

# Signaling of cell fate determination by the TPD1 small protein and EMS1 receptor kinase

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**Sexual reproduction requires the specification of cells with distinct fates in plants and animals. The EMS1 (also known as EXS) leucine-rich repeat receptor-like kinase (LRR-RLK) and TPD1 small protein play key roles in regulating somatic and reproductive cell fate determination in *Arabidopsis* anthers. Here, we show that ectopic expression of TPD1 causes abnormal differentiation of somatic and reproductive cells in anthers. In addition, ectopic TPD1 activity requires functional EMS1. Yeast two-hybrid, pull-down, and coimmunoprecipitation analyses further demonstrate that TPD1 interacts with EMS1 *in vitro* and *in vivo*. Moreover, TPD1 induces EMS1 phosphorylation *in planta*. Thus, our results suggest that TPD1 serves as a ligand for the EMS1 receptor kinase to signal cell fate determination during plant sexual reproduction.**

*Arabidopsis* | sexual reproduction | anther | ligand | signal transduction

Cell fate determination is a critical process in development of all multicellular organisms. A fundamental feature of sexual reproduction in both plants and animals is the specification of distinct types of cells that give rise to eggs and sperm. In animals, primordial germ cells are determined and segregated from their somatic neighbors very early during embryonic development. In contrast, plant sexual reproduction takes place postembryonically (1). In flowering plants, after the switch from vegetative growth to reproductive development, reproductive cells are specified from somatic cells in flowers. So far, little is known about the molecular mechanisms underlying cell fate determination during sexual reproduction in plants (1–3).

Anthers are the male parts of flowers that bear pollen for producing sperm (4, 5). A mature anther is usually a four-lobed structure. Each lobe contains five types of highly specialized cells, which are the epidermis, endothecium, middle layer, tapetum, and microsporocytes (pollen mother cells). Microsporocytes are reproductive cells that generate pollen via meiosis, whereas somatic cells, particularly the tapetum, are required for normal development and the release of pollen. An abnormal tapetum has been shown to cause male sterility in plants. Anther development involves cell division, cell differentiation, and cell death, resulting in the specification of both reproductive and somatic cells in the same organ. Recently the anther has emerged as a prime model system for the study of cell fate determination and receptor-linked signaling, in addition to its central importance to plant breeding and reproduction (2, 3, 5–14).

In *Arabidopsis* the *EXCESS MICROSPOROCTES1* (*EMS1*, also known as *EXTRA SPOROGENOUS CELLS*, *EXS*) and *TAPETUM DETERMINANT1* (*TPD1*) genes play key roles in anther cell fate determination (2, 7, 15). The *ems1* mutant anthers lack the tapetum but produce more microsporocytes at the expense of tapetal cells, suggesting that there is a tradeoff between somatic and reproductive cells (2). The *tpd1* mutant has a phenotype indistinguishable from that of *ems1* (7). The *EMS1* gene encodes a leucine-rich repeat receptor-like kinase (LRR-RLK), whereas *TPD1* encodes a small, putatively secreted protein. Therefore, we hypothesize that TPD1 may act as a key signaling molecule, possibly a ligand, in the EMS1-mediated signal transduction pathway.

In *Arabidopsis*, >600 genes encode RLKs, representing 2.5% of the total genes (16, 17). This number is almost double in the rice genome (18). The LRR-RLKs, with 223 members in *Arabidopsis* ([www4.ncsu.edu/~sclouse/Clouse2010.htm](http://www4.ncsu.edu/~sclouse/Clouse2010.htm)), form the largest family of RLKs. Molecular genetic studies show that LRR-RLKs are involved in a wide range of plant growth and development processes (17, 19), including stem cell maintenance (20, 21), cell fate determination and patterning (2, 8–10, 12, 15, 22, 23), steroid hormone signaling (24–28), organ size and shape regulation (29–31), organ abscission (32), defense responses (33–35), plant transpiration (36), and nodulation (37, 38). However, most signaling molecules, including ligands, remain unidentified even in the known LRR-RLK-linked signal transduction pathways. Here, we report that the ectopic expression of *TPD1* causes abnormal differentiation of somatic and reproductive cells in anthers. Genetic studies demonstrate that TPD1 signaling requires functional EMS1. Furthermore, yeast two-hybrid, pull-down, and coimmunoprecipitation experiments show that TPD1 interacts with EMS1 *in vitro* and *in vivo*. In addition, we found that TPD1 binding activates phosphorylation of the EMS1 kinase. Thus, interaction between EMS1 and TPD1 is required for the specification of somatic and reproductive cells in *Arabidopsis* anthers, which indicates that TPD1 serves as a ligand for the EMS1 receptor kinase.

