

Control of anther cell differentiation: a teamwork of receptor-like kinases

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Received: 2 June 2009 / Accepted: 20 July 2009 / Published online: 6 August 2009
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Abstract Successful sexual reproduction depends on normal cell differentiation during early anther development in flowering plants. The anther typically has four lobes, each of which contains highly specialized reproductive (microsporocyte) and somatic cells (epidermis, endothecium, middle layer, and tapetum). To date, six leucine-rich repeat receptor-like protein kinases (LRR-RLK) have been identified to have roles in regulation of anther cell patterning in *Arabidopsis thaliana*. EXCESS MICROSPOROCTES1 (EMS1)/EXTRA SPOROGENOUS CELLS (EXS) and SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASES1/2 (SERK1/2) signal the differentiation of the tapetum. BARELY ANY MERISTEM1/2 (BAM1/2) defines anther somatic cell layers, including the endothecium, middle layer, and tapetum. Moreover, RECEPTOR-LIKE PROTEIN KINASE2 (RPK2) is required for the differentiation of middle layer cells. In addition to process of anther cell differentiation, conserved regulation of anther cell differentiation in different plant species, this review mainly discusses how these receptor-like kinases and other regulators work together to control anther cell fate determination in *Arabidopsis*.

Keywords Anther · Cell differentiation · Receptor-like kinases · Signal transduction · Evolution

Introduction

The life cycle of flowering plants alternates between the diploid sporophyte and the haploid gametophyte generations. The stamen is the male reproductive organ in a flower. It consists of an anther where the male gametophytes develop, and a filament that anchors the anther to the flower, providing it with water and nutrients. The anther is usually a four-lobed structure (Fig. 1a). Each lobe contains five highly specialized and well-organized cell layers, which are the epidermis, endothecium, middle layer, tapetum, and microsporocytes (pollen mother cells) (Goldberg et al. 1993; Sanders et al. 1999) (Fig. 1a). Microsporocytes are reproductive cells that undergo meiosis and eventually develop into pollen grains. The remaining cell layers are non-reproductive (somatic) cells that are required for the normal development and release of pollen. In particular, the tapetum is critical for pollen development.

In addition to the significance of anthers in the reproductive cycle of flowering plants, studying anther development is important for understanding both fundamental and plant-specific principles. The traits of male sterility are broadly used by plant breeders to produce hybrid varieties for increasing crop yields. Moreover, floral sterility can prevent gene flow from genetically engineered plants through pollen dispersal and increase the overall biomass of plants. Therefore, studying anther development has great impacts on agriculture and the environment. Extensive studies have been conducted to identify genes that control floral organ identity. For example, identity of the six stamens in *Arabidopsis* flowers is controlled by a combination of the B genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*), the C gene *AGAMOUS* (*AG*), and the E genes *SEPALLATA1-4* (*SEP1-4*) (Jack 2004; Ma 2005; Scott et al. 2004; Zhao et al. 2001). However, much less is known about how “organ building”

Communicated by David Twell.

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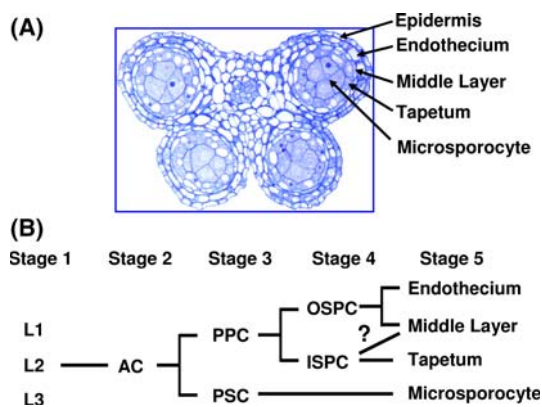


