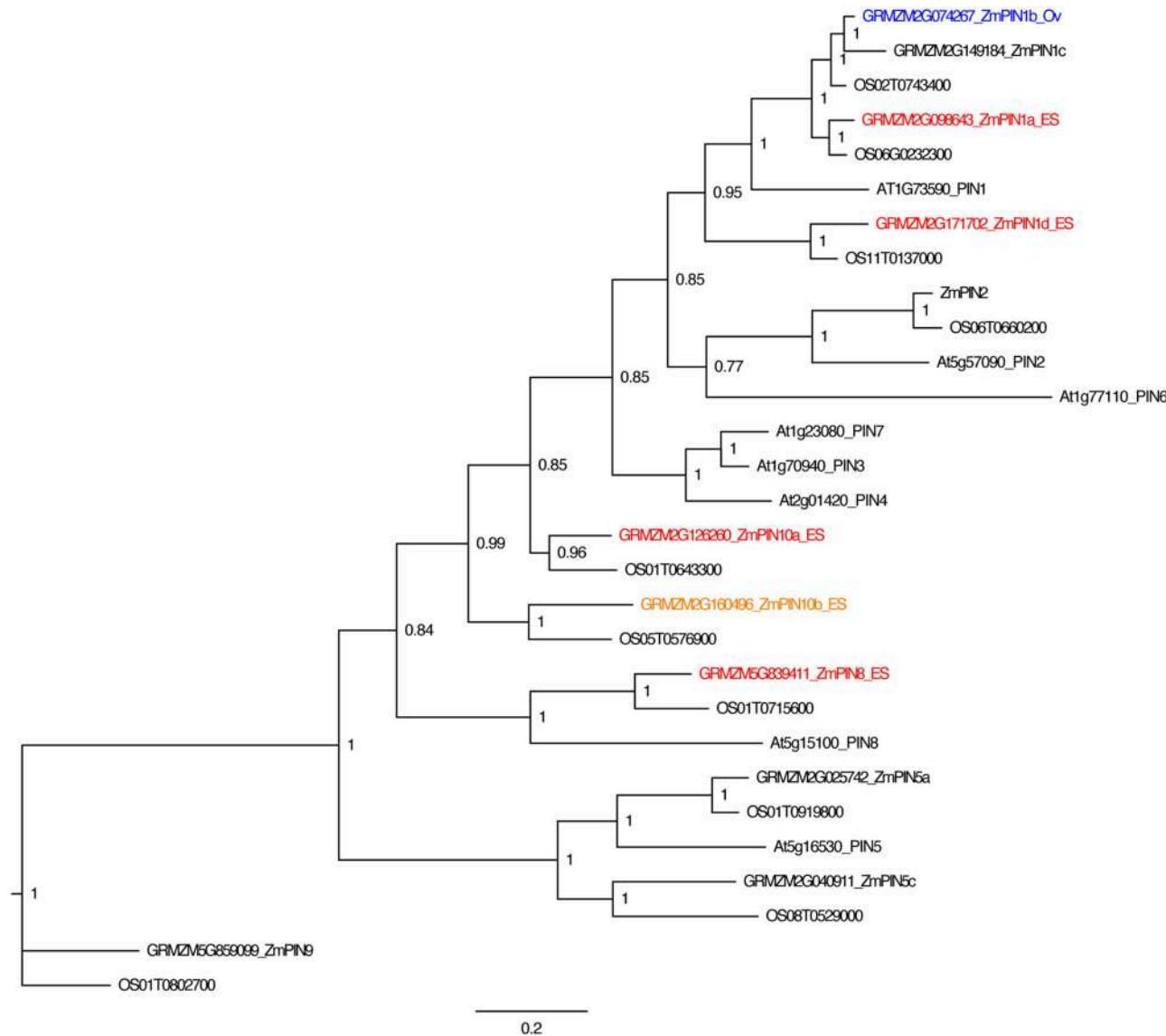
*PIN1a* has complex patterns in the antipodal cell cluster of mature maize embryo sacs (Figure 3 and Table 2). *PIN1a-YFP* is detectable early in antipodal cell development at least as early as the 6–10 cell stage in all but the most chalazal antipodal cells (Figures 3G,H). In later stages, the most common patterns of *PIN1a-YFP* expression are: expression throughout the antipodal cell cluster (Figure 3I), expression in all cells of the antipodal cell cluster except the most chalazal cell (Figure 3J), and expression in the micropylar portion of the antipodal cell cluster with multiple cells at the chalazal end lacking (or with reduced) expression of *PIN1a-YFP* (Figure 3K). Less frequently, *PIN1a-YFP* protein is expressed in all but the micropylar domain of antipodal cells or all but the center of the antipodal cell cluster. The least frequent pattern has *PIN1a-YFP* expression only in the center of the antipodal cell cluster. Using the positions of the cell walls with the strongest expression of *PIN1a-YFP* as a proxy for the direction of auxin flow the two most common patterns are outward from the antipodal cell cluster and away from the central cell or random within the antipodal cell cluster. These patterns suggest that the auxin efflux pattern is dynamic but within the antipodal cell cluster. Whether these patterns represent different stages in mature or nearly mature antipodal cell clusters is difficult to determine without being able to maintain their growth *in vitro*.

*DR5* expression is also detected early after cellularization, at least as early as the 6–10 cell stage in all cells except the most chalazal antipodal cell (Figures 3B,C). At maturity, maximal expression of *DR5* in the embryo sac is detected in the chalazal-most cell of the antipodal cell cluster (Figure 3D). Interestingly, this is the same cell that often has the lowest expression of *PIN1a-YFP*. Increasing the sensitivity for detection of lower levels of *DR5*-driven RFP expression reveals that *DR5* expression is higher in all of the antipodal cells than the surrounding nucellar cells (Figure 3E). Since the *pDR5::RFP* construct is hemizygous, half of the embryo sacs do not carry the transgene and consequently do not express *RFP*. Without the interfering antipodal cell

fluorescence in these ovules, it was possible to reveal *DR5* expression in the nucellus at a lower level than any of the antipodal cells. Nucellar *DR5* expression is located in the cells immediately adjacent to the chalazal end of the embryo sac surrounding the antipodal cells (Figure 3F). Similarly, in the ovules of *pPIN1a::pPIN1a-YFP* hemizygotes in which the embryo sac did not inherit the transgene, low *PIN1a* expression could be detected in the nucellus adjacent to the antipodal cells (Figure 3M). Elsewhere in the ovule, expression of *DR5* and *PIN1a-YFP* is seen in the integuments and the micropylar nucellus between the embryo sac and the micropyle (Figures 3A,B,M).

One model for regulation of embryo sac development is an antagonistic relationship between auxin and cytokinin. Interplay between auxin and cytokinin are involved in other processes (Moubayidin et al., 2009), including an inverse correlation between expression of *DR5* and the cytokinin-responsive TCS promoter in the embryo (Muller and Sheen, 2008). To test if cytokinin signaling antagonizes auxin in the embryo sac the TCS promoter was examined to determine if there is an inverse correlation between *DR5* and TCS in the embryo sac. While no expression from the TCS promoter was detectable within the embryo sac, the nucellar cells immediately chalazal to the antipodal cells express the TCS reporter (Figures 3N,O). These are the same cells that have low-level *DR5* expression (i.e., below the level of the antipodal cells but above that of other nucellar cells).

*DR5* expression reveals that transcriptional responses to auxin are active in the antipodal cells presumably through the release of ARF transcription factors from AUX/IAA proteins in the presence of high auxin. To identify the endogenous transcriptional targets of auxin in the embryo sac it is necessary to determine which ARFs are expressed in these cells to control transcriptional changes in response to auxin. Additionally, to determine if other proteins involved in auxin transport and the local synthesis of auxin contribute to the pattern of auxin responses, transcriptome data from embryo-sac-enriched (ES) tissue samples and from the



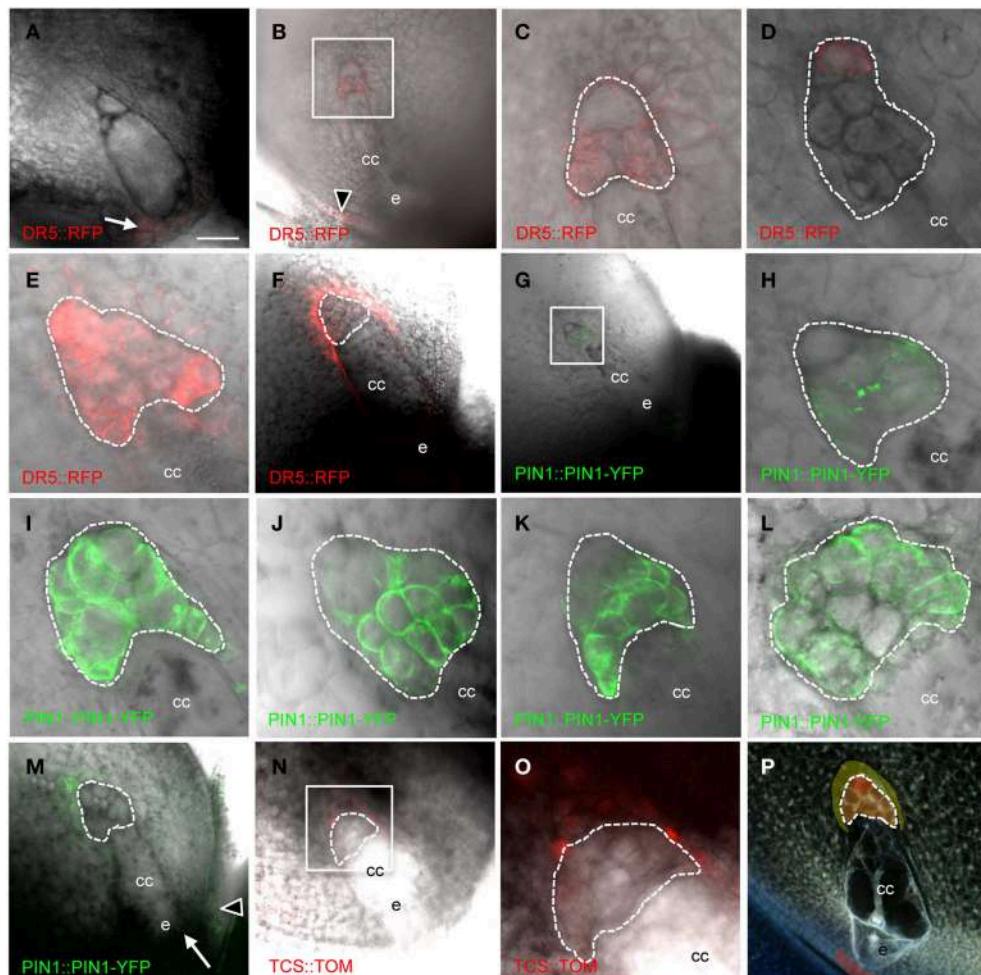
**FIGURE 2 | PIN gene family of maize.** Phylogenetic relationships of maize and Arabidopsis PIN genes. Maize PIN genes up-regulated two-fold in the embryo-sac-enriched samples (and over 0.1 FPKM) compared to the surrounding ovule tissue are indicated in red, while genes with higher expression in the surrounding ovule tissue than the

embryo sac are indicated in blue. Genes indicated in orange have higher expression in the embryo sac than the surrounding ovule but either fall below the 0.1 FPKM cutoff or are only 1.5 to 2.0 fold higher in the embryo sac compared to the ovule. Gene names are according to Forestan et al. (2012).

surrounding ovule tissue without embryo sacs (Ov) were mined for multiple components of auxin signaling and biosynthesis (Table 1 and Tables S2–S9).