## Results

**Ectopic Expression of TPD1 Causes Abnormal Differentiation of Somatic and Reproductive Cells in Anthers.** In wild-type anthers at stage 5 of development, each lobe comprises five types of differentiated cells: the epidermis, endothecium, middle layer, tapetum, and microsporocytes (Fig. 1A). Microsporocytes are located at the center of each lobe, surrounded by a tapetum layer (Fig. 1B). At stage 6, tapetal cells are partially vacuolated, and microsporocytes are separated from each other (Fig. 1C). The *ems1* anther at stage 5 completely lacks tapetum but produces excess microsporocytes (Fig. 1D). At stage 6, microsporocytes are abnormally enlarged and still attached (Fig. 1E). The *tpd1* anther phenotype [see supporting information (SI) Text ] is similar to that of *ems1* (Fig. 1F). Consistent with previous findings (7), our results show that the *ems1 tpd1* double-mutant anther exhibits the identical phenotype to the *ems1* and *tpd1* single mutants (Fig. 1G), which suggests that TPD1 and EMS1 function in the same genetic pathway.

We then tested whether ectopic expression of *TPD1* affects anther cell differentiation by expressing *TPD1* with the cauliflower mosaic virus *CaMV35S* promoter (*CaMV35S*). Both the

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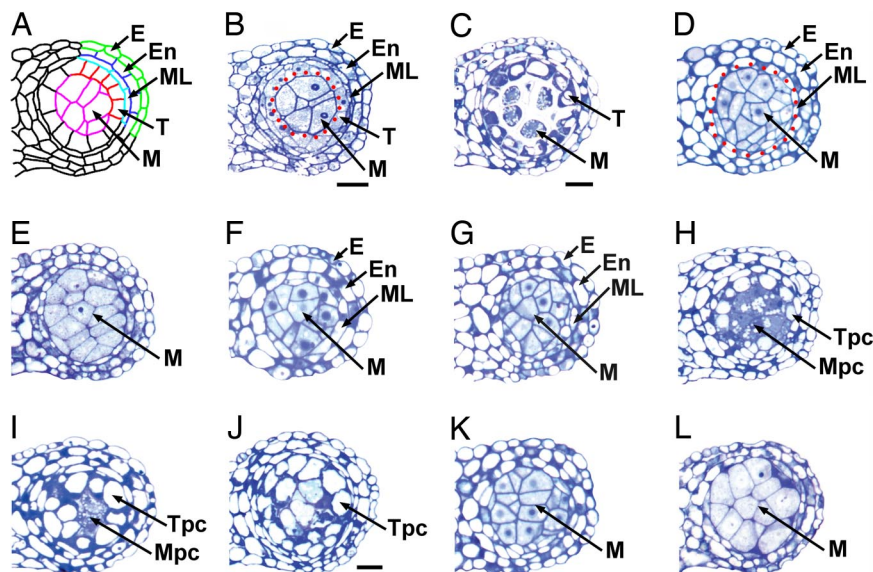
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**Fig. 1.** Ectopic expression of *TPD1* causes abnormal anther cell differentiation, and *TPD1* signaling requires *EMS1*. Semithin sections show one lobe from anthers. (Scale bars: 20  $\mu\text{m}$ .) *B*, *D*, *F*–*H*, and *K*; *C*, *E*, *I*, and *L* have the same magnification. (*A*) Diagram of a mature wild-type anther shows epidermis (*E*), endothecium (*En*), middle layer (*ML*), tapetum (*T*), and microsporocytes (*M*). (*B*) A wild-type anther at stage 5. Enclosed by red dotted line are microsporocytes. (*C*) A wild-type anther at stage 6 shows strongly stained tapetal layer and isolated microsporocytes. (*D*) An *ems1* anther at stage 5 lacks the tapetum layer but has excess microsporocytes (enclosed by red dotted line). (*E*) An *ems1* anther at stage 6 lacks the tapetum layer. Microsporocytes are abnormally enlarged and not isolated. (*F*) A *tpd1* anther at stage 5 has the same phenotype as that of *ems1* (*D*). (*G*) An *ems1 tpd1* double-mutant anther at stage 5 shows the same phenotypes as those of *ems1* (*D*) and *tpd1* (*F*). (*H*–*J*) Anthers of *CaMV35S::TPD1* transgenic plants. (*H*) A stage-5 anther shows vacuolated cells in place of a normal tapetum (tapetum-positioned cells, *Tpc*) and degenerating cells instead of normal microsporocytes (microsporocyte-positioned cells, *Mpc*). (*I*) A stage-6 anther exhibits completely vacuolated *Tpc* and degenerating *Mpc*. (*J*) An anther after stage 6 shows the mix of completely vacuolated *Tpc* and *Mpc*. (*K* and *L*) Anthers from *CaMV35S::TPD1 ems1* plants at stage 5 (*K*) and 6 (*L*) exhibit identical phenotypes to *ems1* (*D* and *E*).

