Regulatory Role of a Receptor-Like Kinase in Specifying Anther Cell Identity

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In flowering plants, sequential formation of anther cell types is a highly ordered process that is essential for successful meiosis and sexual reproduction. Differentiation of meristematic cells and cell-cell communication are proposed to coordinate anther development. Among the proposed mechanisms of cell fate specification are cell surface-localized Leu-rich repeat receptor-like kinases (LRR-RLKs) and their putative ligands. Here, we present the genetic and biochemical evidence that a rice (Oryza sativa) LRR-RLK, MSP1 (MULTIPLE SPOROCYTE1), interacts with its ligand OsTDL1A (TPD1-like 1A), specifying the cell identity of anther wall layers and microsporocytes. An in vitro assay indicates that the 21-amino acid peptide of OsTDL1A has a physical interaction with the LRR domain of MSP1. The ostdl1a msp1 double mutant showed the defect in lacking middle layers and tapetal cells and having an increased number of microsporocytes similar to the ostdl1a or msp1 single mutant, indicating the same pathway of OsTDL1A-MSP1 in regulating anther development. Genome-wide expression profiles showed the altered expression of genes encoding transcription factors, particularly basic helix-loop-helix and basic leucine zipper domain transcription factors in ostdl1a and msp1. Among these reduced expressed genes, one putatively encodes a TGA (TGACGTCA cis-element-binding protein) factor OsTGA10, and another one encodes a plant-specific CC-type glutaredoxin OsGrx_I1. OsTGA10 was shown to interact with OsGrx_I1, suggesting that OsTDL1A-MSP1 signaling specifies anther cell fate directly or indirectly affecting redox status. Collectively, these data point to a central role of the OsTDL1A-MSP1 signaling pathway in specifying somatic cell identity and suppressing overproliferation of archesporial cells in rice.

In flowering plants, germ cells originate from sporophytic tissues during the late developmental stage, which differs from most animals in which their germ cells arise early in embryos (Walbot and Evans, 2003). The anther, the sporophytic male reproductive organ, produces male gametophytes via meiosis and subsequent mitotic divisions to generate the sperm. Anther primordia are generated by the floral meristem and usually contain three meristematic cell layers, L1 to L3. Within each of the anther lobes, the L1 generates the epidermis and the L2 forms both the sporogenous cells (microsporocytes) and three inner anther wall layers: the endothecium, the middle layer, and the tapetum (from outer to inner). The L3 produces the vasculature and connective tissue at the center of each four-lobed anther (Poethig, 1987; McCormick, 1993; Ma, 2005; Wilson and Zhang, 2009; Zhang et al., 2011; Zhang and Yang, 2014).

In the past decade, there have been models explaining the cell division and cell fate specification during anther morphogenesis in Arabidopsis (Arabidopsis thaliana), rice (Oryza sativa), and maize (Zea mays); Ma, 2005; Feng and Dickinson, 2010a, 2010b; Kelliher and Walbot, 2011; Zhang and Yang, 2014). The lineage model proposes that a single hypodermal cell beneath the anther epidermis undergoes sequential asymmetric cell divisions to form three concentric rings of somatic layers and germ cells (Kiesselbach, 1999; Ma, 2005; Feng and Dickinson, 2010a, 2010b). Recent studies in maize dispute the existence of hypodermal cells (Kelliher and Walbot, 2011). By confocal microscopy immature another lobes were shown to contain multiple L2-derived (L2-d) cells. Those cells with only L2-d neighbors respond to hypoxia by differentiating as archesporial cells and subsequent sporogenous cells, while the peripheral L2-d cells differentiate into primary parietal cells (PFCs). The PPC then divides periclinally to form distinctive morphological characteristics of two cell types: the endothecium and secondary parietal cell (SPC) enclosed by the epidermis. Then multipotent SPCs carry on a periclinal division generating daughter cells with the same sizes that further differentiate into the middle layer and the tapetum (Kelliher and Walbot, 2011; Zhang and Yang, 2014).

Successful formation of male gametophytes and gametes requires the nutritive support of neighboring
somatic cells, especially the tapetal cell. Recently, a complex and bidirectional interaction between anther wall layers and microsporocytes was uncovered to play critical roles in determining the cell fate of somatic cells and germ cells. For instance, maize OCL4 (OUTER CELL LAYER4) encodes a HD-ZIP IV transcription factor and expresses exclusively in the epidermis. ocl4 plants have anthers with an extra subepidermal cell layer with endothecial characteristics formed by an aberrant periclinal division in the endothecial cells (Vernoud et al., 2009). In Arabidopsis, the MADS-box transcription factor SPL/NZZ (SPOROCYTELESS/NOZZLE) regulates sporocyte development. The spl/nzz mutant has no formation of microsporocytes and anther somatic wall layers; this AGAMOUS-regulated factor connects floral organ determination with the initiation of anther patterning (Schiethaler et al., 1999; Yang et al., 1999). BAM1 (BARELY ANY MERISTEM1) and BAM2 encode CLAVATA1-related LRR-RLKs; BAM1/2 limit the expression domain of SPL/NZZ, and SPL/NZZ promotes BAM1 expression in the central sporogenous cells, contributing to the correct differentiation of the endothecium, SPc, and the resulting middle layers and tapetal layers (DeYoung et al., 2006; Hord et al., 2006; Sun et al., 2007). ERECTA family genes ER (ERECTA), ERL1 (ERECTA-LIKE1), and ERL2, encoding the LRR-RLKs, redundantly control anther lobe formation and cell patterning (Torii et al., 1996; Shpak et al., 2003; Shpak et al., 2004; Woodward et al., 2005). Furthermore, the signaling of RPK2 (RECEPTOR-LIKE PROTEIN KINASE2), another LRR-RLK, controls the differentiation of the middle layer and maintenance of tapetal cell fate (Mizuno et al., 2007). Besides LRR-RLK proteins, two cytoplasmic mitogen-activated protein kinases (MAPKs), MPK3 and MPK6, function redundantly in anther cell differentiation through a MAPK cascade (Hord et al., 2008).