The set of auxin related genes is over-represented in the ES up-regulated gene set compared either to the whole genome or to Ov up-regulated genes (Table 1). In addition to the PIN proteins, the *AUX1*-like auxin influx carriers, *BR2*-like ABC transporter families, and *PINOID* type protein kinases have members with higher expression in the embryo-sac-enriched tissue than the surrounding ovule suggesting these pathways are operating in the maize embryo sac (Tables S2–S4, Figures S1–S3). In contrast to

Arabidopsis which expresses *AUX1* but not *LAX1*, 2, or 3 in the embryo sac (Litviev et al., 2013) maize embryo sacs express genes in the *LAX2/LAX3* half of this family (Figure S1). Auxin distribution depends on the localization of auxin biosynthesis as well as transport (Zhao et al., 2001). Members of the gene families for both steps of auxin biosynthesis—by TAA1 aminotransferases and *YUCCA* flavin monooxygenases—are up-regulated in the embryo sac compared to the surrounding ovule (Tables S5,S6; Figures S4,S5). Therefore, it is likely that the auxin that accumulates in the antipodal cells is synthesized locally, either in or adjacent to the embryo sac. The *YUCCA* genes up-regulated in



**FIGURE 3 |** Cellularized maize embryo sacs showing expression in the antipodal cells of (A–F) DR5::RFP reporter, (G–M) PIN1a::PIN1a-YFP reporter, and (N–O) TCS::TOMATO reporter. The chalazal tip of the antipodal cell cluster is oriented toward the upper left.

(A) Embryo sac just prior to cellularization. (B,C,G,H) Early post-cellularized embryo sac with 6–10 antipodal cells. (D–F;I–P) Mature embryo sacs. (B–E) DR5::RFP expression in the antipodal cells. (F) DR5::RFP expression in the sporophytic tissues of the nucellus. (C)

Boxed region in (B). (H) Boxed region in (G). (O) Boxed region in (N). (P) Model for auxin and cytokinin signaling in the mature maize embryo sac. Red indicates region of highest auxin signaling; orange indicates moderate auxin signaling; and yellow indicates low auxin signaling plus cytokinin signaling. Dashed lines indicate boundary of antipodal cells. cc, central cell; e, egg cell. Arrow indicates micropylar nucellus, and arrowhead indicates integuments. Scale bar = 100  $\mu\text{m}$  (A,B,F,G,M,N,P) and = 33  $\mu\text{m}$  (C–E,H–L,O).

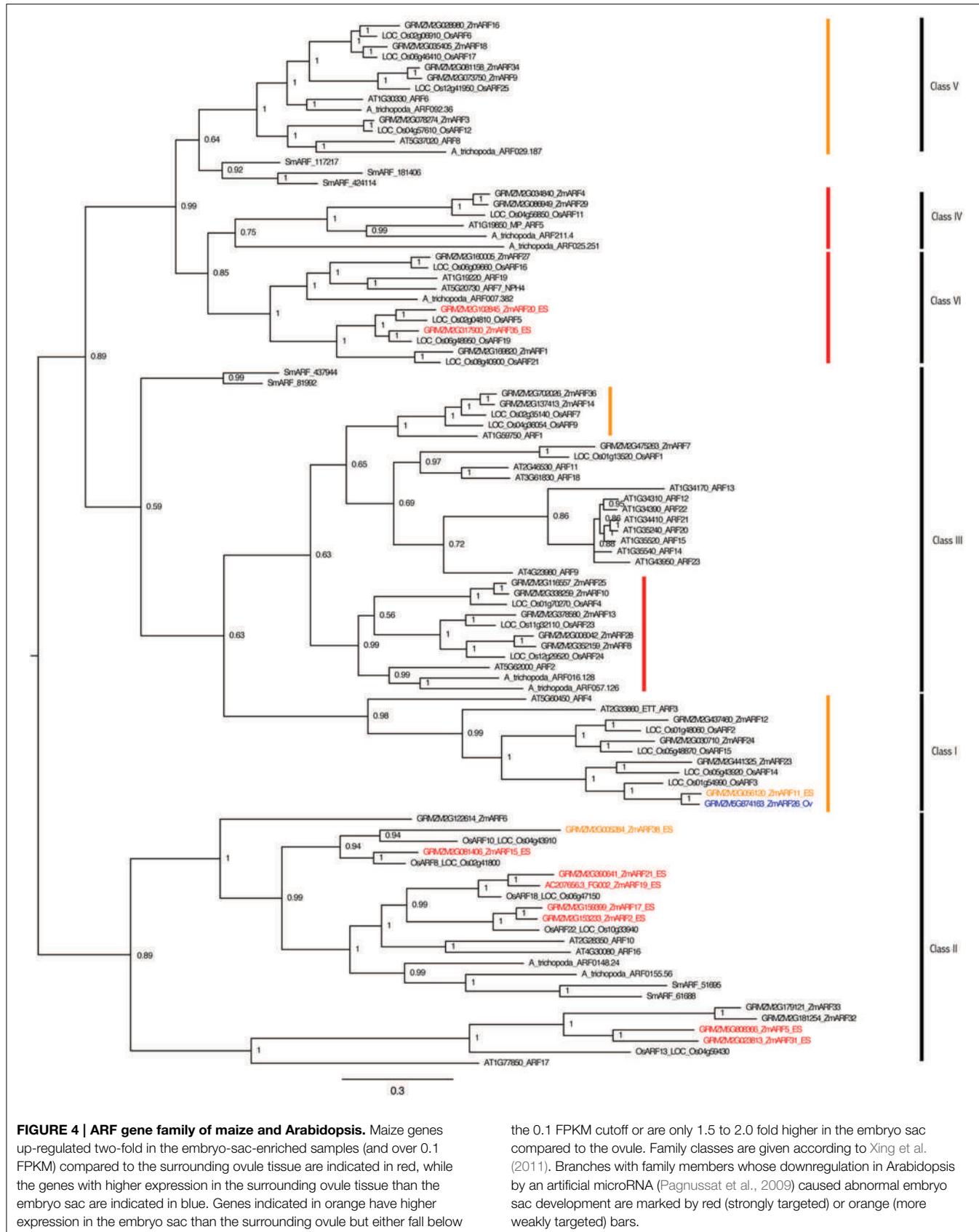
**TABLE 2 |** Patterns of expression of pPIN1::PIN1-YFP in mature antipodal cell clusters.

| All antipodal cells | Expression absent (or Reduced) in the Chalazal-most antipodal cell | Expression absent in multiple antipodal cells at the Chalazal end | Expression absent in the micropylar domain of the antipodal cell cluster | Expression absent in the center of the antipodal cell cluster | Expression only in the center of the antipodal cell cluster |
|---------------------|--|---|--|---|---|
| 35                  | 34   | 31  | 8  | 11  | 2   |

the embryo sac fall into the *YUCCA10* and *YUCCA2/6* branches (Figure S5).

Gene families involved in auxin perception and response were also examined for embryo sac expression. Representatives

of the *TIR1*, *ARF*, and *IAA* gene families have higher expression in the embryo-sac-enriched samples than the surrounding ovule (Tables S7–S9; Figure 4, Figures S6,S7). RNA-seq analysis revealed that nine of the thirty-five *ARF* genes are expressed



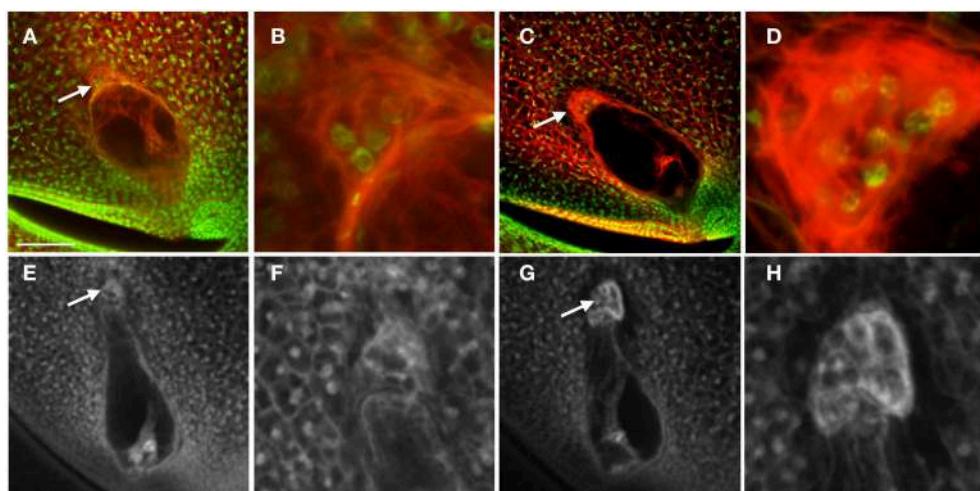
2-fold higher in the embryo-sac-enriched samples than the surrounding ovule tissue plus two more with a weaker increase in the embryo-sac-enriched samples (Table S8). The Class II *ARF* group is over-represented among ES up-regulated genes; seven of the eleven Class II genes have higher expression in the embryo sac enriched samples, while only two of the remaining twenty-four *ARF* genes do, both of which are in Class VI (**Figure 4**). The function of this clade in embryo sac development is unknown. In *Arabidopsis*, combined down-regulation of several *ARF* genes caused abnormal embryo sac development (Pagnussat et al., 2009), but the artificial microRNA targeting *ARFs* in this study did not cover the Class II group, which includes most of the genes with increased expression in the maize embryo-sac-enriched samples.