*TPD1* gene and *TPD1* protein levels were elevated in *CaMV35S::TPD1* transgenic plants (data not shown). Seventy-seven percent of the 581 *CaMV35S::TPD1* transgenic lines studied produced wider and shorter seed pods than wild type (SI Fig. 5), which is in agreement with the findings of Yang *et al.* (39). Fifty-two percent of lines exhibited the greatly reduced fertility. Moreover, anthers in 25% of the lines had no or few viable pollen grains (SI Fig. 6). Our analysis of semithin sections revealed abnormal cell differentiation in these anthers. Well differentiated tapetum layers were not detected. Instead, cells in the position of a tapetum would normally be located are highly vacuolated (Fig. 1 *H*–*J*). Microsporocyte-like cells also appear to have degenerated (Fig. 1 *I* and *J*). High-resolution confocal microscopy confirmed that the tapetal cell and microsporocyte differentiation are impaired in these anthers. In wild-type anthers, tapetal cells typically contain two separated nuclei that result from endomitosis (9, 40) (SI Fig. 7*A*). However, in *CaMV35S::TPD1* anthers, we did not observe two nuclei in the tapetum-positioned cells (SI Fig. 7*B*), suggesting that the differentiation of tapetum is not complete. In wild-type anthers, the tapetum is established at stage 5 (Fig. 1*B*) and begins to degenerate at stage 10. Yang *et al.* (39) found that formation of tapetum is normal in the *TPD1* ectopic expression plants but that degeneration of tapetal cells is delayed. In addition, microsporocytes do not degenerate. Our results, which show more severe defects than the previous findings, indicate that ectopic *TPD1* signaling affects tapetal cell fate determination and inhibits microsporocyte development.

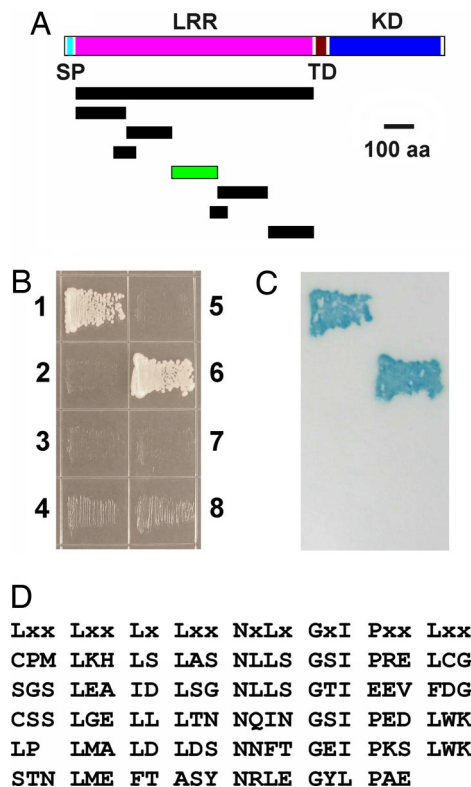
***TPD1* Signaling Depends on Functional *EMS1*.** To test whether *TPD1* signaling requires functional *EMS1*, we introduced the *CaMV35S::TPD1* transgene into the *ems1* mutant. The resulting *CaMV35S::TPD1 ems1* transgenic plants exhibit the typical *ems1* phenotype in seed pod size and fertility (SI Fig. 5). Furthermore, anthers in *CaMV35S::TPD1 ems1* plants at stages 5 and 6 (Fig.