In Arabidopsis, the cell fate decision of tapetal cells and microsporocytes involves a LRR-RLK complex including EMS1/EXS (EXCESS MALE SPOROCYTES1/EXTRA SPOROGENOUS CELLS), TPD1 (TAPETUM DETERMINANT1), and SERK1/2 (SOMATIC EMBRYO RECEPTOR KINASE1/2) proteins (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Albrecht et al., 2005; Colcombet et al., 2005; Yang et al., 2005). Mutants in EMS1/EXS and TPD1 display the same phenotypes of excess microsporocytes and impaired tapetum and therefore are considered to function in the same pathway to regulate cell fate determination (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003; Feng and Dickinson, 2010a). TPD1, a putative secreted ligand, might act as a ligand for the EMS1/EXS receptor kinase (Yang et al., 2003, 2005). Furthermore, the direct interaction between TPD1 and EMS1 has been confirmed in vitro and in vivo (Jia et al., 2008). A model explaining anther cell specification is that the TPD1 signal is secreted from microsporocytes to surrounding tapetal cells and interacts with EMS1 to promote tapetal cell differentiation (Ma and Sundaresan, 2010; Feng and Dickinson, 2010a). The serk1/serk2 double mutant has a phenotype similar to ems1/exs and tpd1, a result suggesting that SERK1/2 and EMS1/EXS may form heterodimeric receptors (Albrecht et al., 2005; Colcombet et al., 2005). The heterodimeric complex may bind TPD1 to activate downstream targets to acquire anther cell fate; yet, the ability of TPD1 to bind heterodimers is unknown. Maize MAC1 (MULTIPLE ARCHESPORIAL CELLS1), a homolog of Arabidopsis TPD1, suppresses archesporial cell proliferation and promotes the periclinal division of subepidermal cells (Sheridan et al., 1996, 1999; Wang et al., 2012). The MAC1 protein is accumulated preferentially in germ cells, and it contains a predicted signal peptide that leads to secretion (Wang et al., 2012). The mac1 mutant exhibits excess proliferation of archesporial cells not only in anthers, but also in ovules (Sheridan et al., 1996, 1999; Wang et al., 2012). This is different from Arabidopsis tpd1, which only exhibits defects in male tissues.

Rice OsTDL1A/MIL2 (MICROSPORELESS2) and MSPI are orthologous to TPD1 (MAC1) and EMS1/EXS (Nonomura et al., 2003; Zhao et al., 2008; Hong et al., 2012a). Mutations in MSPI give rise to a phenotype with excessive sporogenous cells and failure of the subepidermal cells to divide early. In addition, mspl also displays abnormal ovule development, whereas no female defects were reported in Arabidopsis (Canales et al., 2002; Zhao et al., 2002; Nonomura et al., 2003). The expression of MSPI is detectable mainly in neighboring cells surrounding male and female sporocytes (Nonomura et al., 2003; Hong et al., 2012a). A mutant of OsTDL1A/MIL2 displays defects in early anther cell

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patterning, while the RNA interference (RNAi) lines of *OsTDL1A-MIL2* only show ovule defects (Zhao et al., 2008; Hong et al., 2012a). The *OsTDL1A-MIL2* mRNAs are present early in archesporal cells, later radically in inner somatic cells (Hong et al., 2012a). Even though it has been hypothesized that *OsTDL1A-MIL2* may act as a ligand of MSP1, and *OsTDL1A-MSP1* signaling specifies the early anther development (Zhang and Yang, 2014), the mechanism underlying the *OsTDL1A-MSP1* pathway remains largely unknown. Here, we provide more evidence demonstrating that *OsTDL1A* interacts with MSP1 in regulating the specification of cell fate in anthers, and their loss of function profoundly alters expression of a set of genes.

RESULTS

*OsTDL1A-MSP1* Genetic Pathway Specifies Anther Cell Identity and Impacts Ovule Development

To obtain further insights into the molecular mechanism underlying rice sporogenesis, we identified three completely male-sterile mutants called *ostdl1a*, *msp1-4*, and *msp1-6*, named as such because the mutations were in the *OsTDL1A* and *MSP1* genes (see below). *ostdl1a*, allelic to *mil2* (Hong et al., 2012a), was identified to have a 204-kb pair deletion between markers Y1213 and Y1214 on chromosome 12, which was genetically complemented by a 5.27-kb wild-type *OsTDL1A* (*Os12g28750*) genomic fragment (Supplemental Figs. S1A and S2). *msp1-4* was found to be caused by 10 bp deleted within the DNA sequence encoding the LRR domain of MSP1, causing pretermination of MSP1 translation (Supplemental Fig. S1B; Wang et al., 2006). Later gene mapping and sequence analysis identified another novel allele *msp1-6* with a single-base substitution (C to A) and a frameshift-causing insertion of an A in the DNA sequence of the kinase domain (Supplemental Fig. S1B).

To investigate whether *OsTDL1A* and *MSP1* function in the same pathway, we built a double *ostdl1a msp1-4* mutant by crossing. Phenotypic analysis demonstrated that this double mutant had small, white anthers lacking viable pollen grains (Fig. 1, A–F). Cytological analysis indicated that *ostdl1a msp1-4* had excessive archesporial cells enclosed by a single layer of subepidermal PPCs (Fig. 2, A, B, E, and F). Subsequently, subepidermal cells could divide periclinally to form inner somatic layers in the wild type (Fig. 2, C and D). However, there was neither the middle layer nor the tapetum in the double mutant *ostdl1a msp1-4* at stage 5 (Fig. 2, G and H). At this stage, mutant anthers had only one continuous subepidermal layer, which shares the characteristics with endothecium, and some cells with unknown identity were observed. However, the sporogenous cells showed overproliferation (Fig. 2, G–I). These observations indicate that the double mutant phenocopies both *ostdl1a* and *msp1-4* single mutants indicative of the two genes in the same pathway.