Fig. 1 Anther structure (a) and early anther cell differentiation in *Arabidopsis* (b). *L1* L1 layer, *L2* L2 layer, *L3* L3 layer, *AC* archesporial cells, *PPC* primary parietal cells, *PSC* primary sporogenous cells, *OSPC* outer secondary parietal cells, *ISPC* inner secondary parietal cells

genes specify different cell types of an anther. Following the discovery that the EXCESS MICROSPOROCTES1 (EMS1) [also known as EXTRA SPOROGENOUS CELLS (EXS)] leucine-rich repeat receptor-like protein kinase (LRR-RLK) regulates tapetal cell fate determination (Canales et al. 2002; Zhao et al. 2002), five more LRR-RLKs have been found to be involved in anther cell differentiation (Albrecht et al. 2005; Colcombet et al. 2005; Hord et al. 2006; Mizuno et al. 2007). Many excellent reviews have summarized advances in understanding of stamen identity, anther cell differentiation, male meiosis, pollen development, and anther dehiscence (Borg et al. 2009; Feng and Dickinson 2007; Goldberg et al. 1993; Jack 2004; Ma 2005; McCormick 2004; Scott et al. 2004; Walbot and Evans 2003; Wilson and Yang 2004; Wilson and Zhang 2009; Yang and Sundaresan 2000; Zhao and Ma 2000; Zhao et al. 2001; Zik and Irish 2003a). In this review, the regulation of signaling during early anther cell differentiation by LRR-RLKs is summarized, mainly using *Arabidopsis thaliana* as an example.

Anther cell differentiation

Anther development entails cell division, cell differentiation, and cell death, resulting in the specification of both reproductive microsporocytes and somatic cell layers in the same organ. *Arabidopsis* flower development is divided into 12 stages before flowering using morphological landmarks observed by scanning electron microscopy (Smyth et al. 1990). Following the initiation of stamen primordia at stage 5, the establishment of all anther cell types is finished by stage 9. The development of pollen begins at stage 10, and mature pollen grains are released after anther dehiscence at stage 13.

To better understand anther cell differentiation, anther development in *Arabidopsis* has been divided into two phases and 14 stages based on cellular landmarks visible by light microscopy (Sanders et al. 1999). In phase I, represented by stages from 1 to 8 (approximately corresponding to flower stages 5–10), five anther cell types are established and meiosis occurs. In phase II, represented by stages from 9 to 14 (approximately corresponding to flower stages 10–15), pollen grains are developed and released from the anther after anther tissue degeneration and dehiscence. Cell division and differentiation actively occur from stages 1 to 5, resulting in the specification of all five anther cell types in each lobe. The anther originates from three layers of cells (Fig. 1b), designated L1, L2, and L3. The L1 layer forms epidermis and the L2 layer gives rise to most of the anther cells, including somatic cell layers and reproductive microsporocytes in each lobe. The vascular and connective tissues are derived from the L3 layer at the center of the anther. Archesporial cells are generated from the L2 layer at stage 2, and then divide periclinally to form primary parietal cells (PPC) and primary sporogenous cells (PSC) at stage 3 (Fig. 1b). The primary sporogenous cells will differentiate into microsporocytes. The primary parietal cells further divide into two sets of secondary parietal cells (SPC) at stage 4 (Fig. 1b). The inner set of secondary parietal cells adjacent to the sporogenous cells differentiate into tapetal cells, and the outer secondary parietal cells divide periclinally to form the endothecium and middle layer. Before stage 4, cells of the endothecium, middle layer, and precursors of tapetal cells are not yet organized into layers, because they are not formed synchronously (Sanders et al. 1999; Zhao et al. 2002). By stage 5 (approximately corresponding to the flower stage 9), the anther completes the formation of the four-lobed structure and five types of anther lobe cells. From outside to inside the anther cells in each lobe are the epidermis, endothecium, middle layer, tapetum, and microsporocytes (Fig. 1a, b). Without the availability of molecular markers, there is a disagreement over whether the tapetum alone or both tapetum and middle layers are derived from inner set of secondary parietal cells (Fig. 1b) (Ma 2005; Sanders et al. 1999; Sorensen et al. 2002; Yang et al. 1999; Zhao et al. 2002).