1 *K* and *L*) have the same phenotypes as *ems1* (Fig. 1 *D* and *E*). Thus, *TPD1* signaling requires the presence of *EMS1*, and the phenotypes observed in *CaMV35S::TPD1* transgenic plants are the result of ectopic *TPD1* signaling.

***TPD1* Biochemically Interacts with *EMS1* in Yeast Cells.** To test whether *TPD1* interacts with *EMS1*, we conducted yeast two-hybrid experiments that have been used to identify the protein ligand LAT52 for the LePRK2 LRR-RLK in tomato (41). Initially, we did not detect any interaction between *EMS1* and *TPD1* using the entire *EMS1* LRR domain (Fig. 2*A* and *B*). LePRK2 contains 5 LRRs with a low potential for glycosylation, whereas *EMS1* has 29 LRRs with 11 potential *N*-linked glycosylation sites that may have interfered with protein interaction (42). Therefore, we cloned a series of shortened cDNAs that encode truncated *EMS1* LRRs (Fig. 2*A*). We found that one small fragment interacts with *TPD1* (Fig. 2*A*–*C*, SI Fig. 8). We named this small fragment the *TPD1*-interacting region (*TIR*). The *TIR* contains four typical LRRs and one LRR with low similarity to the typical LRR (Fig. 2*D*). Therefore, our results indicate that *TPD1* biochemically interacts with *EMS1*.

***TPD1* Directly Interacts with *EMS1*.** Direct interaction between *TPD1* and *EMS1* was further confirmed by GST pull-down experiments. We produced fusion proteins of GST to the entire *EMS1* LRR domain, *TIR* and non-*TIR* (SI Figs. 9 and 10). The *TPD1* protein was fused to three HA tags. Instead of GST and non-*TIR*, GST-*EMS1* LRR and GST-*TIR* were able to pull down HA-*TPD1* from crude protein extracts (Fig. 3*A*). Thus, our results strongly indicate that *TPD1* directly interacts with *EMS1* in a specific LRR extracellular region, which is *TIR*.

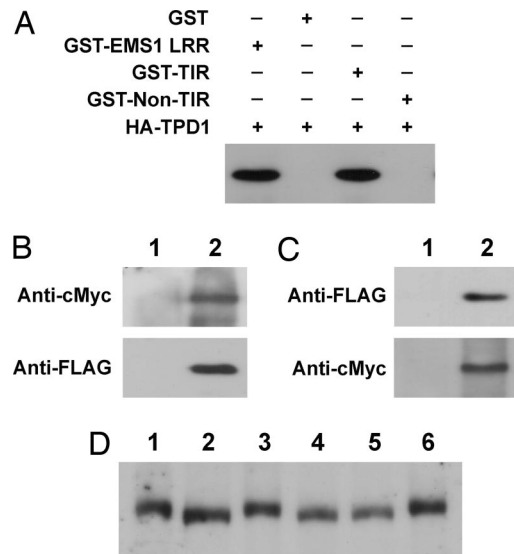
***TPD1* Interacts with *EMS1* in Planta.** To determine whether *TPD1* interacts with *EMS1* *in vivo*, we performed coimmunoprecipitation experiments. *EMS1::EMS1-cMyc* and *TPD1::TPD1-FLAG*



**Fig. 2.** TPD1 interacts with EMS1 in yeast two-hybrid assays. (A) Diagram shows constructs for mapping the TPD1 interacting region (TIR) of EMS1. Bar with green color represents the TIR. (B) Yeast grown on synthetic complete (SC) medium without Leu, Trp, and His. (1) Positive control. (2) Negative control. (3) The entire EMS1 LRR domain and TPD1. (4) The entire EMS1 LRR domain. (5) TPD1 alone. (6) TIR and TPD1. (7) TIR alone. (8) Non-TIR and TPD1. (C) Verification of the interaction by filter lift assay showing blue color of yeast. (D) The amino acid sequence of TIR. The consensus LRR sequence is shown at the top.

transgenic plants were created to express EMS1-cMyc and TPD1-FLAG proteins by their native promoters. Both transgenes complemented phenotypes of their corresponding mutants. EMS1-cMyc and TPD1-FLAG proteins were also detected in double-transgenic plants (Fig. 3B). Membrane protein extracts from young inflorescences of double-transgenic plants were immunoprecipitated with an anti-cMyc antibody. We detected TPD1-FLAG by Western blot with an anti-FLAG antibody (Fig. 3C Upper). In the reciprocal coimmunoprecipitation experiment, we also detected EMS1-cMyc from the anti-FLAG immunoprecipitates (Fig. 3C Lower). In conclusion, our results strongly indicate that TPD1 interacts with EMS1 *in planta*.