**OsTDL1A Directly Binds to MSP1**

Consistent with the fact that *OsTDL1A* genetically interacts with *MSP1*, these two genes exhibit a distinct but overlapping expression pattern (Supplemental Fig. S4A; Nonomura et al., 2003; Hong et al., 2012a). To further investigate the regulatory relationship between *OsTDL1A* and *MSP1*, we performed qRT-PCR (quantitative reverse transcription PCR) to analyze their...
expression and found that the expression level of OsTDL1A and MSP1 did not show significant changes in msp1 and ostdl1a respectively (Supplemental Fig. S4, C and D). Therefore, OsTDL1A and MSP1 do not regulate each other at transcriptional level. A protein subcellular localization assay showed that MSP1 was localized at plasma membrane (Supplemental Fig. S4B). Given that MSP1 is a membrane kinase protein and OsTDL1A is a putative secretory protein containing an N-terminal signal peptide, it was proposed that OsTDL1A is the ligand of MSP1 (Zhao et al., 2008; Hong et al., 2012a; Zhang and Yang 2014).

To test the feasibility of this hypothesis, we conducted yeast two-hybrid (Y2H) experiments. When the entire LRR domain of MSP1 was used as a bait, no interaction was detected for OsTDL1A (Fig. 3C). The MSP1 protein has 28 LRRs with potential N-linked glycosylation sites that may interfere with its protein interaction as it is produced in yeast Saccharomyces cerevisiae (van der Hoorn et al., 2005; Jia et al., 2008; Supplemental Fig. S5A). Therefore, we made a series of shortened truncated cDNA fragments encoding the LRRs of MSP1 (Fig. 3A). We observed that only one fragment, named MSP1-LRR4 encoding the 20 to 28th LRRs, interacted with OsTDL1A (Fig. 3, A and C). The OsTDL1A encodes a predicted protein of 226 amino acids with a predicted cleavable signal peptide of 1 to 35 amino acids. Multiple sequence alignments showed that the C-terminal segment of OsTDL1A is well conserved in land plants (Supplemental Fig. S5B). To check whether OsTDL1A encodes a small functional peptide, the full-length OsTDL1A and a C-terminal truncation encoding the peptide OsTDL1A-2 (110–226 amino acids) were tested via Y2H assays, where both were found to be capable of interacting with MSP1-LRR4. In contrast, this was not a case for the N-terminal truncation OsTDL1A-1 (1–107 amino acids; Fig. 3, B and D). Further assays confirmed that a 21-amino acid long peptide of OsTDL1A-2 called OsTDL1A-4 (161–181 amino acids) could interact with MSP1-LRR4 in vitro (Fig. 3, B and D). Surprisingly, this peptide was also well conserved in land plants, including multiple monocot and eudicot species, a liverwort, and a moss (Supplemental Fig. S5B), implying that the conserved OsTDL1A may secrete a 21-amino acid peptide serving as a ligand to the membrane-bound LRR-RLK MSP1 in reproductive development.

A direct interaction between OsTDL1A and MSP1 was further confirmed in vitro. A GST-OsTDL1A fusion protein isolated from bacteria was able to pull down transiently expressed MSP1 in tobacco (Nicotiana tabacum) leaves from the total soluble proteins (Fig. 3E). The in vivo physical interaction between OsTDL1A and MSP1 was confirmed by coimmunoprecipitation in tobacco leaves that transiently coexpressed MSP1-Myc and OsTDL1A-FLAG fusion proteins. Membrane protein extract from transgenic leaves was immunoprecipitated with an anti-Myc antibody, and OsTDL1A-FLAG was detected by a western blot using an anti-FLAG antibody (Fig. 3F). In the reciprocal coimmunoprecipitation
experiment, we also detected MSP1-Myc from the anti-FLAG immunoprecipitates (Supplemental Fig. S6). In conclusion, these results strongly suggest that OsTDL1A directly interacts with the specific LRR extracellular region of MSP1 in planta.

A Wide Range of Genes Are Coregulated by OsTDL1A-MSP1 Signaling Pathway

To identify genes involved in the OsTDL1A-MSP1 signaling, we collected spikelets from wild-type, ostdl1a, and msp1-4 plants with three biological replicates. Two key stages were selected for analysis: stage 3 anthers in which L2-d cells have already differentiated into archesporial cells and primary parietal cells; and stage 5 in which middle layers and tapetal cells have been generated by periclinal division of secondary parietal cells (Fig. 4A). Total RNA was extracted from these spikelets and subject to RNA-seq analysis to identify differentially expressed genes. In total, we identified 2,395 genes with substantial differential expression in ostdl1a and/or msp1-4 compared with the wild type (Supplemental Table S1). Remarkably, a large number of genes were down-regulated relative to the wild type at stage 3 and stage 5, and 195 and 147 genes were up-regulated (Fig. 4C). These findings support the hypothesis that OSTDL1A and MSP1 share a common molecular pathway for anther development, which is concomitant with the above genetic and biochemical analysis. Although a large number of common genes was shared between ostdl1a and msp1-4, there were ~400 and ~300 genes differentially expressed only in ostdl1a and msp1-4, respectively (Fig. 4C). Given the diverse expression patterns between OsTDL1A and MSP1, they may also trigger signaling in different molecular pathways during reproductive development.

Considering the great number of common genes with differential expression shared between ostdl1a and msp1-4, hierarchical clustering analysis of significant Gene Ontology (GO) terms was performed to identify...
molecular processes that are affected by the mutation of OsTDL1A or MSP1 simultaneously (Fig. 4D). The enriched processes were classified into four groups, namely, G1, G2, G3, and G4 (Fig. 4D). G1 cluster included enriched processes shared in ostdl1a and msp1-4 at stage 3 and stage 5, such as hormone-mediated signaling pathways, multiorganism processes, DNA-templated transcription, cell death, and reactive oxygen species (ROS) metabolic process, etc. The identification of hormone-mediated signaling pathways is consistent with the fact that hormones play critical roles in determining cell fate in anther development (Ye et al., 2010; Song et al., 2013); however, it is not clear that whether they were directly or indirectly affected by OsTDL1A-MSP1 signaling. The enrichment in DNA-templated transcription suggests a great number of genes encoding transcription factors are involved in OsTDL1A-MSP1 signaling to regulate anther development. Noteworthy, ROS metabolic process was observed, supporting the recent evidence that redox state specifies anther cell fate in plants (Kelliher and Walbot, 2012; Zhang and Yang, 2014). In the G2 clade, there is an enrichment of genes associated with signaling cascades, like signal transduction, cell communication, and MAPK cascade at stage 5 in ostdl1a and msp1-4, which is consistent with the biological function of kinase protein MSP1. Nucleobase-containing compound metabolic process and photosynthesis were enriched in clade 3. Furthermore, differentially expressed genes in the G4 clade associated with cell division were specifically observed in ostdl1a, possibly due to its role in cell proliferation (Fig. 4D).