At stage 6, several major events occur. Microsporocytes enter meiosis and become detached from each other. Callose is deposited within the primary cell wall of microsporocytes. In addition, the middle layer becomes thinner and elongated (Owen and Makaroff 1995). Moreover, tapetal cells become vacuolated. Meiosis followed by cytokinesis is completed at stage 7, resulting in the formation of tetrads of microspores. Stage 8 is marked by the release of microspores due to the degeneration of the callose wall surrounding tetrads. At stage 9, surrounded by an exine

wall, microspores become vacuolated. In addition, septum is formed. The degeneration of tapetum is initiated at stage 10 and completed at stage 12. At stage 11, pollen undergoes mitotic divisions. Furthermore, cells in the endothelial layer are enlarged in size. To prepare for anther dehiscence, the degeneration of septum cells is initiated and stomium cells start to differentiate. At stage 12, pollen grains become tricellular. The septum is completely degenerated. At stage 13, followed by the breakage along stomium, anther dehiscence occurs, allowing the release of pollen grains. The anther shrinks at stage 14. Although anther morphology was studied by sectioning in *Arabidopsis* and other species more than a decade ago, only recently have some key genes been identified that directly regulate early anther cell differentiation (Feng and Dickinson 2007; Goldberg et al. 1993; Ma 2005; Sanders et al. 1999; Scott et al. 2004; Walbot and Evans 2003; Wilson and Zhang 2009).

EMS1/EXS and SERK1/2 determine the tapetum

EMS1/EXS is the first LRR-RLK that was identified to play important roles in signaling anther cell differentiation (Canales et al. 2002; Zhao et al. 2002). The *ems1/exs* mutant anther lacks tapetum, but produces a greater number of microsporocytes than the wild type (Fig. 2). The number of microsporocytes in *ems1* mutant anthers is close to the sum of microsporocytes and tapetal cells in wild-type anthers (Zhao et al. 2002). The fact that *ems1* anthers produce excess microsporocytes at the expense of tapetal cells indicates that there is a trade-off between somatic and reproductive cells. The *EMS1/EXS* gene encodes an LRR-RLK, suggesting that EMS1/EXS mediates signals to control tapetal cell fate determination during early anther development.

Although neither *somatic embryogenesis receptor-like kinase1* (*serk1*) nor *serk2* single mutant exhibits detectable anther phenotypes, anther defects in the *serk1 serk2* double mutant resembles that seen in *ems1/exs* anthers (Fig. 2). (Albrecht et al. 2005; Colcombet et al. 2005). Both *SERK1* and *SERK2* encode LRR-RLKs with a high level of similarity. Therefore, SERK1 and SERK2 function redundantly in determining tapetal cell fate during anther development. Moreover, SERK1/2 may be involved in the same signaling pathway as EMS1/EXS. Mutation of the *TAPETUM DETERMINANT1* (*TPD1*) gene results in the absence of tapetum and the formation of excess microsporocytes, which is nearly identical to the phenotypes of the *ems1/exs* single mutant and the *serk1 serk2* double mutant (Fig. 2) (Yang et al. 2003). Genetic analysis supports the idea that both *TPD1* and *EMS1/EXS* function in the same genetic pathway (Jia et al. 2008;

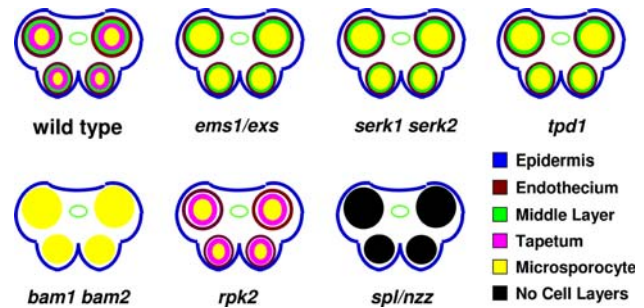


Fig. 2 Diagrams showing the anther structure in wild type and anther phenotypes of *ems1/exs*, *serk1 serk2*, *tpd1*, *bam1 bam2*, *rpk2*, and *spl/nzz* mutants. Anthers in *ems1/exs*, *serk1 serk2*, and *tpd1* mutants lack tapetum, but produce excess microsporocytes. The *bam1 bam2* double mutant anther does not form endothecium, the middle layer, or tapetum, instead it produces extra microsporocytes. The *rpk2* mutant anther has no middle layer. The *spl/nzz* mutant anther fails to produce microsporocytes and anther walls, including the endothecium, middle layer, and tapetum. All the mutants are male sterile