### **Laxmidrib1 Mutants Interfere with Proliferation and Auxin Signaling in the Antipodal Cell Cluster**

To determine a potential function for the auxin maximum in the antipodal cells, we then examined the effect of an antipodal cell mutant on the expression of the *DR5* and *PIN1* fluorescent reporters. Because the *indeterminate gametophyte1* (*ig1*) mutation affects both leaf polarity and embryo sac development (Evans, 2007), other leaf polarity mutants were examined for effects on embryo sac morphology. The *Laxmidrib1-O* (*Lxm1-O*) mutant is a dominant mutant with adaxialized leaves. Sectors of adaxial tissue are produced on the abaxial side of leaves with ectopic leaf flaps produced on either side of these sectors (the opposite leaf polarity defect as *ig1*) (Schichnes et al., 1997; Schichnes and Freeling, 1998). Approximately one third of the embryo sacs in *Lxm1-O/+* heterozygotes in a W23 inbred background are abnormal (39/122) (**Figure 5**). These embryo sacs have fewer antipodal cells than their wild-type siblings (six antipodal cell nuclei in **Figures 5A,B**), indicating that proliferation of the antipodal cells in mutant embryo sacs is reduced or

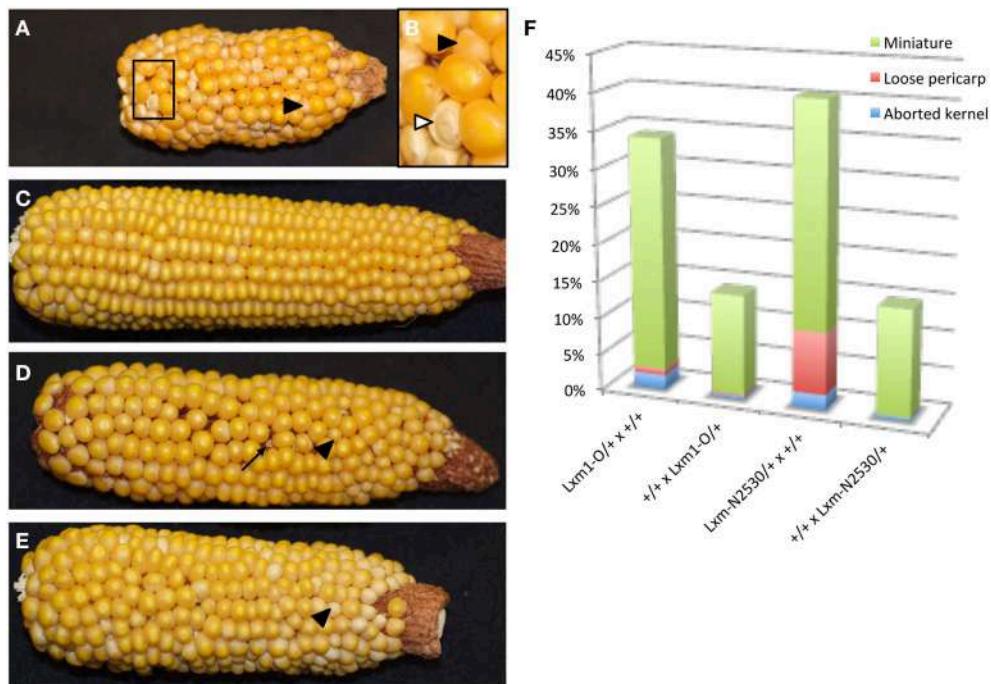
in some cases absent. The size and morphology of these antipodal cells are similar to wild type, however, and, like wild type, the nuclei have speckled staining with Propidium Iodide and lack prominent nucleoli. The overall size and morphology of the central cell, egg cell, and synergids are not affected by *Lxm1-O*. A second mutation, *Lxm\*-N2530*, which has similar effects on leaf development as *Lxm1-O*, also produces embryo sacs with smaller antipodal cell clusters than wild type, supporting the argument that this is a result of the *Lxm* mutations rather than a second mutation segregating in the background (**Figures 5E–H**). The normal and mutant antipodal cells of the *Lxm\*-N2530* line are slightly larger and more vacuolated than those of the *Lxm1-O* line, but these mutations are in different inbred backgrounds.

Heterozygotes for both *Lxm* mutations also produce miniature seeds of different severity and frequency depending upon the direction of the cross (**Figure 6**). *Lxm1-O/+* and *Lxm\*-N2530/+* females segregate kernels that are small and pale with a loose pericarp. Progeny testing of kernels from crosses of *Lxm/+* females by homozygous wild-type males revealed that inheritance of the mutation (i.e., the fertilization of mutant embryo sacs) is correlated with the miniature kernel phenotype (19/21 miniatures that were tested had inherited *Lxm* but only 2/31 normal kernels tested had inherited *Lxm*). Although the crosses of *Lxm/+* males onto wild type do not produce the reduced endosperm, loose pericarp class of kernels, some crosses do produce a less severe miniature kernel type (**Figure 6E**), particularly in crosses using *Lxm* plants with the most severe leaf phenotype. When crossed as females, these severe *Lxm* heterozygotes produce some miniatures and some early aborting kernels and have partial sterility (**Figure 6D**). All abnormal kernel types are more common in crosses with *Lxm* females than males, especially the most severe classes (**Figure 6F**). The loose pericarp and aborted



**FIGURE 5 | Effect of *Lxm* mutations on embryo sac development.** **(A–D)** Embryo sacs from a *Lxm1-O/+*; W23 heterozygote fixed in FAA and stained with Acriflavine and Propidium Iodide and **(E–H)** Embryo sacs from a *Lxm\*-N2530* heterozygote in a hybrid genetic background fixed in FAA. **(A,B,E,F)**

Embryo sacs with abnormal antipodal cell clusters. **(C,D,G,H)** Normal sibling embryo sacs for each mutant line. **(B,D,F,H)** are magnifications of the antipodal cells in **(A,C,E,G)**, respectively. Arrows indicate antipodal cell cluster. Scale bar = 100  $\mu$ m **(A,C,E,G)** and = 33  $\mu$ m **(B,D,F,H)**.



**FIGURE 6 | Reciprocal crosses between *Lxm1*/+ and wild-type siblings.** Black arrowheads point to miniature kernels. (A) *Lxm1*/+ female with mild leaf phenotype crossed by wild-type male. (B) Enlargement of boxed region in (A). White arrowhead points to a kernel with a loose pericarp. (C) Wild-type female crossed by *Lxm1*/+ male with mild leaf

phenotype. (D) *Lxm1*/+ female with strong leaf phenotype crossed by wild-type male. Arrow points to an aborted kernel. (E) Wild-type female crossed by *Lxm1*/+ male with strong leaf phenotype. (F) Frequency of abnormal kernel types in reciprocal crosses between *Lxm1*-O or *Lxm-N2530* heterozygotes and homozygous wild type. Female genotypes are listed first.

kernel phenotypes are rarely seen in crosses with *Lxm1*-O or *Lxm*<sup>\*</sup>-N2530 males but are found in females of both mutants.

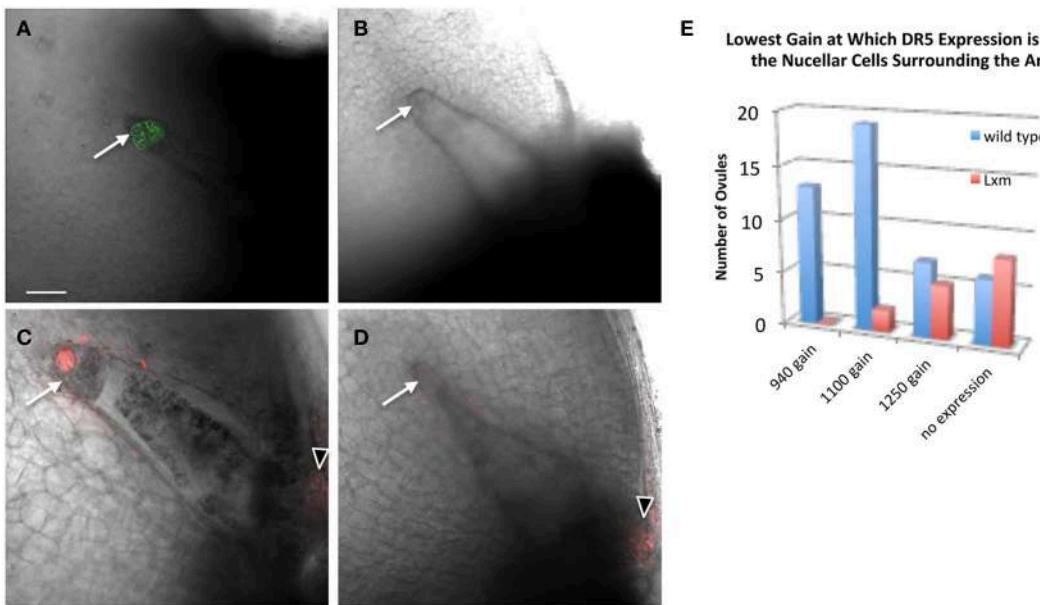
To test whether *Lxm1*-O affects auxin distribution in the embryo sac, *pPIN1a::PIN1a-YFP* and *pDR5::RFP* were crossed with *Lxm1*-O/+ mutant lines. Plants heterozygous for *Lxm1*-O and hemizygous for either *pPIN1a::PIN1a-YFP* or *pDR5::RFP* were examined for effects of *Lxm1*-O on auxin signaling in the embryo sac. Examination of mutant plants revealed that the *Lxm1*-O mutation interferes with expression of both *PIN1a* and *DR5* in maize antipodal cells (Figure 7 and Table 3). In plants heterozygous for *Lxm1*-O and hemizygous for *pPIN1a::PIN1a-YFP*, approximately half of the normal embryo sacs express the transgene, as expected, but none of the abnormal embryo sacs express the transgene. The effect of *Lxm1*-O on *pDR5::RFP* expression is essentially the same as for *pPIN1a::PIN1a-YFP*, with only 1 of 17 abnormal embryo sacs expressing the transgene, with the one exceptional individual having an intermediate antipodal cell phenotype. For *pDR5::RFP* fewer than half of the wild type embryo sacs express the transgene, perhaps reflecting silencing of this construct, but still a significantly higher frequency of normal embryo sacs than mutant embryo sacs are positive for *DR5*.

The effect of a small antipodal cell cluster on *DR5* expression in the surrounding nucellar cells was also examined in the *Lxm1* mutant. A comparison was made between ovules which surrounded either normal or mutant embryo sacs, from the same heterozygous *Lxm1*/+ mutant. The settings of the microscope

needed to detect *DR5* expression were used as a proxy for the relative expression level of *DR5* in these ovules. Single mid-plane images were collected at three different gain settings to reduce the effects of photo-bleaching and each ovule was evaluated for the lowest setting at which *DR5* expression could be detected. *Lxm1* has a quantitative effect on the expression of *DR5* in the nucellar cells surrounding the antipodal cell cluster (Figure 7E). A higher gain is necessary to detect *DR5* in the nucellar cells around the small antipodal cell clusters of *Lxm1* mutant embryo sacs than around those of wild-type embryo sacs, indicating that *DR5* has lower expression around mutant embryo sacs than wild-type embryo sacs despite the fact that these two sets of nucellar cells are from ovules of the same plant and are therefore genetically identical.