Previous transcriptome data sets generated using microarray analysis in Arabidopsis ems1/exs and maize mac1 showed a wide range of changes in gene regulatory networks (Wijeratne et al., 2007; Zhang et al., 2014). Given the conserved function of OsTDL1A-MSP1, MAC1, and EMS1 in male organ formation, a comparison of these three transcriptome sets was performed here (Supplemental Fig. S7A). Of the differentially

Figure 4. Differential gene expression profiles of ostdl1a and msp1-4. A, Developmental progression of an anther lobe for RNA-seq from the establishment the archesporial cells to post cell fate specification of four anther wall layers. B, Heat map for the expression profiles of differentially expressed genes in ostdl1a and msp1-4 compared with the wild type. C, Differentially expressed genes shared between ostdl1a and msp1-4 at stage 3 and stage 5. Red arrows represent genes up-regulated, and green arrows represent genes down-regulated. D, GO analysis for differentially expressed genes shared between ostdl1a and msp1-4. Venn diagrams above each set of columns are shaded to represent differentially expressed genes shared between the two mutants (from left to right): differentially expressed genes in ostdl1a only, in ostdl1a and msp1-4, and in msp1-4 only. G, group; S3, stage 3; S5, stage 5.
expressed genes in Arabidopsis ems1 mutant, 10% overlapped with their corresponding rice orthologs in osd11a and msp1-4 mutants, while a higher proportion (21%) was observed between maize and rice (Supplemental Fig. S7A and Supplemental Table S2). Further GO analysis revealed that these commonly expressed genes in rice, maize, and Arabidopsis are involved in similar processes, including transcription factor activity, oxidoreductase activity, and phosphatase activity, etc. (Supplemental Fig. S7B). This suggests that OsTDL1A (or MSP1) and its orthologs are able to regulate similar biological processes during anther development.

Altered Expression of Genes Encoding Transcription Factors

To identify early signatures of transcriptional regulation in specifying anther cell fate, we analyzed the expression of transcription factors in osd11a and msp1-4 (Supplemental Table S3). In total, there are 25 families of transcription factors, such as APETALA2, bHLH, MYB, and bZIP, enriched in the two mutants analyzed (Supplemental Fig. S8A). Overall, osd11a and msp1-4 had similar numbers of differentially expressed transcription factors during anther development. Among these, 70% were down-regulated in the two mutants, such as bHLH, MYB, NAC, HB (homeobox domain), and bZIP families. Only some genes encoding members of the MADS, B3 (ABI3VP1 protein), OFP (ovate family protein), and HMG (high mobility group protein) families were up-regulated (Supplemental Fig. S8B). The DBB (double B-box) and ARR-B (the type-B plant male reproduction (Kelliher and Walbot, 2012; Zhang and Yang, 2014). Among the genes with altered expression in osd11a and msp1-4, we observed numerous genes associated with redox modulation (Fig. 6A; Supplemental Table S4), and a great overlap was observed between osd11a and msp1-4 (Fig. 6A, red dotted line). These differentially expressed genes encode ROS-related enzymes and proteins, including putative peroxidases, oxidoreductases, cytochrome P450s, thioderodoxins, and glutaredoxins, which are involved in the ROS-producing and ROS-scavenging pathway. In agreement with the expression change of genes associated with redox status, the result of 3,3′-diaminobenzidine (DAB) staining analysis for the presence of hydrogen peroxide showed the production of the probes for OsbHLH167 and OsbHLH042 were absent (Fig. 5B). OsbHLH006 and OsbHLH148, which are specifically expressed at early stages of anther development, were identified in the same cluster with TIP2 and TDR. In the UDT1 cluster, OsbHLH035 and OsbHLH063 showed relatively higher expressions at premeiosis stages, while the expression of OsbHLH010 was high at early meiosis stages (Fig. 5B). qRT-PCR analysis further confirmed that OsbHLH6, OsbHLH148, and OsbHLH10 were down-regulated in the two single mutants and the double mutant at stage 3 and stage 5, whereas TIP2 showed reduced expression at stage 5 (Fig. 5, D–G). In addition, the expression of OsbHLH063 decreased only in osd11a msp1-4 at stage 5, while OsbHLH035 and OsbHLH036 did not show significant changes in the single mutants (Fig. 5H). These observations suggest that the bHLHs regulated by OsTDL1A-MSP1 signaling play a diverse function in regulating cellular events during anther development.

Genes for Redox Modulation Are Impacted by OsTDL1A-MSP1 Pathway

Redox regulation has recently emerged as a crucial mechanism for managing significant stages during plant male reproduction (Kelliher and Walbot, 2012; Zhang and Yang, 2014). Among the genes with altered expression in osd11a and msp1-4, we observed numerous genes associated with redox modulation (Fig. 6A; Supplemental Table S4), and a great overlap was observed between osd11a and msp1-4 (Fig. 6A, red dotted line). These differentially expressed genes encode ROS-related enzymes and proteins, including putative peroxidases, oxidoreductases, cytochrome P450s, thioderodoxins, and glutaredoxins, which are involved in the ROS-producing and ROS-scavenging pathway. In agreement with the expression change of genes associated with redox status, the result of 3,3′-diaminobenzidine (DAB) staining analysis for the presence of hydrogen peroxide showed the production...
of hydrogen peroxide only in the middle layer of wild-type anthers after the differentiation of the four cell wall layers at stage 5 (Fig. 6, C–F). No staining was observed in ostdl1a and msp1-4, suggesting that ostdl1a and msp1-4 have defects in the production of hydrogen peroxide during the anther development probably because they lack a middle layer (Fig. 6, G–J). In support of this, there were two differentially expressed genes encoding plant-specific CC-type glutaredoxins, which function as small oxidoreductases of the thioredoxin family proteins. Glutaredoxin OsGrx-II was significantly downregulated in the two single mutants and the double mutant at stage 3 and stage 5 (Fig. 6B). In addition, glutaredoxin MIL1 (MICROSPORELESS1), which was previously reported to control the meiosis initiation in microsporocytes and differentiation of the inner
secondary parietal cells into the middle layer and tapetal cells (Hong et al., 2012b), decreased in the mutants at stage 5 (Fig. 6B). These observations suggest that tightly regulated redox status plays an important role in the process of anther development. In addition, ROS accumulation is reduced in mutants defective in formation of the middle layer, suggesting that the middle layer cells might be the source of ROS production.