Yang et al. 2005). Further studies demonstrate that ectopic expression of *TPD1* causes abnormal differentiation of tapetal cells and microsporocytes. The abolishment of anther phenotypes caused by the ectopic expression of *TPD1* in the *ems1* mutant indicates that the *TPD1* signaling requires a functional EMS1/EXS. *TPD1* encodes a novel small putatively secreted protein and *TPD1* interacts with EMS1/EXS in vitro and in vivo (Jia et al. 2008). Furthermore, *TPD1* induces the phosphorylation of EMS1/EXS. Therefore, *TPD1* could serve as a ligand for the EMS1/EXS receptor kinase.

In animals, signals from somatic cells play critical roles in influencing sex cell fate determination (Santos and Lehmann 2004; Zhao and Garbers 2002). Messenger RNA in situ hybridization experiments show that the *EMS1/EXS* gene is primarily expressed in the tapetum, while *TPD1* is mainly restricted to microsporocytes (Yang et al. 2003; Zhao et al. 2002). Therefore, the *TPD1* small protein may be secreted from microsporocytes or their precursors (Fig. 3). *TPD1* binds to the EMS1/EXS receptor that is localized on the cell membrane of tapetum precursor cells. Signals relayed by EMS1/EXS might direct tapetum formation by activating the expression of genes that promote tapetal cell fate determination, and oppositely by repressing the expression of genes required for differentiation of microsporocytes. Without the EMS1/EXS receptor in the *ems1/exs* mutant or the *TPD1* ligand in the *tpd1* mutant, genes promoting microsporocyte differentiation might be expressed in tapetum precursor cells at a higher level than normal, which consequently results in tapetal precursor cells adopting a microsporocyte fate. Furthermore, the EMS1/EXS-*TPD1* signaling complex may contain the SERK1/2 LRR-RLK.

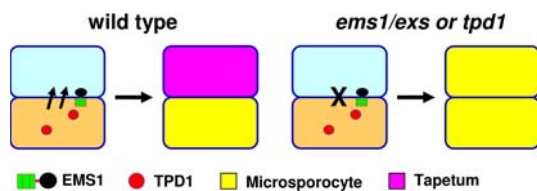


Fig. 3 A model for the EMS1/EXS-TPD1 signaling pathway in regulating anther cell fate determination. The TPD1 protein is secreted from microsporocytes or their precursors and then binds to the EMS1/EXS receptor that is localized on the cell membrane of tapetum precursor cells. Signals elicited by EMS1/EXS activate a pathway to promote fate determination of tapetal cells and conversely repress a pathway for differentiation of microsporocytes. In either *ems1/exs* or *tpd1* mutant, the EMS1/EXS-TPD1 signaling pathway is blocked. Therefore, the pathway for the differentiation of microsporocytes is not restrained in tapetum precursor cells, resulting in tapetal precursor cells adopting a microsporocyte fate

BAM1/2 signals the differentiation of anther somatic cell layers

BAM1 (for BARELY ANY MERISTEM) and BAM2 LRR-RLKs, which share high levels of identity with CLAVATA1, are involved in regulating early anther cell differentiation in *Arabidopsis* (DeYoung et al. 2006; Hord et al. 2006; Shiu and Bleecker 2001). Neither *bam1* nor *bam2* single mutants have detectable phenotypes (DeYoung et al. 2006). However, *bam1 bam2* double mutants exhibit various developmental abnormalities, including reduced meristem size and aberrant leaves, as well as irregular male and female fertility (DeYoung et al. 2006). Additional detailed morphological analysis of anther development reveals that the *bam1 bam2* double mutant anther does not form somatic cell layers, including endothecium, middle layer, and tapetum (Fig. 2) (Hord et al. 2006). Nevertheless, the double mutant anther produces only microsporocytes. Both *BAM1* and *BAM2* genes are expressed in archesporial cells at as early as stage 2, while in later stages they are preferentially expressed in sporogenous cells and microsporocytes. Therefore, BAM1- and BAM2-mediated signaling is required for the specification of parietal cells that generate cell layers of the anther wall.