## Discussion

Little is known about angiosperm antipodal cell function and development. The only evidence for antipodal cell function is based on implications from ultrastructural data (Diboll, 1968). The antipodal cells have many features that distinguish them from their sibling embryo sac cells, including: nuclear morphology, sucrose synthase activity, and cell wall invaginations. Some genetic evidence is also available for regulation of antipodal cell development including the influence of the central cell on antipodal cell persistence in *Arabidopsis* (Kagi et al., 2010) and of the egg cell on antipodal cell identity in maize (Krohn et al.,



**FIGURE 7 | Effect of *Lxm1*-O on embryo sac expression of *pPIN1::PIN1-YFP* and *pDR5::RFP*.** Live cell imaging of sibling (**A,C**) wild-type and (**B,D**) *Lxm1*-O embryo sacs from *Lxm1*-O heterozygotes segregating either (**A,B**) *pPIN1::PIN1-YFP* or (**C,D**) *pDR5::RFP*. (**A**) Embryo sac expressing *pPIN1::PIN1-YFP*. (**C**) Embryo sac expressing *pDR5::RFP*. The small antipodal cell cluster distinguishes *Lxm1*-O from wild-type. Neither *DR5* nor *PIN1* are expressed in mutant *Lxm1*-O embryo sacs. Arrows indicate antipodal cell cluster. Arrowheads indicate

*DR5* expression in the micropylar nucellus. Scale bar = 100  $\mu$ m. (**E**) Embryo sacs were visualized at three different sensitivity settings for *DR5* expression: a gain of 940, 1100, or 1250. This was used as a proxy for relative *DR5* expression between embryo sacs. For most of the normal embryo sacs, *DR5* expression could first be detected at the lower gain settings used, while for most of the mutant embryo sacs *DR5* expression either was not detected at all or was only detected at the highest setting.

2012). Auxin signaling has been shown to occur in the maize antipodal cells (Lituvie et al., 2013), but no function for auxin in the embryo sac of maize was shown.

Localization of PIN1a protein suggests that auxin is transported away from the central cell and often toward the chalazal tip of the cluster, where the highest expression of *DR5* is located. Lower levels of *DR5* expression are found throughout the antipodal cells and an even lower level in the surrounding nucellar cells. Indeed, PIN1a localization suggests that auxin is also transported from the antipodal cells into the surrounding cell layers. Analysis of antipodal cell morphology and *DR5* and *PIN1a* expression patterns reveals a dynamic pattern within the antipodal cell cluster. The chalazal-most antipodal cell of the cluster is unique, with unusual morphology and absence of *DR5* and *PIN1a* expression early in antipodal cell development and the highest level of *DR5* expression late. Several patterns of *PIN1a* expression were also seen in multicellular, mature or nearly mature, antipodal cell clusters. Whether or not these different patterns represent a development progression within antipodal cell clusters that are morphologically similar is unclear.

One hypothesis for regulation of embryo sac development is an antagonistic relationship between auxin and cytokinin with an inverse correlation between *DR5* and *TCS* expression as occurs during development of the embryonic root pole (Muller and Sheen, 2008). Interaction between auxin and cytokinin has been shown to act in root development (Moubayidin et al., 2009) shoot apical meristem development (Lee et al., 2009) and shoot

branching (Shimizu-Sato et al., 2009). However, neither *DR5* nor *TCS* are expressed in the micropylar or central domains of the embryo sac (e.g., the central cell or the egg apparatus), suggesting that embryo sac cell identity is not regulated by antagonism of auxin and cytokinin signaling within the embryo sac. However, *TCS* expression was detected in the nucellus surrounding the antipodal cells, a region that also has weak auxin signaling as revealed by *DR5* expression. It may be that interplay between cytokinin and auxin in these cells is important to prevent proliferation of the nucellus next to the growing embryo sac. Promotion of cell division by auxin in the antipodal cells and antagonism of this action by cytokinin would be similar to the effects of auxin and cytokinin in promoting and inhibiting, respectively, the early divisions that establish lateral root primordia (Laplaze et al., 2007). The overlap of *TCS* expression with the lower expression of *DR5* in the nucellus around the antipodal cells and the absence of *TCS* from the antipodal cells that have higher expression of *DR5* is similar to the relationship between the expression of *TCS* and *DR5* in the embryonic root stem cell lineage with *TCS* and low *DR5* expression in the lenticular cell and *DR5* expression in the basal cell (Muller and Sheen, 2008).

In the mature maize embryo sac both *DR5* and *PIN1* are expressed strongly and specifically in the antipodal cell cluster, in stark contrast to Arabidopsis. This raises the possibility that the difference in auxin levels in the maize and Arabidopsis embryo sacs may be responsible for their different antipodal cell biology. One prediction based on these possibilities is that impaired auxin

**TABLE 3 | Expression of *PIN1* and *DR5* reporters in embryo sacs of plants heterozygous for *Lxm1* and hemizygous for the transgene.**

|   | Wild type without<br>fluorescent reporter expression | Wild type with fluorescent<br>reporter expression | <i>Lxm1</i> without fluorescent<br>reporter expression | <i>Lxm1</i> with fluorescent<br>reporter expression |
|---|--|---|--|---|
| <i>Lxm1</i> -O/+ <i>pPIN1::PIN1-YFP</i> - | 16   | 16  | 13   | 0   |
| <i>Lxm1</i> -O/+ <i>pDR5::RFP</i> -       | 51   | 34  | 17   | 1*  |

\*intermediate phenotype, either mild mutant phenotype or wild-type with smaller antipodal cluster.

signaling or reduced auxin levels in the antipodal cells could disrupt their identity and/or proliferation. The dominant *Lxm1*-O mutation blocks proliferation of and auxin signaling in the antipodal cells. Despite failing to proliferate, *Lxm* mutant antipodal cells have normal morphology based on size, staining, and fluorescent properties. The phenotype of *Lxm1* mutants supports a model in which auxin promotes antipodal cell growth rather than antipodal cell identity. *Lxm1*-O plants also have defects in leaf polarity, leaf primordia size, and flowering time (Schichnes et al., 1997; Schichnes and Freeling, 1998). It has yet to be determined if these other *Lxm1*-O phenotypes are associated with defects in auxin signaling, but all of these processes are impacted by auxin (Reinhardt et al., 2000; Ellis et al., 2005; Okushima et al., 2005; Garcia et al., 2006; Richter et al., 2013; Qi et al., 2014).

The nucellar cells of *Lxm1*-O/+ ovules surrounding wild-type embryo sacs express *DR5* normally, but those surrounding mutant embryo sacs do not. This is consistent with a model in which the nucellar *DR5* expression depends on the antipodal cell cluster rather than being cell-autonomous or dependent on other nucellar cells. Under this model, the *Lxm1*-O mutation would interfere with auxin signaling in the nucellus by reducing the source of auxin for these cells from the antipodals. The rate-limiting step for auxin synthesis is performed by the YUCCA class of flavin monooxygenases (Dai et al., 2013). Maize genes in *YUC10* and *YUC2/6* branches of this family show higher expression in the embryo sac enriched samples than the surrounding ovule tissue, consistent with local auxin synthesis, perhaps in the antipodal cells themselves.

Since the antipodal cells persist after fertilization in maize and other grasses (Randolph, 1936; Engell, 1994), they may even act as a transfer tissue for early seed development. One prediction for this model is that a reduction of antipodal cell transfer ability, possibly by reducing cluster size, would reduce growth of the seed and/or embryo sac. The maize *stunter1* mutant causes a reduction in antipodal cell number, central cell size, and seed size, but also causes a reduction in early stage female gametophytes before cellularization, suggesting that the *stt1* gene product is involved in growth of the embryo sac and antipodal cells independently of each other rather than a reduced antipodal cell cluster causing embryo sac and seed size reduction (Phillips and Evans, 2011). Consequently, the effects of *Lxm1* on antipodal cell development and seed size were also analyzed. Reciprocal crosses between *Lxm1* and wild type revealed that mutant females produce small kernels at a greater severity and frequency than mutant males.

The causes of the miniature kernel phenotype of *Lxm* mutants are potentially complex, however. The fact that the miniature kernel phenotype correlates with inheritance of the *Lxm* mutation

through the embryo sac demonstrates that this miniature phenotype is not an incompletely penetrant maternal sporophyte effect (e.g., variable ovule morphology causing some seeds to develop abnormally), but rather a consequence of the genotype of the embryo sac or the endosperm. Seed growth may be sensitive to dosage of the dominant *Lxm* mutations in the endosperm rather than being a consequence of fertilization of abnormal embryo sacs. This is consistent with the milder effect on seed size when using *Lxm* as a male than a female since there would be only one copy of *Lxm1*-O in the endosperm compared to two copies of *Lxm1*-O in the endosperm when used as a female parent. However, it is also possible that the stronger effect of *Lxm* through the female is a combination of a post-fertilization effect of *Lxm* in the endosperm plus a maternal effect of *Lxm*, potentially because of the abnormal antipodal cells. To determine if the antipodal cells play a role in seed size, additional experiments are necessary, such as the targeted ablation of the antipodal cells or analysis of less pleiotropic mutants to determine if antipodal cell defects cause maternal gametophyte effects on seed development.

The antipodal cell growth pattern that is widespread in the grasses suggests that the antipodal cells serve a common function in the grasses. The presence of cell wall invaginations on the sides of antipodal cells facing the nucellus (Diboll, 1968) suggests that the antipodal cell cluster functions as a transfer tissue for the embryo sac. Auxin may function to promote the growth of the antipodal cells for this function. In contrast, the lack proliferation and auxin signaling in these cells may correlate with the lack of this function in *Arabidopsis*. However, as in maize, the antipodal cells of *Arabidopsis* can persist through embryo sac development and after fertilization (Randolph, 1936; Song et al., 2014). Perhaps the antipodal cells serve as a signaling center providing positional information for the embryo sac or developing endosperm in maize and *Arabidopsis*, while the transfer tissue function has a more limited distribution.

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## Supplementary Material

The Supplementary Material for this article can be found online at: <http://www.frontiersin.org/journal/10.3389/fpls.2015.00187/abstract>

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# Establishment of embryonic shoot–root axis is involved in auxin and cytokinin response during *Arabidopsis* somatic embryogenesis

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Auxin and cytokinin signaling participates in regulating a large spectrum of developmental and physiological processes in plants. The shoots and roots of plants have specific and sometimes even contrary responses to these hormones. Recent studies have clearly shown that establishing the spatiotemporal distribution of auxin and cytokinin response signals is central for the control of shoot apical meristem (SAM) induction in cultured tissues. However, little is known about the role of these hormones in root apical meristem (RAM) initiation. Here, we found that the expression patterns of several regulatory genes critical for RAM formation were correlated with the establishment of the embryonic root meristem during somatic embryogenesis in *Arabidopsis*. Interestingly, the early expression of the *WUS-RELATED HOMEOBOX 5* (*WOX5*) and *WUSCHEL* genes was induced and was nearly overlapped within the embryonic callus when somatic embryos (SEs) could not be identified morphologically. Their correct expression was essential for RAM and SAM initiation and embryonic shoot–root axis establishment. Furthermore, we analyzed the auxin and cytokinin response during SE initiation. Notably, cytokinin response signals were detected in specific regions that were correlated with induced *WOX5* expression and subsequent SE formation. Overexpression of the *ARABIDOPSIS* RESPONSE REGULATOR genes *ARR7* and *ARR15* (feedback repressors of cytokinin signaling), disturbed RAM initiation and SE induction. These results provide new information on auxin and cytokinin-regulated apical–basal polarity formation of shoot–root axis during somatic embryogenesis.