Glutaredoxins are important for redox control in normal anther development and are confirmed to act by posttranslationally modifying TGA transcription factors through a direct biochemical interaction. The physical interaction between OsTGA10 and OsGrx_I1 was detected by Y2H analysis (Fig. 7A). To gain further insights into the role of OsTGA10 and OsGrx_I1 in early anther development, RNA in situ hybridization was performed. OsTGA10 transcripts were detectable in the primary parietal cells at early anther development stage, and subsequently OsTGA10 expression was strongly detectable in the middle layer and tapetal cells, whereas its signal was almost invisible during meiosis (Fig. 7, B–G). Overall, the expression pattern of OsTGA10 was similar to that of MSP1, which specifically expressed in anther inner somatic layers, implying that OsTDL1A-MSP1 may specify anther cell fate by directly or indirectly affecting the expression of OsTGA10. Supportively, the signal from OsTGA10 was barely observed in ostdl1a and msp1-4 (Fig. 7, H–M). Given the previous discovery that glutaredoxins act as interacting partners of TGA transcription factors (Murmu et al., 2010; Hong et al., 2012b), we speculate that the CC-type glutaredoxin OsGrx_I1 may modify the conserved Cys residues within OsTGA10, thereby affecting its transcriptional activity. Interestingly, OsTGA11 and OsTGA12 were found to be expressed in...
the inner parietal layer and archesporial cells at stage 3 and strongly in the middle layer, tapetal cells, and sporogenous cells at later stage 5 (Supplemental Fig. S9, D–K). Expression pattern analysis showed that the mRNA levels of OsTGA11 and OsTGA12 were high at premeiosis stages, and they are homologous to Arabidopsis TGA9/10. Although the mutations of OsTDL1A and MSP1 did not affect the expression of OsTGA11 and OsTGA12, the expression of these two genes partially overlap with that of OsTGA10. It is possible that TGA members may function redundantly in anther development.

Figure 7. Expression pattern of OsTGA10 and its interaction with OsGrx_I1. A, The interaction between OsTGA10 and OsGrx_I1 in yeast two hybrid assays. B to G, In situ analyses of OsTGA10 in wild-type anthers from stage 2 to stage 7. OsTGA10 is transcribed mainly in primary parietal at early development stage and later strongly in the middle layer and tapetal cells. H to J, ostdl1a anthers hybridized with OsTGA10 antisense probe from stage 3 to stage 5. K to M, msp1-4 anthers hybridized with OsTGA10 probe from stage 3 to stage 5. No obvious expression of OsTGA10 in ostdl1a and msp1-4 anthers. ML, middle layer; T, tapetum. Bars = 15 μm.

OSTDL1A-MSP1 Pathway Affects Signal Transduction for Cell Fate Specification

RLKs are associated with signal perception and transduction in organisms, and several LRR-RLKs were reported to play major roles during anther development. Therefore, we were interested in whether there were additional genes encoding LRR-RLK or RLKs, whose expression was affected by the ostdl1a and/or msp1-4. In total, we observed 25 RLKs with changed expression levels, including LRR-RLKs (8), S-locus domain RLKs (6), WAK (wall-associated kinase)-RLKs (4), lectin-like RLKs (3), and other RLKs (4) (Supplemental Table S5). Among all the LRR-RLKs genes, MSP1 decreased only in the msp1-4 mutant, not in the ostdl1a. Moreover, three LRR-RLK genes (Os05g51070, Os01g42294, and Os01g51400) belonging to the same subfamily of MSP1 were up-regulated only in msp1-4. One LRR-V subfamily member (Os12g43640) was down-regulated in ostdl1a and msp1-4. Besides, one gene (Os06g35850) in the lectin-like RLK group showed 2- to 3-fold increase in the two mutants at stage 3 and stage 5. In addition to RLKs, six genes encoding MEK kinases (MAPKKKs) and one MAPK were down-regulated in ostdl1a and/or msp1-4 (Supplemental Table S5). qRT-PCR analysis further confirmed that the expression of OsMAPKK55 and OsMAPKK62 decreased in the mutants at stage 3 and stage 5, while OsMAPKK63 decreased at stage 5 (Supplemental Fig. S10, B–D). Furthermore, one MAPK OsMAPK3, the ortholog of Arabidopsis MPK3 functioning in early anther development, showed reduction in ostdl1a and msp1-4 at stage 5 (Supplemental Table S5). Previous reports indicated that OsMAPK3 and OsMAPK6 have roles in rice immunity (Kim et al., 2012), and whether they have similar functions in anther development as Arabidopsis MAPK3/6 needs further study. Interestingly, the corresponding MAPKK homologs from maize were down-regulated in mac1 microarray data (Supplemental Table S2). These results suggest that perturbations in OsTDL1A-MSP1 signaling affect the protein kinase signaling pathways during anther development.

DISCUSSION

OsTDL1A-MSP1 Represents a Conserved and Diversified Signaling Pathway in Specifying Cell Fate during Plant Reproduction

Establishment of new cell types is essential for the formation of organized and functional organs. Development of male organs and reproductive cells requires the initiation of the anther primordium, cell division, and differentiation, all of which when properly executed result in the development of concentric rings of somatic layers and germinal cells. In this study, our data support that the small secretory peptide OsTDL1A is able to bind a LRR-RLK receptor, MSP1, and OsTDL1A-MSP1 signaling pathway specifies the early
anther cell fate by triggering the formation of the middle layer and tapetal layer from meristematic parietal cells and proper number of reproductive microsporocytes in rice.