It appears that BAM1/2, as well as EMS1/EXS and SERK1/2 play a role that is opposite to that of the *SPOROXYTELESS (SPL)/NOZZLE (NZZ)* transcription factor in regulating anther cell differentiation. The *spl/nzz* mutant anther fails to produce microsporocytes and anther walls, suggesting that the *SPL/NZZ* gene promotes the formation of microsporocytes and anther walls, including the tapetum (Fig. 2) (Schiefthaler et al. 1999; Yang et al. 1999). The anther phenotype of the *bam1 bam2* double mutant suggests that *BAM1/2* may repress the proliferation of sporogenous cells, but promote the differentiation of

adjacent parietal cells. In addition, EMS1/EXS together with SERK1/2 may inhibit the formation of microsporocytes. However, it is not clear whether *SPL/NZZ* is the target gene of the BAM1/2, EMS1/EXS, or SERK1/2 signal transduction pathway. Without the availability of molecular markers for different anther cell types, the morphological analyses of *spl* and *nzz* anthers lead to somewhat different interpretations. It is believed that the *spl* anther can produce secondary parietal cells and primary sporogenous cells (Yang et al. 1999), while the *nzz* anther stops cell differentiation after forming a group of archesporial cells (Schiefthaler et al. 1999). *SPL/NZZ* is expressed in both parietal and sporogenous cells during early stages of anther development and then it is restricted to microsporocytes when anther cells are fully differentiated (Yang et al. 1999). However, the expression of *SPL/NZZ* in the *bam1 bam2* anther is expanded to all, or most of the L2-derived cells (Hord et al. 2006). Therefore, BAM1/2 signaling seems to repress the expression of *SPL/NZZ*. This might be true for the EMS1/EXS and SERK1/2 signal transduction pathways. According to this assumption, the signal relayed by EMS1/EXS or SERK1/2 represses the expression of genes required for microsporocyte differentiation in tapetal precursors, such as *SPL/NZZ*. In the *ems1/exs* single or *serk1 serk2* double mutant, *SPL/NZZ* should be persistently expressed in the tapetal precursor cells, which eventually leads to the formation of excess microsporocytes. The *SPL/NZZ* gene could also be involved in regulating the expression of *EMS1/EXS*, *SERK1* or *SERK2* in parietal cells. In the *spl/nzz* mutant, the abnormal expression of *EMS1/EXS*, *SERK1* or *SERK2* causes the lack of tapetum. Therefore, it is possible that the genetic interactions between the EMS1/EXS or SERK1/2 signal transduction pathway and the *SPL/NZZ* gene establish a negative feedback loop to control anther cell differentiation. It is also possible that the lack of microsporocytes in the *spl/nzz* mutant causes the failed differentiation of tapetal cells, since the TPD1 protein is likely produced in microsporocytes.

RPK2 defines the middle layer

The *RECEPTOR-LIKE PROTEIN KINASE2 (RPK2)* gene, which encodes an LRR-RLK, is required for differentiation of the middle layer during anther development (Fig. 2) (Mizuno et al. 2007). The disruption of *RPK2* function results in the formation of anthers without the middle layer. Moreover, the tapetum is affected, displaying hypertrophy. Different from *ems1/exs*, *serk1 serk2*, and *bam1 bam2* mutants, the *rpk2* mutant anther produces tetrads and microspores, although the pollen maturation is abnormal. The expression of *RPK2* is strongly detected in the tapetum.

Therefore, RPK2 signaling not only controls the differentiation of the middle layer, but also maintains the tapetum.