**Keywords:** shoot–root axis, root apical meristem, cytokinin response, auxin response, somatic embryogenesis, *Arabidopsis*

## INTRODUCTION

The most critical event during embryogenesis appears to be the formation of the shoot apical meristem (SAM) and root apical meristem (RAM), from which almost the entire plant is post-embryonically established (Meinke, 1991; Scheres, 2007). In the SAM of *Arabidopsis*, *WUSCHEL* (*WUS*) is a critical regulator, and encodes a homeodomain protein that is required for stem cell formation and maintenance (Laux et al., 1996). *WUS* is switched on in the four inner cells of the pro-embryo at the 16-cell globular stage, and is an early molecular marker for SAM initiation in the embryo (Weigel and Jürgens, 2002).

Root growth and development are sustained by the RAM, which is formed during embryogenesis (Sabatini et al., 2003; Petricka et al., 2012). Embryonic RAM formation is initiated at the globular stage, when the uppermost cell of the suspensor—the hypophysis—is recruited in the embryo proper. After asymmetric division of the hypophysis, the small descendant cell gives rise to the quiescent center (QC), which maintains stem cell identity in the surrounding cells of the RAM to produce a set of differentiated tissues (Möller and Weijers, 2009; Peris et al., 2010). Mutants that fail to form the hypophysis often produce rootless seedlings (Möller and Weijers, 2009). An element required in the QC to

maintain columella stem cells is *WUS-RELATED HOMEOBOX 5* (*WOX5*), a putative homeodomain transcription factor (Haecker et al., 2004). In the QC, *WOX5* acts in a similar way to *WUS* in the organizing center (OC) of the SAM, highlighting molecular and developmental similarities between the stem cell niches of both root and shoot meristems (Perilli et al., 2012). In addition, several other putative transcription factors have been shown to contribute to embryonic RAM formation. *PLETHORA* (*PLT*) genes, which belong to the AP2-type transcription factor family, play a key role in the specification and maintenance of root stem cells from early embryogenesis onward (Aida et al., 2004; Galinha et al., 2007). Ectopic *PLT* expression in the embryo induces transformation of apical domain cells into root stem cells (Aida et al., 2004). The *SCARECROW* (*SCR*)/*SHORTROOT* (*SHR*) transcription factors are required to maintain stem cell activity within the RAM (Di Laurenzio et al., 1996; Helariutta et al., 2000; Sabatini et al., 2003).

Auxin and cytokinin are required for cell differentiation and specification during embryogenesis (Müller and Sheen, 2008; Möller and Weijers, 2009). Asymmetric distribution of auxin mediated by auxin polar transport establishes the apical–basal axis of the embryo, showing that auxin is required for pattern

formation of the embryo (Friml et al., 2003; Möller and Weijers, 2009). Cytokinin signaling components function in the hypophysis at the early globular stage of the embryo (Müller and Sheen, 2008). After the first division of the hypophysis, the apical daughter cell maintains the phosphorelay activity of cytokinin signaling, whereas cytokinin signaling is repressed in the basal daughter cell. In early embryogenesis, auxin antagonizes cytokinin signaling through direct transcriptional activation of *ARABIDOPSIS RESPONSE REGULATOR (ARR)7* and *ARR15*, feedback repressors of cytokinin signaling in the basal cell (Hwang and Sheen, 2001; Müller and Sheen, 2008; Buechel et al., 2010).

Somatic embryogenesis is generally believed to be mediated by a signaling cascade triggered by exogenous auxin (Skoog and Miller, 1957; Sugiyama, 1999, 2000). Indeed, our previous work has shown that the establishment of auxin gradients is correlated with induced *WUS* expression and subsequent embryonic SAM formation during somatic embryogenesis (Su et al., 2009). It has also been suggested that the establishment of the RAM in somatic embryos requires an appropriate auxin gradient (Bassuner et al., 2007). However, the mechanism by which root stem cell specification occurs during early somatic embryogenesis is far from understood. Here, we analyzed the expression patterns of a few critical marker genes involved in RAM formation and development during somatic embryogenesis. Besides the auxin gradients that are established in specific regions of embryonic callus, we found that the spatiotemporal cytokinin response was correlated with RAM formation. Such cytokinin response patterns were critical for spatial induction of RAM-specific genes, such as *WOX5* and *PLT*, and subsequent RAM establishment in the embryonic callus. Our results reveal the distinct functions of cytokinin and auxin signaling required for RAM and SAM induction and shoot–root axis establishment during early somatic embryogenesis.

## MATERIALS AND METHODS

### PLANT MATERIALS

All *Arabidopsis* mutants and transgenic lines used in this study were Columbia ecotypes. The *pWOX5::GFP*, *pPLT2::RFP*, *pSCR::GFP* reporter lines and *plt2-1* mutants were kindly provided by Dr. C. Li (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences). *pWUS::DsRED-N7* and *DR5rev:3XVENUS-N7* seeds were kindly provided by Dr E. M. Meyerowitz (Division of Biology, California Institute of Technology, Pasadena, CA, USA). The *ahk2 ahk4* and *ahk3 ahk4* mutants were obtained from Dr. C. Ueguchi (Bioscience and Biotechnology Center, Nagoya University, Nagoya, Japan). The *DR5rev::GFP* lines were provided by Dr J. Friml (Zentrum für Molekulare Biologie der Pflanzen, Universität Tübingen, Germany). *pARR7::GFP* and *pARR15::GFP* seeds were provided by Dr J. Sheen (Harvard Medical School, USA). Double reporter lines were generated as follows: *pWOX5::GFP* lines were crossed with *pWUS::DsRED-N7* lines; *DR5rev:3XVENUS-N7* lines were crossed with *pWOX5::GFP* lines, and *pPLT2::RFP* lines were crossed with *DR5rev::GFP* lines; *pARR7::GFP* lines were crossed with *pWUS::DsRED-N7* lines.

### ANTISENSE *WOX5* cDNA PLASMID CONSTRUCTION

To determine the role of *WOX5* during somatic embryo induction, a 754 bp cDNA fragment of the *WOX5* coding region was amplified

using the primers 5'-ATACTAGTAAACAGTTGAGGACTTACA TC-3' (forward) and 5'-ATCTCGAGTACGCATTCCATAACATAG ATT-3' (reverse). The *WOX5* antisense cDNA was then cloned into the estradiol inducible XVE binary vector (Zuo et al., 2000), and transformed into *Arabidopsis* plants.

### GROWTH CONDITIONS, SE INDUCTION AND CHEMICAL TREATMENTS

The growth conditions for *Arabidopsis* plants and somatic embryo (SE) induction followed Su et al. (2009). To induce the transcription of inserted *WOX5* antisense cDNAs, the primary somatic embryos (PSEs) were cultured in embryonic callus-inducing medium (ECIM) with 10 µM estradiol (prepared in DMSO as 10 mM stock; Sigma) for 14 days. Then, the cultured tissues were transferred to somatic embryo-inducing medium (SEIM) with 10 µM estradiol for another 8 days. Estradiol was added every 2 days. The embryonic calli in SEIM were collected for phenotype observation.

### IN SITU HYBRIDIZATION

Embryonic calli were fixed in FAA (10% formaldehyde: 5% acetic acid: 50% alcohol) overnight at 4°C. After dehydration, the fixed callus tissues were embedded in paraffin (Sigma) and sectioned at 8 µm. Antisense and sense *WOX5* probes were used for hybridization as previously described by Zhao et al. (2006). The primers used to amplify the 440-bp *WOX5* probes were 5'-CATCATCATCAACCATCAACT-3' (forward) and 5'-CCATAACATAGATTCTTATATC-3' (reverse).

### IMAGING CONDITIONS

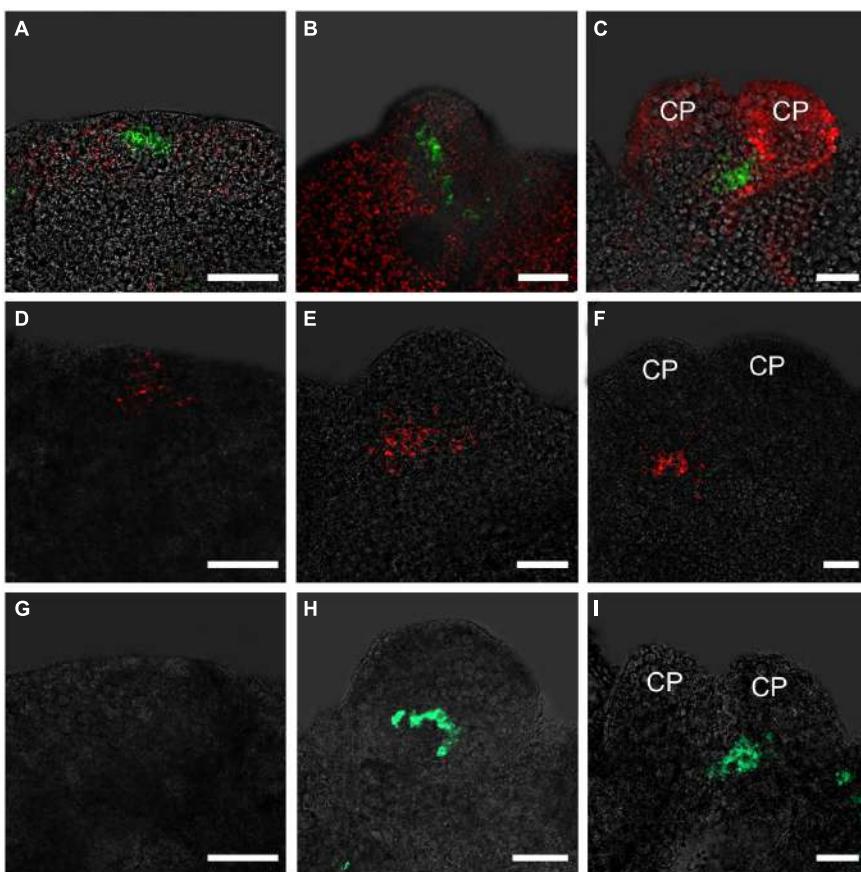
Somatic embryo morphology was photographed using an Olympus JM dissecting microscope. To detect the fluorescence signals of the marker lines, a Zeiss 510 Meta laser scanning confocal microscope with a 20 × air objective and a 40 × oil-immersion lens was used. Specific sets of filters were selected as described previously by Heisler et al. (2005). The Zeiss LSM software was used to analyze the confocal images. At least 80 samples of each marker line were imaged to confirm the expression patterns at each stage.

## RESULTS

### GENES FOR RAM SPECIFICATION ARE INDUCED DURING EARLY SOMATIC EMBRYOGENESIS

Previously, we described a highly reproducible somatic embryogenesis system in *Arabidopsis* in detail (Su et al., 2009). Green PSEs can be generated from explants (immature zygotic embryos), and then disk-like embryonic calli are produced from PSEs in ECIM containing 2,4-dichlorophenoxyacetic acid (2,4-D). After the calli are transferred to 2,4-D-free SEIM, secondary somatic embryos (SSEs) are induced.