Our genetic and cytological analysis revealed that ostdl1a msp1-4 double mutant shows defects including the lack in the middle layer and tapetal cells and an increased number of microsporocytes and partially aborted ovule development similar to the single mutant ostdl1a or msp1-4 (Figs. 1 and 2). This result indicates that OsTDL1A functions in the same signaling pathway with MSP1 in specifying the anther cell fate. Biochemically, the OsTDL1A-MSP1 interaction was confirmed by coimmunoprecipitation assays (Fig. 3), which is consistent with previous observation that OsTDL1A binds to the LRR domain of MSP1 by bimolecular fluorescence complementation in onion cells; however, OsTDL1B, the paralog of OsTDL1A, has no physical interaction with MSP1 (Zhao et al., 2008). OsTDL1A mRNA accumulates in both inner somatic cells and sporogenous cells, while MSP1 mRNA was detectable early in parietal cells and later in middle layers and tapetal cells. OsTDL1A/MIL2 protein was previously seen to localize on the margin of the tapetum and middle layer (Hong et al., 2012a). It is likely that OsTDL1A acts as a developmental signal secreted from sporogenous cells and parietal cells. The membrane-bound MSP1 perceives this signal and promotes the periclinal division of parietal cells to form the endothecium, SPC, and subsequent the middle layer and the tapetum, simultaneously suppressing the proliferation of reproductive cells (Fig. 8). In agreement with the placement of OsTDL1A and MSP1 in the same pathway, our transcriptional analysis revealed that OsTDL1A and MSP1 shared a variety of common downstream genes with altered expression levels in ostdl1a and msp1-4, even though there are some genes displaying differential expression changes between ostdl1a and msp1-4 (Fig. 4). With the respect to the differentially expressed genes involved in signal transduction enriched in msp1-4 at stage 5, in ostdl1a at stage 3 (Fig. 4D), we speculate that MSP1 may have an additional partner(s) except for OsTDL1A, and vice versa. Consistently, more up-regulated LRR-RLKs were preferentially detected in msp1-4 (Supplemental Table S5).

To date, a few LRR-RLKs have been identified in perceiving signals such as steroids, peptides, and secreted proteins (Larsson et al., 2007). Phytosulfokine is a sulfated peptide five amino acids in length that serves as a ligand of Arabidopsis phytosulfokine receptor in promoting cellular growth (Matsubayashi et al., 2002, 2006). The 22-amino acid flagellin-derived peptide flg22 binds to Arabidopsis FLAGELLIN SENSITIVE2 and

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**Figure 8.** Proposed model of OsTDL1A-MSP1 signaling in specifying early anther development. The small secreted peptide OsTDL1A serves as a ligand that interacts with the LRR domain of MSP1 localized on the outer surface of inner somatic layers. Rice OsTDL1A-MSP1 signaling promotes the division and differentiation of primary parietal cells for the formation of middle layer and tapetum, and limits the proliferation of reproductive sporogenous cells. MAPK cascades and putative RLKs may act downstream of OsTDL1A-MSP1 complex to transduce the anther developmental signal. OsTDL1A-MSP1 signaling may indirectly affect the expression of the TGA transcription factor OsTGA10 and glutaredoxins OsGrx_I1 and MIL1. Moreover, the expression of genes encoding transcription factors such as OsbHLH members is affected by OsTDL1A-MSP1. Positive and negative regulatory actions are indicated by arrows and lines with bars. Lines ending with diamonds represent interactions. Ar, archesporial cell; KD, kinase domain; LRR, leu-rich repeat; ML, middle layer; Sp, signal peptide; Sp, sporogenous cell; T, tapetum; TM, transmembrane domain.
regulates defense response (Gómez-Gómez and Boller, 2000). The small secreted EPIDERMAL PATTERNING FACTOR-LIKE proteins are 45 to 76 amino acids in size and can bind to ER receptor kinases in Arabidopsis to regulate stomatal development (Kondo et al., 2010; Sugano et al., 2010). CLV3 (CLAVATA3) is a secreted peptide producing the 12-amino acid hydroxylated peptide as an in vivo ligand for the CLV1-CLV2 receptor kinase complex, with a role in regulation of the size of the shoot apical meristem (Fletcher et al., 1999; Kondo et al., 2006). Here, we showed that a 21-amino acid peptide of OsTDL1A interacts with MSP1 in vitro (Fig. 3). Moreover, the 21-amino acid peptide sequence of OsTDL1A is evolutionarily conserved from lower plants (such as liverwort and moss) to seed plants. It is highly possible that OsTDL1A may be secreted and cleaved into a small functional peptide that acts as the ligand of MSP1, but the exact size and sequence of the functional peptide remains to be elucidated.

Morphologically, monocots (including rice and maize) and eudicots (including Arabidopsis) have similar and distinct aspects of anther development and pollen formation (Wilson and Zhang, 2009; Kelliher and Walbot, 2011; Zhang et al., 2011), suggesting that both conserved and divergent mechanisms underlie plant male reproduction. In the grasses, the related small secreted ligands OsTLD1A (and its receptor MSP1) and MAC1 are required for early anther development. Mutations of OsTLD1A or MAC1 cause the defective somatic patterning. Furthermore, MAC1 has been shown to have a function in male and female development similar to that of OsTLD1A (Wang et al., 2012), suggesting that the functional conservation of OsTDL1A-MSP1 in maize, even though the MSP1 homolog in maize has not been characterized. In contrast, in the absence of the ortholog TPD1 or EMS1/EXS in Arabidopsis, the PPC could perform a periclinal division to establish a bilayer (the endothecium and SPC), although tapetal layers cannot be formed because of the failure of subsequent symmetric cell divisions of SPCs (Canales et al., 2002; Zhao et al., 2002; Yang et al., 2003). It is possible that a related (but not yet defined) ortholog or other factors are essential for differentiation of the PPC in Arabidopsis.