Integration of multiple LRR-RLK-linked signaling pathways

So far, six LRR-RLKs have been identified that play important roles in signaling early anther cell differentiation. A crucial difference between plant and animal cells is that plant cells have a rigid cell wall, which does not allow cells to move. Therefore, intercellular signaling using diffusible molecules is particularly important for plant cell–cell communication. Receptor-like kinases (RLKs) are key components in plant cell signaling. In *Arabidopsis*, more than 600 genes encode RLKs, representing 2.5% of the total genes (Shiu et al. 2004; Torii 2004). This number is almost doubled in the rice genome (Shiu et al. 2004). LRR-RLKs, with 223 members in *Arabidopsis* (www4.ncsu.edu/~sclouse/Clouse2010.htm), form the largest family of RLKs. LRR-RLKs are involved in a wide range of plant growth and developmental processes, including stem cell maintenance, cell fate determination and patterning, steroid hormone signaling, organ size and shape regulation, organ abscission, defense responses, plant transpiration, and nodulation (Becraft 2002; Clouse 2002; Dievart and Clark 2004; Johnson and Ingram 2005; McCarthy and Chory 2000; Morillo and Tax 2006; Morris and Walker 2003; Nakajima and Benfey 2002; Torii 2004; Vert et al. 2005; Waites and Simon 2000). Although mutations of *EMS1/EXS*, *SERK1* and *SERK2*, *BAM1* and *BAM2*, as well as *RPK2* genes cause male sterility, it is not clear how these LRR-RLK-linked signaling pathways integrate to regulate anther cell differentiation.

The *bam1 bam2* anther is unable to produce three of the normal layers of the anther wall—the endothecium, middle layer, and tapetum. Instead it produces extra microsporocytes (Hord et al. 2006). The cell layers that are absent from the *bam1 bam2* anther wall are derived from parietal cells, suggesting that *BAM1/2* signaling is required for the specification of parietal cells from archesporial cells at an early stage of anther development (Fig. 4). The division of secondary parietal cells seems normal in the *ems1* mutant (Zhao et al. 2002). This indicates, therefore, that *EMS1/EXS* signaling acts later than *BAM1/2* signaling in determining the fate of tapetal cells, which are generated from the secondary parietal cells. Both BRASSINOSTEROID-INSENSITIVE 1 (*BRI1*) and *EMS1/EXS* belong to the LRR-RLK X subfamily, while *SERK1*, *SERK2*, and *BRI1*-ASSOCIATED RECEPTOR KINASE (*BAK1*, also known as *SERK3*) are members of LRR-RLK II subfamily (Albrecht et al. 2005; Albrecht et al. 2008; Colcombet et al. 2005). *BAK1* and *BRI1* form a signaling complex via

heterodimerization to mediate plant steroid signaling (Albrecht et al. 2008; Li et al. 2002; Nam and Li 2002; Wang et al. 2005; Wang et al. 2008). The phenotype of the *serk1 serk2* double mutant anther resembles that of *ems1/exs*. Thus, *SERK1/2* may function in the same pathway as *EMS1/EXS* to determine the tapetum (Fig. 4). The *rpk2* mutant anther fails to form the middle layer, while the development of tapetum is affected (Mizuno et al. 2007). *RPK2* signaling may affect the division of inner or outer secondary parietal cells. *RPK2* and *EMS1/EXS1* should act in similar developmental stages (Fig. 4). Moreover, it is possible that *RPK2* and *EMS1/EXS* play antagonistic roles in specifying the middle layer and tapetum from their parietal cell precursors. Future molecular genetic analyses hold promise to address how these LRR-RLKs work together to define different cell types during early anther development.

Conserved regulation of early anther cell differentiation

Different plants may use similar mechanisms to control early anther cell differentiation. The rice *multiple sporocyte1 (msp1)* mutant lacks tapetum, but produces excess microsporocytes in the anther, which is similar to the phenotype of *ems1/exs* (Canales et al. 2002; Nonomura et al. 2003; Zhao et al. 2002). The *MSP1* gene encodes an LRR-RLK with a high level of identity to *EMS1/EXS*, suggesting that *MSP1* is an *EMS1/EXS* ortholog (Nonomura et al. 2003). Although the mutant gene is not known, the maize mutant *multiple archesporial cells (mac1)* has a phenotype nearly identical to that of *msp1* (Sheridan et al. 1999). Rice has at least two *TPD1*-like genes, *OsTDL1A* and *OsTDL1B* (Zhao et al. 2008). Furthermore, in maize the *male sterile converted anther1 (msca1)* mutant has a similar phenotype to *splnzz*, which