**TPD1 Induces the Phosphorylation of EMS1.** We investigated whether the binding of TPD1 to EMS1 triggers a downstream signaling event. EMS1 is predicted to be a receptor-like serine/threonine kinase. Based on the well established paradigm for mammalian receptor kinase action, ligand binding can elicit receptor kinase phosphorylation. Such phosphorylation often causes a change in mobility in SDS/PAGE (43). We generated *EMS1::EMS1-cMyc tpd1* plants and found that EMS1-cMyc proteins from *EMS1::EMS1-cMyc tpd1* plants (Fig. 3D, lane 2) migrated faster in SDS/PAGE than those from *EMS1::EMS1-cMyc* plants (Fig. 3D, lanes 1 and 3). To test whether the mobility shift was caused by phosphorylation, we treated proteins with calf intestinal alkaline phosphatase (CIP). This treatment shifted the slower migrating band (Fig. 3D, lane 4) to the same position as the faster



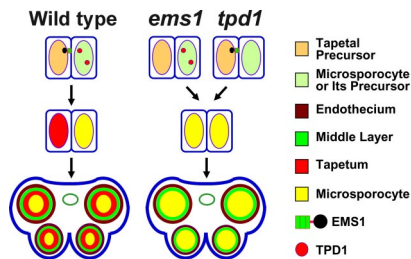
**Fig. 3.** *In vitro* and *in vivo* interaction between TPD1 and EMS1 and TPD1 induces the phosphorylation of EMS1. (A) TPD1 interacts with EMS1 in GST pull-down assay. Three micrograms of GST and GST fusion proteins were used to pull down the same amount of crude protein (200  $\mu$ g) extracts containing HA-TPD1, respectively. (B and C) TPD1 interacts with EMS1 *in planta* in coimmunoprecipitation assay. (B) EMS1-cMyc (Upper) and TPD1-FLAG (Lower) were detected in *EMS1::EMS1-cMyc TPD1::TPD1-FLAG* double-transgenic plants, respectively, by Western blot. (1) Wild type. (2) *EMS1::EMS1-cMyc TPD1::TPD1-FLAG* double-transgenic plant. (C) TPD1-FLAG was detected when proteins from *EMS1::EMS1-cMyc TPD1::TPD1-FLAG* double-transgenic plants were immunoprecipitated with an anti-cMyc antibody (Upper). EMS1-cMyc was also detected when the same proteins were immunoprecipitated with an anti-FLAG antibody (Lower). (1) *TPD1::TPD1-FLAG* (Upper) and *EMS1::EMS1-cMyc* (Lower) single-transgenic plants. (2) *EMS1::EMS1-cMyc TPD1::TPD1-FLAG* double-transgenic plant. (D) TPD1 binding induces EMS1 phosphorylation. Lanes 1, 3, 4, and 6: *EMS1::EMS1-cMyc* transgenic plants; lanes 2 and 5: *EMS1::EMS1-cMyc tpd1* transgenic plants, in which TPD1 is not present. Proteins in lanes 4 and 5 were treated with calf intestinal alkaline phosphatase (CIP). EMS1-cMyc protein from *EMS1::EMS1-cMyc tpd1* plants (lane 2) migrated faster than those from *EMS1::EMS1-cMyc* plants (lanes 1 and 3). A shift in mobility of EMS1-cMyc did not occur (lane 4) after the CIP treatment (lanes 5 and 6 are controls).

band (Fig. 3D, lane 5, lane 6 is the control). Therefore, our results suggest that TPD1 binding leads to EMS1 phosphorylation.

In summary, our studies indicate that interaction between TPD1 and EMS1 is required for cell fate determination during plant sexual reproduction. Furthermore, TPD1 interacts with EMS1 *in vitro* and *in vivo*, and TPD1 binding activates EMS1 phosphorylation. Thus, we propose that the TPD1 small protein serves as a ligand for the EMS1 receptor kinase.