Role of Redox Status in Specifying Anther Cell Fate

It is well known that cellular redox states critically control the fate of anther cells (Kelliher and Walbot, 2012; Zhang and Yang, 2014). In this work, transcriptional analysis revealed that many genes involved in redox process are differentially expressed in both mutants at stages 3 and 5, confirming the role of redox status in controlling male gametogenesis (Fig. 6A). Previously, we showed that ROS molecules are highly detectable in wild-type anthers at stages 8 to 9, invisible at stages 10 and 11, then observed again at stage 12, which corresponds the initiation of tapetal programmed cell death and then degeneration (Hu et al., 2011). In this work, DAB staining analysis revealed the presence of hydrogen peroxide in the middle layer (in wild-type anthers) after the differentiation of four another wall layers (Fig. 6, C–F). The function of hydrogen peroxide in the middle layer is not clear, but ROS (hydrogen peroxide) could be a marker for the middle layer. It is possible that ROS has a critical role in promoting the differentiation from parietal cells to inner anther wall layers. During the process of anther development, we can infer that spatiotemporal production of ROS is triggered by developmental signal, accompanying the temporal expression of redox-related factors. Thus, the redox status is a significant determinant of cell fate in early anther development.

Glutaredoxins (GRXs) have long been known as small oxidoreductases that are involved in various cellular processes and responses to oxidative stress, such as Arabidopsis ROXY1 and ROXY2 (Xing and Zachgo, 2008; Murmu et al., 2010), maize MSCA1 (MALE STERILE CONVERTED ANther1; Chaubal et al., 2003), and rice MIL1 (Hong et al., 2012b). In this work, we showed that two CC-type glutaredoxins OsGrx_I1 and MIL1 had down-regulation in ostdl1a and msp1-4 (Fig. 6B). Glutaredoxins have been recently revealed to posttranslationally modify TGA transcription factors, thereby affecting their transcriptional activity (Li, 2014). In Arabidopsis, CC-type glutaredoxins ROXY1/2 play a redundant role in regulating early anther lobe formation and anther wall differentiation by interacting with TGA9/10 (Xing and Zachgo, 2008; Murmu et al., 2010). MSCA1, the ROXY1/2 homologs in maize, plays a role in interpreting hypoxia to trigger archesporial cell fate acquisition. msca1 has no progression of archesporial cell differentiation and forms vasculature instead (Chaubal et al., 2003; Kelliher and Walbot, 2012). Furthermore, MSCA1/Abph2 (Aberrant phylloxytax 2) can interact with FEA4 (FASCIA TATE EAR4) a TGA protein, and regulate the shoot apical meristem size in maize (Pautler et al., 2015; Yang et al., 2015). Rice MIL1 is the maize ortholog of MSCA1, but the mil1 mutant microsporangiun exhibits normal differentiation of the archesporial cells and the development of two secondary parietal layers, different from those of msca1 and roxy1/2 (Hong et al., 2012b). Furthermore, mil1 has the defects in the differentiation of the inner secondary parietal cells into middle and tapetal layers as well as the meiosis initiation, and MIL1 has a interaction with OsTGA1 (Hong et al., 2012b). These findings suggest that there is functional divergence among GRXs and TGAs in regulating the differentiation of both germinal and somatic anther tissues.

In this work, we showed that OsGrx_I1 has a physical interaction with OsTGA10 (Fig. 7A), which is consistent with the previous report that GRXs bind to bZIP-type TGA transcription factors to activate the expression of downstream genes (Li, 2014). Moreover, OsTGA10 has a dramatically reduced expression in ostdl1a and msp1-4 (Fig. 5I). Strikingly, OsTGA10 shares an expression pattern similar to that of MSP1 in the primary parietal cell fate acquisition.
cells and then peaking in middle layers and tapetal cells, implying that OsTGA10 might have a role in regulating anther cell specification directly or indirectly dependent on OsTDL1A-MSP1 pathway. Consistently, almost no mRNA accumulation of OsTGA10 was detected in ostdl1a and msp1-4 (Fig. 7, B–M). Therefore, we speculate that OsTGA10 may be modified by the redox modulator OsGrx_I1, activating the transcription of downstream targets. Collectively, these results provide new evidence that redox signaling plays a critical role in anther specification, broadening our understanding of how redox-regulated TGA factors contribute to plant development.

The Regulatory Pathway of OsTDL1A-MSP1 Signaling

Increasing evidence reveals the key role of transcription factors in regulating tapetal fate and normal microsporogenesis. For instance, four bHLH members UDT1 (Jung et al., 2005), TIP2 (Fu et al., 2014), TDR (Li et al., 2006), and EAT1 (ETERNAL TAPETUM1; Ni et al., 2013) were shown to be key regulators for rice anther development (Zhang and Liang, 2016). UDT1 specifies the differentiation of secondary parietal cells to functional tapetal cells (Jung et al., 2005). The expression signal of TIP2 is strongly present in the middle layer and tapetum and weakly in the endothecium, and TIP2 promotes cell differentiation of three inner somatic layers. The tip2 mutant displays undifferentiated inner three anther wall layers and aborted tapetal programmed cell death (Fu et al., 2014). Genetic and biochemical evidence indicates that TIP2 functions upstream of TDR and EAT1, and TDR interacts with both TIP2 and EAT1, respectively, forming a TIP2-TDR-EAT1 regulatory cascade for anther development (Ni et al., 2013; Fu et al., 2014). Our transcriptome analysis showed that the expression of UDT1, TIP2, and TDR is reduced in ostdl1a and msp1-4, suggesting that UDT1, TIP2, and TDR are downstream of OsTDL1A-MSP1 signaling. In support of this, in situ hybridization showed the reduced expression pattern of UDT1 in ostdl1a/mil2-1 anthers (Hong et al., 2012a). On the other hand, the expression levels of MSP1 and OsTDL1A were not significantly changed in tip2, while UDT1 was up-regulated in tip2 (Fu et al., 2014). Furthermore, we observed 21 bHLH members with changed expression in ostdl1a and/or msp1-4 (Fig. 5). It will be interesting to investigate the function of these bHLHs affected by OsTDL1A-MSP1 in early anther development.