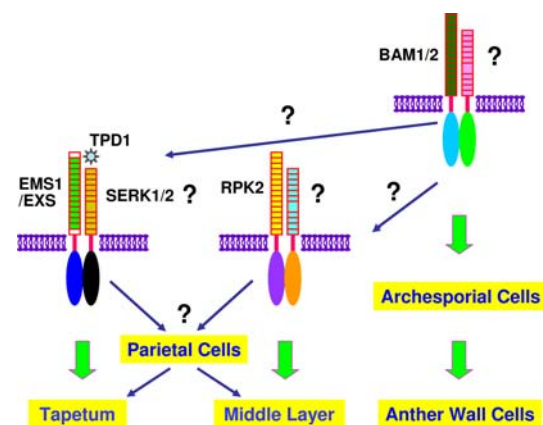


Fig. 4 A diagram showing *BAM1/2*, *EMS1/EXS*-*SERK1/2*-*TPD1*, and *RPK2* signal transduction pathways and their possible integrations in regulating early anther cell differentiation in *Arabidopsis*

forms archesporial cells, but fails to produce microsporocytes (Chaubal et al. 2003). These results support the idea that eudicots and monocots show conservation in regulating early anther cell differentiation, although they diverged at least 120 million years ago.

Plants have evolved to have different complexities of microsporangium structures. For example, the existence of tapetum is universal in all land plants. Although the tapetum has similar function, the structure varies among species (Pacini et al. 1985). The *leptosporangiate* ferns have two layers (inner and outer) of tapetum (Hord et al. 2006), while in *Psilotum nudum* ferns tapetal cells are intermingled with microsporocytes (Pacini et al. 1985). It will be interesting to investigate whether signaling molecules identified in *Arabidopsis* have conserved functions in controlling microsporangium development in other plant species.

Perspective

The anther is not only vitally important for plant sexual reproduction, but also provides an excellent system to elucidate many fundamental principles, such as the molecular mechanisms of cell fate determination. Normal early anther cell differentiation ensures normal male meiosis, pollen development, and anther dehiscence. Many genes have been found to be linked to anther development based on expression studies using microarray techniques (Alves-Ferreira et al. 2007; Hennig et al. 2004; Lu et al. 2006; Ma et al. 2007; Ma et al. 2008; Wellmer et al. 2004; Wijeratne et al. 2007; Zik and Irish 2003b). However, so far only a few genes have been identified that play direct roles in controlling anther cell differentiation, particularly in cell fate specification of tapetum and microsporocytes. Future studies should focus on identifying genes that regulate cell differentiation in the whole process of anther development. For example, little is known about how the differentiation of archesporial cells is controlled.

Although six LRR-RLKs have been found to signal early anther cell differentiation, significant gaps remain in these LRR-RLK-linked signal transduction pathways. It is not clear what kinds of signaling molecules are involved in relaying signals from the cell surface to the cell interior, and from cell to cell. Furthermore, nothing is known about crosstalk among these signaling networks. Therefore, it will be necessary to identify the signaling components and determine how they are integrated using a combination of molecular genetic, cell biological, genomic, and proteomic approaches. Furthermore, computational and imaging technologies should be employed to study the dynamics of anther cell patterning in the future.

Acknowledgments I would like to thank Hong Ma for giving me the opportunity to study anther development when I was in his laboratory. His insight greatly influenced my decision to continue my research in this area. I also thank H. A. Owen and C. Starrett for critical comments on this manuscript, and thank G. Jia for providing the image of the anther section. This work was supported by a grant from the University of Wisconsin-Milwaukee Research Growth Initiative (RGI) Program (to D.Z.), an NSF grant IOS-0721192 (to D.Z.), and the Shaw Scientist Award (to D.Z.).

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