## Discussion

Our results indicate that TPD1 is a ligand for the EMS1 LRR-RLK. So far, studies have identified only a few ligands for some LRR-RLKs with known functions. Steroid brassinolide is the ligand for the *Arabidopsis* BRI1, BRL1, and BRL3 LRR-RLKs (24, 43, 44). Small peptides are found to serve as ligands for LRR-RLKs, such as PSK for carrot and *Arabidopsis* PSKR1 (45, 46), f1g22 for *Arabidopsis* FLS2 (33), and EF-Tu for *Arabidopsis* EFR (35). CLV3, a small secreted protein (47), acts as the ligand of CLV1 (48, 49). Recent studies show that the major active domain of CLV3 is a 12- to 15-aa peptide (50–53). Genetic evidence supports the idea that the EPF1 small secreted peptide acts as a ligand for ERECTA family receptor kinases (54). Small protein LAT52 is a protein ligand for the tomato LRR-RLK, LePRK2 (41). TPD1 is a small protein with 176 aa that contains a predicted N-terminal-secreted signal peptide, followed by a



**Fig. 4.** A model for EMS1-TPD1 signaling in anther cell fate determination. In the wild-type anther, TPD1 small protein is secreted from microsporocytes or their precursors and then bind to EMS1 receptor kinases that are localized to tapetal precursors. EMS1-TPD1 signaling ensures specification of tapetal cell fate by activating the downstream signaling cascade. In the absence of TPD1 ligand or EMS1 receptor in the *tpd1* or *ems1* mutant, signals directing tapetum differentiation are blocked. Consequently, tapetal precursors adopt a microsporocyte fate, resulting in the formation of excess microsporocytes.

predicted cleavage site between the 21st and 22nd amino acid residues ([www.cbs.dtu.dk/services/SignalP](http://www.cbs.dtu.dk/services/SignalP)). Our results provide several lines of evidence to strongly support the idea that TPD1 serves as a ligand of EMS1. Determination of whether secretion of TPD1 is required for its function remains for future study as well as identification of additional signaling molecules in the EMS1 signal transduction pathway.

Very little is known about the signaling mechanisms governing cell fate determination during reproduction in plants and in many other organisms. Among LRR-RLKs with known functions, EMS1 specifically directs the determination of somatic and reproductive cell fates (2). The *EMS1* gene is predominantly expressed in the tapetum, whereas *TPD1* expression is restricted to microsporocytes (2, 7). Therefore, we hypothesize that TPD1 could be secreted from microsporocytes or their precursors and then bind to EMS1 localized on the surface of tapetum precursors (Fig. 4). Signals relayed via EMS1, in turn, direct tapetum formation. In the absence of EMS1-TPD1 signaling, genes promoting microsporocyte differentiation might be persistently expressed in tapetum precursors, resulting in tapetal precursors adopting a microsporocyte fate (Fig. 4). It is also possible that tapetal cells inhibit further proliferation of microsporocytes. Our studies will provide insights into molecular mechanisms of cell fate determination during sexual reproduction in plants and other organisms.

Abnormal differentiation of the tapetum and microsporocytes in *CaMV35S::TPD1* anthers could possibly be due to a negative feedback regulation between the EMS1-TPD1 signal transduction pathway and its target genes, such as *SPL/NZZ* (55, 56). The *spl/nzz* mutant fails to produce microsporocytes and anther wall layers, and *SPL/NZZ* is expressed in both parietal and sporogenous cells, indicating that *SPL/NZZ* promotes anther cell differentiation, including the tapetum during early stages (55, 56). The fact that both *ems1* and *tpd1* anthers form excess microsporocytes suggests that EMS1-TPD1 signaling may repress microsporocyte formation (2, 7). Our unpublished data (G.J. and D.Z.) show that *SPL/NZZ* is epistatic to *EMS1/EXS*. The expression of *EMS1/EXS* was significantly increased after the *SPL/NZZ* function was induced by dexamethasone treatment. Therefore, *SPL/NZZ* possibly controls the expression of genes required for differentiation of both microsporocyte and tapetum, for example *EMS1/EXS*.

On the other hand, EMS1-TPD1 signaling may direct tapetum formation by repressing genes required for microsporocyte differentiation in tapetum precursors. For example, without EMS1-TPD1 signaling, *SPL/NZZ* expression might be persistent in tapetum precursors (i.e., parietal cells), which reverses their cell fate and results in the formation of excess microsporocytes.