To analyze the spatiotemporally regulated formation of the root stem cell niche at the early stages of somatic embryogenesis, we examined the expression patterns of genes that play critical roles in root stem cell specification. Weak *pWOX5::GFP* signals were detected in a few internal regions but not in the edge regions of embryonic callus grown in ECIM for 14 days (data not shown). In contrast, after the embryonic calli were transferred to SEIM, stronger GFP signals started to be detected in some small edge regions at around 24 h (**Figure 1A**). At this time, the pro-embryos



**FIGURE 1 | Expression patterns of *WOX5*, *PLT2*, and *SCR* genes in embryonic calli during somatic embryogenesis. (A–C)** Expression patterns of *WOX5* indicated by *pWOX5::GFP* in embryonic calli induced in SEIM for 24 h (**A**; 83.72%,  $n = 86$ ), 2 days (**B**; 84.27%,  $n = 89$ ) and 3 days (**C**; 80.21%,  $n = 96$ ). **(D–F)** Expression patterns of *PLT2* indicated by *pPLT2::RFP* in embryonic calli induced in somatic embryo-inducing medium

(SEIM) for 24 h (**D**; 87.65%,  $n = 81$ ), 2 days (**E**; 84.95%,  $n = 93$ ) and 3 days (**F**; 90.53%,  $n = 95$ ). **(G–I)** Expression patterns of *SCR* indicated by *pSCR::GFP* in embryonic calli induced in SEIM for 24 h (**G**; 87.36%,  $n = 87$ ), 2 days (**H**; 89.58%,  $n = 96$ ) and 3 days (**I**; 86.90%,  $n = 84$ ). CP, cotyledon primordia. Red signals in **A–C** represent chlorophyll autofluorescence. Scale bars = 80  $\mu$ m.

were not identifiable morphologically, but the callus cells with *WOX5* activity might have been the initial QC. Later, GFP signals were observed in the basal regions of the globular pro-embryos and then pro-embryos with cotyledon primordia (CP; Figures 1B,C). *PLT2* exhibited similar spatial expression patterns to *WOX5* at these stages (Figures 1D–F). Different from *WOX5* expression, *PLT2* expression was observed in a relatively large group of cells within the callus, which represented the root stem cell niche of the SE. We also determined the expression patterns of *SCR*, whose expression defines the position of the QC (Figures 1G–I). The GFP signals of *SCR* were first detected at 36–48 h after induction in SEIM, which was later than *WOX5* and *PLT2* expression. The expression patterns of genes for the QC and stem cell formation indicated that the RAM is established during SE induction.

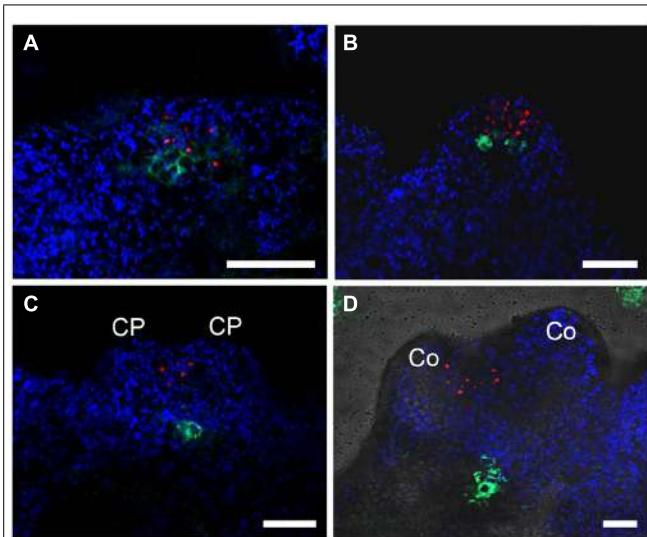
#### THE EMBRYONIC SHOOT–ROOT AXIS OF THE SE IS ESTABLISHED AT EARLY SOMATIC EMBRYOGENESIS

To determine the relative expression domains of *WUS* and *WOX5*, we analyzed their co-localization using a *pWUS::DsRED-N7 pWOX5::GFP* marker line. *WUS* and *WOX5* transcription

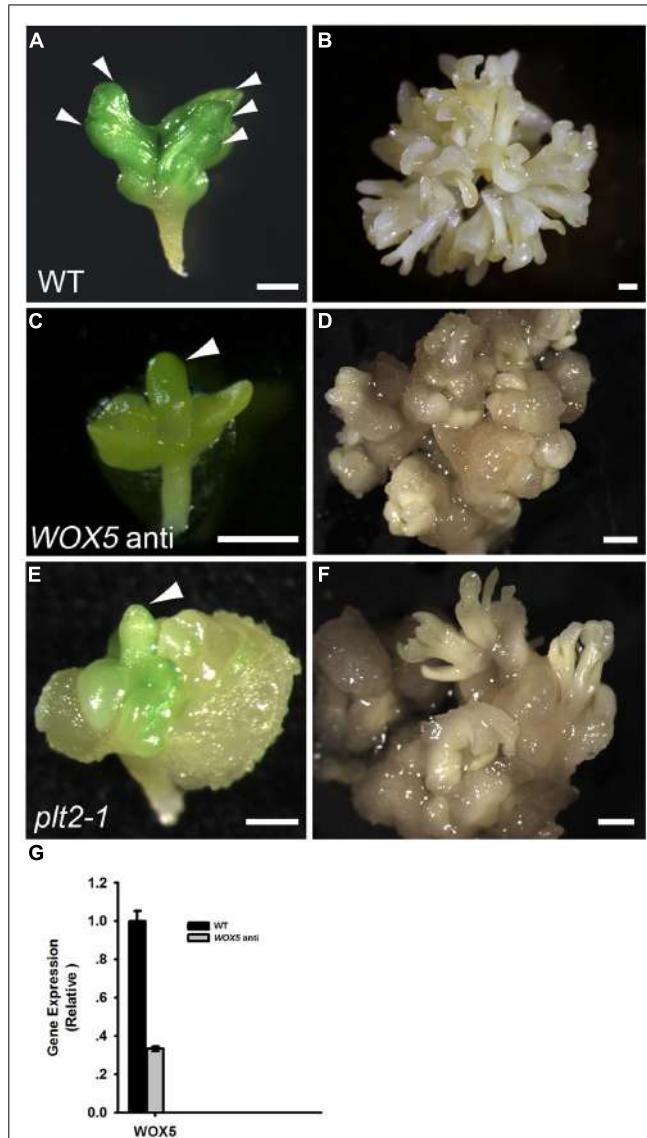
signals were first detected nearly overlapped at the edge regions of callus grown in SEIM for around 24 h (Figure 2A). At 36 h, the *WOX5* expression domain was just below and adjacent to that of *WUS* (Figure 2B). Subsequently, *WUS* transcripts were localized at the top regions between the CP of the pro-embryo, whereas *WOX5* transcripts were localized at the basal regions (Figures 2C,D). Thus, the SAM and RAM were initiated early and nearly overlapped in the edge regions of the callus, indicating that the apical–basal polarity of SE is determined and an embryonic shoot–root axis is established at the early stages of somatic embryogenesis.

#### RAM-SPECIFIC *WOX5* AND *PLT* EXPRESSION IS REQUIRED FOR EMBRYONIC ROOT FORMATION AND SE INDUCTION

To determine the roles of *WOX5* during somatic embryogenesis, we constructed a vector carrying antisense *WOX5* driven by an estradiol receptor-based transactivator, XVE (Zuo et al., 2000), and transferred it into plants. To monitor estradiol-induced production of cDNA-encoded transcripts, quantitative real-time PCR (qRT-PCR) was performed to detect expression levels of *WOX5*



**FIGURE 2 | Relative expression domains of *WOX5* and *WUS* genes.**  
 $pWOX5::GFP$  (green) and  $pWUS::DsRed-N7$  (red) signals in embryonic calli induced in SEIM for 24 h (**A**; 86.81%,  $n = 91$ ), 2 days (**B**; 85.39%,  $n = 89$ ), 3 days (**C**; 89.66%,  $n = 87$ ) and 4 days (**D**; 87.37%,  $n = 95$ ); CP, cotyledon primordia; Co, cotyledons. Blue signals represent chlorophyll autofluorescence. Scale bars = 80  $\mu$ m.



**FIGURE 3 | Functional analysis of both *WOX5* and *PLT2* during somatic embryogenesis.** (**A,C,E**) Phenotypes of primary somatic embryos (PSE) induction from wild type (**A**), *WOX5* antisense (**C**) and *plt2-1* mutant (**E**) explants. Arrowheads indicate the PSE. (**B,D,F**) Phenotypes of SSE induction from WT (**B**), *WOX5* antisense (**D**) and *plt2-1* mutant (**F**) calli grown on SEIM for 8 days. (**G**) Expression levels of *WOX5* in estradiol-induced 15 days shoots of WT and *WOX5* antisense plants. Scale bars = 0.5 mm (**A,C,E**) and 1.2 mm (**B,D,F**).

in 15 days shoots of wild type (WT) and *WOX5* antisense plants (Figure 3G). Estradiol was added every 2 days in medium. Immature zygotic embryos of the transgenic plants were used as explants. Green PSEs were induced on the shoot meristems of 84.47% of the explants after 10 days of culture on B5 agar medium containing 4.5  $\mu$ M 2,4-D in light, without estradiol in the medium (Figure 3A; Table 1). After the PSEs were transferred from ECIM to SEIM, 62.26% of untreated calli produced SSEs and each embryonic callus generated  $52.6 \pm 7.6$  normal SSEs (Figure 3B; Table 1). However, most explants carrying the antisense *WOX5* construct produced abnormal PSEs with deficient hypocotyl elongation and embryonic root formation in the presence of estradiol (Figure 3C; Table 1). Only 8.61% of embryonic calli carrying the antisense *WOX5* construct produced SSEs, and each embryonic callus generated only  $3.3 \pm 2.2$  normal SSEs (Figure 3D; Table 1). We also examined *PLT2*'s function during SE induction. The *plt2-1* mutants generated PSEs with abnormal hypocotyls and embryonic roots (Figure 3E), as described for *plt1-1 plt2-1* double mutants (Su and Zhang, 2014). Embryonic calli of *plt2-1* mutants produced severely abnormal SSEs, without cotyledons or SAMs (Figure 3F; Table 1). Likewise, no hypocotyl- or root-like structures were observed on these SSEs. These results suggested that both *WOX5* and *PLT2* are required for SE formation.

#### SPATIOTEMPORAL DISTRIBUTION OF AUXIN RESPONSES IN EARLY SE INDUCTION

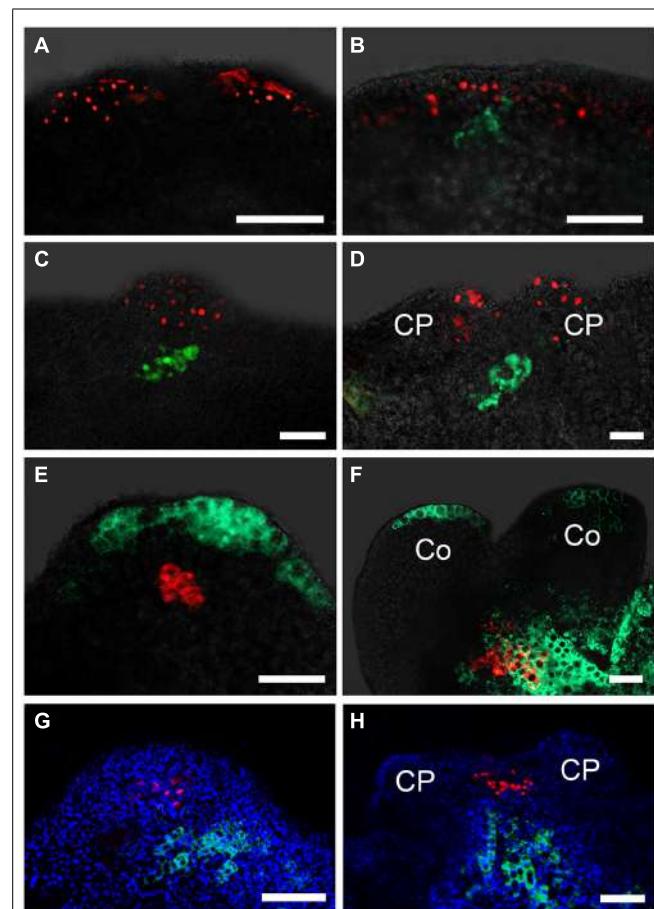
Previously, we reported that auxin response gradients were established in specific regions of the embryonic callus, and were responsible for SE formation (Su et al., 2009). Furthermore, we showed that the spatiotemporal distribution of the auxin response was correlated with the induced *WUS* expression at

early somatic embryogenesis. Interestingly, we observed hardly any auxin response signals in the basal part of the somatic pro-embryo. We analyzed the auxin response signals and *WOX5* expression within the callus by double labeling with *DR5rev:3XVENUS-N7* and  $pWOX5::GFP$ . After 16 h incubation in SEIM, auxin response signals were detectable at the edge regions of the callus, but no *WOX5* signal could be detected (Figure 4A). At 24 h after induction, *WOX5* signals were detected in the region just beneath the outermost cell layers, where auxin response signals were identified (Figure 4B). After 48 h incubation,

**Table 1 | Somatic embryo (SE) regeneration frequencies of different mutants and transgenic lines.**

| Mutant <sup>a</sup> | Wild type  | WOX5 anti | plt2-1     | 35S::ARR7  | 35S::ARR15 | ahk2 ahk4  | ahk3 ahk4  |
|---------------------|------------|-----------|------------|------------|------------|------------|------------|
| Ratio <sup>b</sup>  | 84.47%     | 18.79%    | 25.56%     | 34.14%     | 26.67%     | 32.23%     | 30.54%     |
| Ratio <sup>c</sup>  | 62.26%     | 8.61%     | 23.50%     | 21.05%     | 17.70%     | 15.95%     | 15.15%     |
| Number <sup>d</sup> | 52.6 ± 7.6 | 3.3 ± 2.2 | 17.2 ± 7.3 | 15.3 ± 5.6 | 13.5 ± 7.1 | 15.5 ± 7.6 | 12.2 ± 4.3 |

<sup>a</sup>Mutant name; <sup>b</sup>Proportion of explants that produced normal PSEs; <sup>c</sup>Proportion of embryonic calli that produced normal SEs following culture in SEIM for 8 days; <sup>d</sup>Number of normal SEs produced per embryonic callus following culture in SEIM for 8 days (mean ± SD, n ≥ 90).

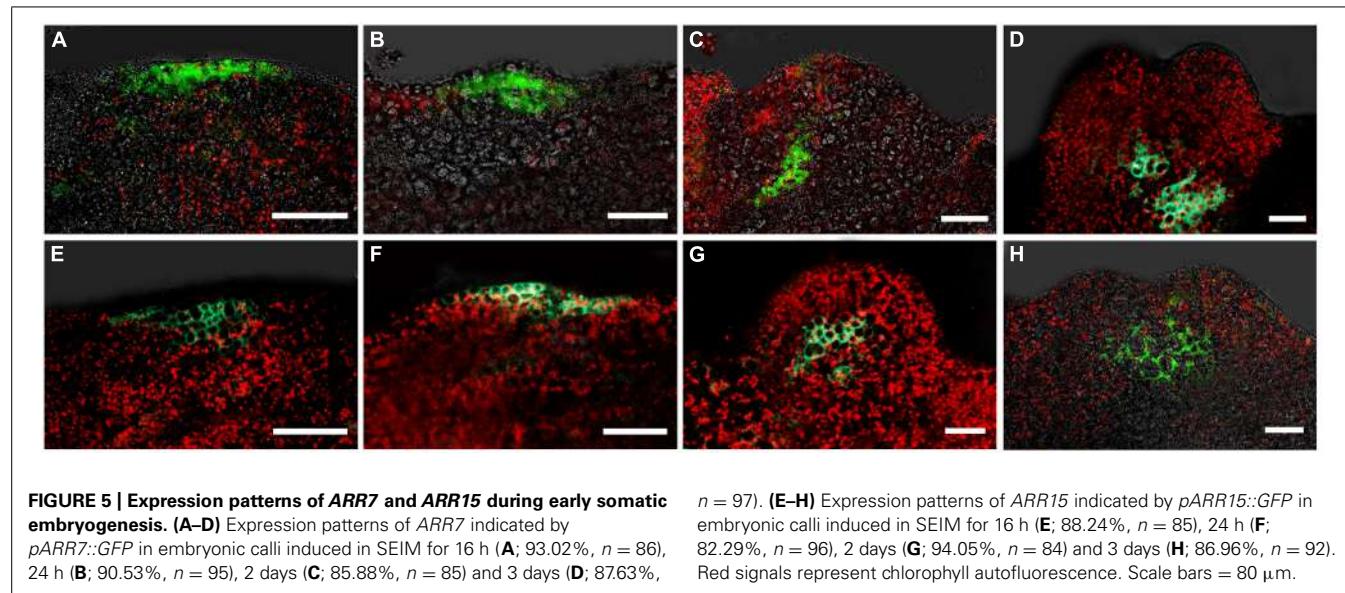


**FIGURE 4 | Auxin and cytokinin responses in early somatic embryogenesis.** (A–D) Auxin response represented by *DR5rev:3XVENUS-N7* correlated with *WOX5* induction represented by *pWOX5::GFP* in embryonic calli induced in SEIM for 16 h (A; 88.24%, n = 85), 24 h (B; 83.33%, n = 96), 2 days (C; 87.50%, n = 88) and 3 days (D; 89.66%, n = 87). *pWOX5::GFP* signals are in green, *DR5rev:3XVENUS-N7* fluorescence signals are in red. (E,F) Auxin response represented by *DR5rev::GFP* correlated with *PLT2* induction represented by *pPLT2::RFP* in embryonic calli induced in SEIM for 3 days (E; 86.73%, n = 98) and 4 days (F; 90.91%, n = 77). *DR5rev::GFP* fluorescence signals are in green, *pPLT2::RFP* signals are in red. (G,H) Cytokinin response represented by *pARR7::GFP* correlated with *WUS* induction represented by *pWUS::DsRed-N7* in embryonic calli induced in SEIM for 2 days (G; 90.43%, n = 94) and 3 days (H; 85.37%, n = 82). *pARR7::GFP* fluorescence signals are in green, *pWUS::DsRed-N7* signals are in red, and chlorophyll autofluorescence is shown in blue. CP, cotyledon primordia; Co, cotyledons. Scale bars = 80 μm.

strong auxin response signals were detected at the upper part of the pro-embryo, but *WOX5* signals were localized at the basal part (**Figure 4C**). Later, auxin signals were redistributed to the top regions of the CP, and *WOX5* was continuously expressed in the basal part of the pro-embryo (**Figure 4D**). We also examined the auxin response in relation to *PLT2* expression through double labeling with *DR5rev::GFP* and the *PLT2* reporter *pPLT2::RFP*. Until 4 days after induction in SEIM, there were auxin signals distributed at the basal region where *PLT2* was expressed (**Figures 4E,F**). These results suggested that auxin response gradients were established in the SAM but not in the RAM of the pro-embryo during the early stages of somatic embryogenesis.

#### CYTOKININ RESPONSES ARE SPATIOTEMPORALLY CORRELATED WITH RAM FORMATION

It has been reported that both auxin and cytokinin responses are critical for specifying the root stem cell niche in embryos (Müller and Sheen, 2008). To determine how the cytokinin response occurs in callus when SEs are induced, we analyzed the spatiotemporal expression patterns of *ARR7* and *ARR15*, which are primary responsive genes in cytokinin signaling and can be rapidly induced by cytokinin (To and Kieber, 2008; Werner and Schmülling, 2009). Signals of *pARR7::GFP* were first detected at some small regions of the calli near the edge at 24 and 36 h after induction in SEIM (**Figures 5A,B**), which was similar to the auxin response at these stages. Interestingly, after 2 days induction in SEIM, the signals were restricted to the basal part of the pro-embryo rather than the top (**Figures 5C,D**). We also used the *pARR15::GFP* reporter to examine the expression patterns of *ARR15*, and found similar distribution patterns of GFP signals to those of *ARR7* (**Figures 5E–H**). These results showed that the cytokinin response occurs in the regions of SE initiation, but the cytokinin response patterns are different from those of the auxin response. To examine whether the cytokinin response is correlated with the establishment of the embryonic SAM, we visualized the cytokinin response using a *pARR7::GFP pWUS::DsRED-N7* marker line. The distribution regions of cytokinin signaling were quite different from those of *WUS* expression, which was localized in the opposite pole of the pro-embryos (**Figures 4G,H**). We further examined the cytokinin response in relation to *WOX5* expression through double labeling with the *pARR7::GFP* and *pWOX5::RFP* reporters (Su and Zhang, 2014). Strong cytokinin responses were induced in the restrictive regions substantially overlapping with the *WOX5* signals. Thus, the results suggest that establishment of the cytokinin response is



correlated not with *WUS* but with *WOX5* induction within the callus, implying that cytokinin is required for embryonic RAM initiation.