However, ectopic EMS1-TPD1 signaling might repress the expression of *SPL/NZZ*, which could affect parietal and sporogenous cell differentiation and subsequently cause aberrant differentiation of tapetum and microsporocytes. It is also possible that functions of other genes, such as *DYT1* and *AMS*, are affected by ectopic expression of *TPD1* (11, 57). In the shoot apical meristem (SAM), the CLV3 ligand interacts with its CLV1/CLV2 receptor complex to elicit a negative signal that represses the *WUS* expression (48, 58). Ectopic expression of *CLV3* causes down-regulation of *WUS*, leading to termination of the SAM (48, 59). The abnormal tapetal layer in some *CaMV35S::TPD1* anthers might possess characteristics of outer layers. In this case, other signaling pathways, like the one mediated by RPK2, could be affected (12). *TPD1* might also restrict its own expression domain. It will be necessary to perform more experiments to test these hypotheses.

The anther primordium (stage 1) consists of L1, L2 and L3 three layers of cells from outer to inner (2). L1 cells form the epidermis, whereas vascular tissues are derived from L3 cells. The L2 cells, which give rise to the majority of anther cells, first differentiate into archesporial cells at stage 2, then develop into primary sporogenous cells and primary parietal cells at stage 3. The primary sporogenous cells eventually become microsporocytes by stage 5. The primary parietal cells divide into secondary parietal cells at stage 4, and subsequently develop into endothecium, middle layer and tapetum by stage 5.

So far, six LRR-RLKs have been identified that play important roles in signaling anther cell differentiation. Without detectable defects before stage 4, the *serk1 serk2* double-mutant anther has a phenotype same as that of *ems1/exs* (2, 8, 9). Similar to BRI1 and BAK1 (also known as SERK3) (26, 27), EMS1/EXS and SERK1/2 could be involved in the same signaling pathway possibly by heterodimerization to control anther cell fate determination after the formation of sporogenous and parietal cells. Mutations in the *RPK2* gene result in failure of middle layer formation and abnormal degeneration of the tapetum after meiosis (12). Therefore, RPK2 signaling might function early to control middle layer determination and later to maintain the tapetum. RPK2 and EMS1/EXS1 might also elicit antagonistic signals to determine the middle layer or tapetum from parietal cells. The *bam1 bam2* mutant, which has a more severe phenotype than *ems1/exs*, *serk1 serk2*, and *rpk2* mutants, lacks the endothecium, middle layer, and tapetum, but produces extra microsporocytes (10). Thus, BAM1/2 might signal differentiation of archesporial cells, which functions earlier than EMS1/EXS, SERK1/2 and RPK2 do. It will be interesting to determine how these LRR-RLK-linked signaling pathways coordinate to regulate anther cell differentiation.

EMS1-TPD1 signaling appears to be a conserved mechanism for anther cell differentiation, because an apparent ortholog to EMS1 exists in rice and possibly in maize (6, 60). We also found several genes that encode proteins similar to TPD1 in the rice genome (D.Z., unpublished data). Eudicots and monocots diverged at least 120 million years ago and together represent >90% of angiosperms, so the presence of the EMS1-TPD1 signal transduction pathway in both suggests that this pathway might have existed in early angiosperms. Land plants produce sporangia that vary in the complexity of structures, such as the tapetum. Ferns, which are nonflowering plants, form a simple sporangium with an outer wall and a tapetum. Thus, studies of EMS1-TPD1 signaling in other vascular plants will shed light on evolution of male reproductive structures in plants.

## Materials and Methods

**Plant Materials and Growth Condition.** *Arabidopsis thaliana* plants were of the Landsberg *erecta* (*Ler*) ecotype and were grown in Metro-Mix 360 soil (Sun-Gro Horticulture) in growth chambers under a 16-h light/8-h dark photoperiod at 22°C and 50% humidity.

**Microscopy.** Semithin sections were made according to the previous procedure (2). Images were photographed with an Olympus BX51 microscope by using an Olympus DP 70 digital camera.

**Yeast Two-Hybrid Assay.** In this study, all DNA and cDNA fragments for cloning were PCR-amplified by the Phusion High-fidelity DNA polymerase (SI Table 1) (New England Biolabs). The resulting constructs were verified by sequencing.

The *TPD1* cDNA was cloned into pAD-GAL4 vector (HybriZAP 2.1 Two-Hybrid System; Stratagene). cDNAs encoding the entire EMS1 LRR domain and shortened EMS1 LRRs were cloned into the pBD-GAL4 vector (Fig. 2A). Yeasts containing a combination of bait and prey constructs were grown on synthetic complete (SC) medium without Leu, Trp, His, and SC medium with a series of concentrations (5, 10, and 30 mM) of 3-Amino-1,2,4-Triazole (3AT). The filter lift assay was performed according to the manufacturer's manual. Positive and negative controls were provided by the manufacturer.

**Pull-Down Assay.** *EMS1* cDNA fragments encoding the EMS1 LRR domain, TPD1-interacting region (TIR), and a non-TIR were PCR-amplified, respectively (SI Fig. 9 and SI Table 1), and then cloned into pGEX-4T-2 vector (Amersham) to produce GST fusion proteins (SI Fig. 10). The *TPD1* cDNA was cloned into pWS93 vector to produce HA-TPD1 proteins in yeast. Three  $\mu$ g each of GST and GST fusion proteins bound to Glutathione-Sepharose-4B beads (Amersham) were incubated with 200  $\mu$ g of crude protein extract overnight at 4°C. After washing three times, the pull-down mixtures were analyzed by Western blot using an anti-HA antibody (Roche).

**Generation of Transgenic Plants.** The Gateway binary vectors were used to generate transgenic plants. The 1740-bp *EMS1* promoter and full-length *EMS1* cDNA were PCR-amplified (SI Table 1), and then cloned into the pENTR TOPO vector (Invitrogen). The *EMS1::EMS1* fragment was cloned into the Gateway binary vector pGWB16 with cMyc tag by an LR reaction (Invitrogen). The 2,770-bp *TPD1* promoter was PCR-amplified (SI Table 1) and then cloned into a vector harboring the *TPD1* cDNA. Similarly, the *TPD1::TPD1* fragment was cloned the Gateway binary pGWB10 vector with the FLAG tag. To ectopically express *TPD1*, the *TPD1* cDNA was cloned into the Gateway vector pGWB2 with a *CaMV35S* promoter.

The resulting constructs were introduced into *Agrobacterium* strain GV3101. After plant transformation, transformants were screened with 50

mg/liter kanamycin and hygromycin. Forty individual *EMS1::EMS1-cMyc* and *TPD1::TPD1-FLAG* transgenic lines were crossed to their corresponding mutants to examine for complementation of the phenotype. *EMS1::EMS1-cMyc* and *TPD1::TPD1-FLAG* plants were then crossed to produce double-transgenic plants. Forty *CaMV35S::TPD1* transgenic lines that occasionally produced a few pollen grains were also crossed to the *ems1* mutant.

**Coimmunoprecipitation.** Young inflorescences harvested from wild-type, single- and double-transgenic plants were ground into fine powder by using liquid N<sub>2</sub> and then in cold grinding buffer [20 mM Tris-HCl (pH 8.8), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 100  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>, 20% glycerol, and protease inhibitor cocktails]. Samples were spun at 6,000  $\times$  g for 15 min at 4°C, and supernatants were further spun at 100,000  $\times$  g for 40 min at 4°C to pellet membrane fractions (26, 61). The pellets were sonicated and resuspended in membrane protein extraction buffer [10 mM Tris-HCl (pH 7.3), 150 mM NaCl, 1 mM EDTA, 50 mM NaF, 100  $\mu$ M Na<sub>2</sub>VO<sub>4</sub>, 10% glycerol, 2% Triton X-100, and protease inhibitors]. After centrifugation at 100,000  $\times$  g for 25 min at 4°C, supernatants were incubated with prewashed anti-FLAG M2 affinity gel (Sigma-Aldrich) overnight at 4°C. After three washes, the eluted immunoprecipitates were used in Western blots to detect EMS1-cMyc (Anti-cMyc; Roche). The reciprocal coimmunoprecipitation was performed by using the Profound c-Myc Tag IP/Co-IP kit (Pierce).

**Kinase Phosphorylation Assay.** Proteins extracted from *EMS1::EMS1-cMyc* and *EMS1::EMS1-cMyc tpd1* plants were separated on a 5% SDS/PAGE gel (43), followed by Western blotting to detect EMS1-cMyc proteins. To confirm whether the mobility shift was caused by phosphorylation, proteins were treated with 10 units of calf intestinal alkaline phosphatase (New England Biolabs) at 37°C for 60 min (61).

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