#### CYTOKININ SIGNALING IS REQUIRED FOR EMBRYONIC RAM REGENERATION AND SE INDUCTION

The spatial distribution of cytokinin responses detected through *ARR7* and *ARR15* transcriptional signal profiles prompted us to confirm whether a functional cytokinin signaling mechanism is necessary for normal somatic embryogenesis. *ARR7* and *ARR15* act as negative regulators of cytokinin signaling by repressing type-B *ARRs* via unknown mechanisms (To and Kieber, 2008; Werner and Schmülling, 2009). Overexpression of *ARR7* and *ARR15* can attenuate cytokinin signaling to a sufficiently low level, resulting in reduced sensitivity to cytokinin in root elongation and shoot formation or an early flowering phenotype (Werner and Schmülling, 2009). To facilitate functional analysis of cytokinin signaling, we generated transgenic plants overexpressing *ARR7* or *ARR15* under the control of the CaMV 35S promoter. The *ARR7*-overexpressing explants showed a severely defective PSE phenotype without normal elongated hypocotyls or obvious embryonic roots (Su and Zhang, 2014). Subsequently, 78.95% of PSEs generated severely abnormal SSEs after induction in SEIM (Figure 6A; Table 1). Similar to *ARR7*-overexpressing plants, *ARR15*-overexpressing plants also generated abnormal PSEs with defective hypocotyls and embryonic roots, and subsequently, abnormal SSEs (Su and Zhang, 2014; Figure 6B). In *Arabidopsis*, three histidine kinases (AHKs), AHK2, AHK3, and AHK4, positively regulate cytokinin-signaling as direct receptors of cytokinin (To and Kieber, 2008). Thus, we further analyzed the developmental characteristics of *ahk2 ahk4* and *ahk3 ahk4* double mutant calli during SE regeneration. The phenotypes of both double mutants were consistent with previous descriptions of *ARR7*- and *ARR15*-overexpressing plants (Figures 6C,D; Table 1). Interestingly, we found that SE regeneration was impaired with defective cytokinin signaling, which was similar to *WOX5*-antisense plants and *plt2-1* mutants.

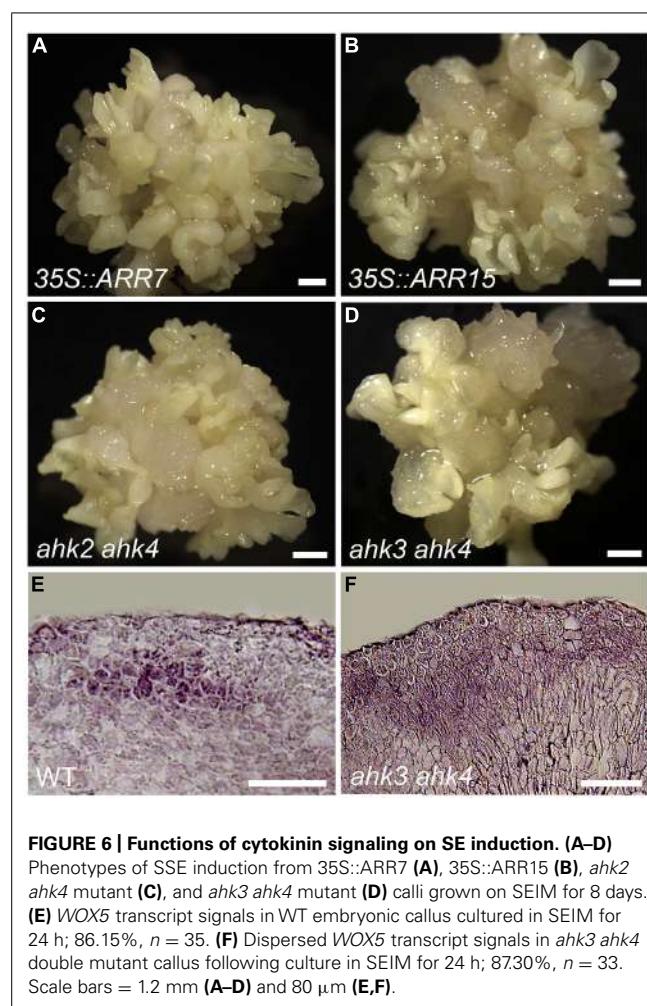
Because the expression patterns of *WOX5* were quite similar to the cytokinin response distribution, we hypothesized that cytokinin signaling might regulate *WOX5* expression for early SE induction. We performed *in situ* hybridization to analyze *WOX5* expression in the *ahk3 ahk4* double mutant. Indeed, the *WOX5* expression pattern was greatly disrupted in the *ahk3 ahk4* double mutant compared with the WT after the callus was transferred into SEIM (Figures 6E,F). *WOX5* signals were restricted to the site of the future embryonic root meristem in the WT (Figure 6E), whereas the localization of *WOX5* signals was stronger and more dispersed in the *ahk3 ahk4* mutant (Figure 6F). These results indicated that cytokinin signaling negatively regulates *WOX5* expression in the proper pattern for initiation of the embryonic RAM.

#### DISCUSSION

During somatic embryogenesis, the developmental process from the globular stage to the torpedo stage shares considerable similarity with that of zygotic embryogenesis (Meinke, 1991; Zimmerman, 1993). Although there are many similarities in the morphological and cellular programs of both zygotic and somatic embryogenesis, the mechanisms determining the initiation of these two processes might be different. The specific characteristics of somatic embryogenesis could be due to the origination of the somatic embryo from embryonic callus, not a zygote as in zygotic embryogenesis.

#### THE ORIGINS OF EMBRYONIC SAM AND RAM WERE QUITE DIFFERENT BETWEEN SOMATIC EMBRYOS AND ZYGOTIC EMBRYOS

In *Arabidopsis*, the mature embryo displays a main shoot–root axis of polarity, with the correct relative positioning of the embryonic SAM at the top and RAM at the opposite pole, separated by the hypocotyl (embryonic stem; Jürgens, 2001; Friml et al., 2003). The origin of this apical–basal pattern of shoot–root axis has been traced back to early embryogenesis, when zygotic division generates a smaller apical and a larger basal cell. After the apical domain



of the pro-embryo has been specified, the embryonic SAM is initiated by the onset of *WUS* expression in the four subepidermal apical cells of the 16-cell embryo (Mayer et al., 1998; Laux et al., 2004). Subsequently, the QC of the RAM is established at approximately late globular embryo stage and marked by the expression of *WOX5* (Wysocka-Diller et al., 2000; Haecker et al., 2004).

During somatic embryogenesis, *WUS* and *WOX5* were simultaneously activated in nearly overlapped callus cells, when somatic pro-embryos could not be identified morphologically (Figure 2; Su et al., 2009). The nearly overlapped spatial relationship between regenerated embryonic SAM and RAM during SE initiation represents the different origins of apical–basal pattern between somatic embryos and zygotic embryos. It is likely that SEs initiate from specific embryonic callus cells which acquire features similar to meristematic cells. These specific embryonic cells of callus are reprogrammed and determined to form cells of both OC and QC for embryonic SAM and RAM formation. In addition, early defects in RAM initiation with inhibited *WOX5* expression also affected the initiation of the SAM, probably by disrupting the apical–basal pattern of early somatic embryogenesis. These results suggest that QC signaling not only maintains stem cell identity in the RAM but also is crucial for OC cells initiation, implying that the stem cell

niches of the RAM and the SAM share developmental correlations during SE initiation.

#### CYTOKININ RESPONSE WAS INVOLVED IN INDUCING CORRECT *WOX5* EXPRESSION AND RAM FORMATION

The patterns of embryonic SAM and RAM establishment in SE initiation suggest the presence of inductive hormonal signals to position them within the embryonic callus. Given the positive effects of auxin on *WUS* expression and SE induction (Su et al., 2009; Figure 4), it is likely a candidate factor that is required for embryonic RAM formation. Here, we found a cytokinin response distribution established in the regions where *WOX5* and *PLT2* were initiated (Figures 1 and 5). RAM formation and SE regeneration were severely inhibited in transgenic plants overexpressing *ARR7* or *ARR15* and in the *ahk* mutants, in which cytokinin signaling was inhibited (Figure 6). Moreover, in cultured tissues of the *ahk* mutants, *WOX5* expression patterns were seriously disturbed compared with control tissues (Figure 6). Thus, we hypothesize that removal of exogenous auxin may be a stress factor that causes cytokinin polar distribution and responses in specific regions, which induce correct *WOX5* expression and subsequent SE initiation. Induced *WOX5* transcripts were continuously detectable in areas of high cytokinin response (Su and Zhang, 2014), suggesting that cytokinin functions in the initiation and maintenance of the embryonic RAM during somatic embryogenesis. The positive action of cytokinin in SAM regeneration has been reported in several studies (Pernisová et al., 2009; Buechel et al., 2010). Treatment with high levels of exogenous cytokinin induces cell proliferation and stimulates shoot regeneration (Skoog and Miller, 1957). Cytokinin induces *WUS* expression during *in vitro* establishment of the SAM from cultured root explants (Gordon et al., 2009). A cytokinin response occurs in the center of the regenerated SAM, overlapping with *WUS* expression regions (Cheng et al., 2013). In contrast, an opposite effect of cytokinin in root regeneration has been observed. Cytokinin influences auxin-induced RAM regeneration via regulation of PIN-mediated auxin polar transport (Pernisová et al., 2009). Therefore, the functions of cytokinin in RAM establishment during SE initiation differ from those in shoot and root regeneration.

#### SPATIOTEMPORAL DISTRIBUTION OF AUXIN AND CYTOKININ RESPONSE IN EMBRYONIC CALLUS DETERMINES ESTABLISHMENT OF SE SHOOT–ROOT AXIS

Cytokinin and auxin appear to be the most important hormones in the regulation of organ regeneration (Moubayidin et al., 2009; Su et al., 2011). A high exogenous auxin/cytokinin ratio induces root regeneration, whereas a low ratio promotes shoot induction (Skoog and Miller, 1957). Recent studies have suggested that exogenous hormones treatment is the critical factor triggering biosynthesis and response of endogenous hormones in early developmental events of *in vitro* regeneration. Specialized endogenous hormonal signaling is required for specific cell differentiation that determines the developmental fate of callus cells (Gordon et al., 2007; Su et al., 2009, 2011). During early somatic embryogenesis, removal of exogenous auxin triggers the regional distribution of endogenous auxin response

in callus surrounding areas of *WUS* expression initiation (Su et al., 2009). Following *WUS* induction, distribution of auxin response was re-established in the SAM region. In contrast, the distribution of cytokinin-response signal in callus overlapped with the areas of *WOX5* expression (Su and Zhang, 2014). These results imply that establishment of auxin and cytokinin response patterns within callus plays an important role in *WUS* and *WOX5* regional expression and shoot–root axis formation. Furthermore, auxin response signals accumulated at the basal region of pro-embryos following prolonged incubation in SEIM (**Figures 4E,F**). The redistributed auxin response corresponded to *PLT2* expression at the later stages of SE development. Therefore, our results suggest that cytokinin and auxin are key players in axial patterning of the SE, especially in shoot and root meristem initiation. The mechanisms of hormonal regulation in SE initiation are quite different from those in shoot or root regeneration individually, which remains a major challenge for the future.

## AUTHOR CONTRIBUTIONS

Ying Hua Su and Xian Sheng Zhang designed the research. Ying Hua Su and Yu Bo Liu performed the research. Bo Bai analyzed the data. Ying Hua Su and Xian Sheng Zhang wrote the paper.

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