Genetic studies have uncovered that RLKs regulate various steps in anther patterning. Although a few genes encoding LRR-RLKs have expression alteration in ostdl1a and/or msp1-4, their biological function remains unclear (Supplemental Table S5). In addition, in ostdl1a and msp1-4, we observed the down-regulations of mRNA levels of several OsMAPKKs and OsMAPK3, whose counterparts also exhibited reduced expression in msp1 (Supplemental Table S2), suggesting possible common and conserved regulatory pathways involving OsTDL1A-MSP1 in anther development. Consistent with the hypothesis that MAPK proteins function downstream of OsTDL1A-MSP1, Arabidopsis MAP kinases MPK3/6 and the LRR-RLKs ER/ERL1/ERL2 were shown to act in the same pathway required for normal anther lobe formation and cell differentiation (Hord et al., 2008). Future work may reveal which MAPKs receive the developmental signal from OsTDL1A-MSP1 pathway and coordinate the development of anther cell wall layers and germ cells in the future.

CONCLUSION

In summary, we used genetic and biochemical approaches to support that the peptide OsTDL1A binds to a LRR-RLK MSP1 and then promotes the development of parietal cells into the middle layer and tapetal layer. These proteins also limit the extra number of sporogenous cells during rice anther development. The 21-amino acid domain of OsTDL1A that binds MSP1 is evolutionarily conserved from lower plants to higher plants. The OsTDL1A-MSP1 signaling pathway may specify anther cell fate by changing the expression of numerous genes encoding bHLH, bZIP transcription factors, and redox-related proteins directly or indirectly (Fig. 8). In particular, OsTDL1A-MSP1 signaling dramatically affects the expression of OsTGA10, which could interact with a glutaredoxin OsGrx_I1. Intriguingly, the presence of hydrogen peroxide in the middle layer highlights a new role of reactive oxygen species in specifying anther cell identity. These findings provide insights into signaling pathway important for the specification of cell identity during early anther development in plants.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

All the rice (Oryza sativa) plants used in this study are in the background of 9522 cultivar (O. sativa ssp. japonica) and were grown in the paddy field of Shanghai Jiao Tong University. Male sterile mutants ostdl1a, msp1-4, and msp1-6 are from our rice mutant library made by 60Co γ-ray radiation. The ostdl1a msp1-4 double mutant was isolated by crossing and verified by genotyping.

Characterization of the Mutant Phenotype

 Morphology of flowers and anthers was photographed with a Leica S8APO stereomicroscope. Pollen viability analysis was conducted using iodine-potassium iodide solution and then photographed with a Leica DM2500 Microscope. The 4’,6-diamino-phenylindole staining of microspores and semithin section analysis were performed according to a previous study (Li et al., 2006). Anthers from different developmental stages, as defined by Zhang et al. (2011), were collected based on spikelet length and lemma/palea morphology, and the developmental stages of wild-type anthers were further confirmed by semithin sections.

Map-Based Cloning

The F2 mapping population was generated from a cross between ostdl1a/msp1-6 (japonica) and 9311 (indica) for gene mapping. To map the OsTDL1A and
MSP1 locus, bulked segregating analysis was used as described by Liu et al. (2005), and insertion-deletion (inDel) molecular markers were designed based on the sequence difference between rice sps. *japonica* and rice sps. *indica* described in the National Center for Biotechnology Information. Further fine-mapping was performed using the previously published method (Chu et al., 2005). Markers used for cloning are listed in Supplemental Table S6.

### Yeast Two-Hybrid Analysis

In this study, all full-length cDNA and cDNA fragments for cloning were amplified using primers listed in Supplemental Table S6. PCR sequences were cloned into pGBKTT7 and pGADT7 (Clontech). Yeast two-hybrid assays were performed according to instructions of the Matchmaker GAL4 two-hybrid system (Clontech). Specific bait and prey constructs were transformed into yeast strain AH109 simultaneously. Protein interactions were tested by the growth conditions on selective synthetic defined (SD) media (Trp-/Leu-/His-/Ade+/Xa-ergal).

### Antibody Preparation and Purification

The specific fragment of MSP1 cDNA was synthesized for antigen and was cloned into pGEX-6P-1 vector at the BamHI and NotI sites. The construct was transformed into *Escherichia coli* BL21 (DE3; Novagen) and protein expressed and purified using Affinity Resin (Clontech) from a soluble fraction under natural conditions, according to the manufacturer’s instructions. Recombinant proteins were separated by SDS-PAGE, and gel slices containing the protein were used directly for the production of rabbit polyclonal antibodies. MSP1 antibodies were purified as described previously (Ritter, 1991).

### Pull-Down Assay and Coimmunoprecipitation

The full-length cDNA of OsTDL1A was synthesized by Sangon and was cloned into pGEX-6P-1 vector at the BamHI and NotI sites. Recombiant proteins were expressed in the DE3 strain induced by isopropyl-β-D-thiogalactoside (IPTG) at 30°C. The purified GST-OsTDL1A and GST proteins were immobilized onto glutathione Sepharose beads (Amersham). Then total soluble protein extract of *Escherichia coli* transformed into *Escherichia coli* strain induced by isopropyl-β-D-thiogalactoside (IPTG) was added to the beads. The beads were washed three times with wash buffer (150 mM NaCl, 1% Triton X-100, 2.5 mM EDTA, 2 mM benzamidine [Sigma-Aldrich], 10 mM 3-mercaptoethanol, 20 mM NaF, 1 mM PMSF, 1% Protease Cocktail [Sigma-Aldrich], and 10% glycerol). After centrifugation at 13,000g for 10 min at 4°C, the supernatant was incubated with anti-FLAG or anti-Myc antibodies and IgG-bound to Protein A/G Sepharose beads (Sigma-Aldrich) for 1 h at 4°C, and the beads were washed three times with wash buffer. Proteins were eluted by boiling the beads in 2× SDS sample buffer and separated on SDS-PAGE before immunoblotting using anti-FLAG or anti-Myc antibodies.

### qRT-PCR and in Situ Hybridization

Total RNAs were isolated from rice tissue using the Trizol reagent (Invitrogen) following the manufacturer’s instructions. cDNA was synthesized from 1 μg of total RNA per sample using the Primerscript RT reagent kit with genomic DNA eraser (Takara). qRT-PCR was performed on a CFX96 (Bio-Rad) machine using SYBR Green qRT-PCR Mix (Bio-Rad) according to the manufacturer’s instructions. *ACTIN* was used as an internal control, and all the primers for qRT-PCR are listed in Supplemental Table S6. qRT-PCR results shown are representative of experiments performed at three biological repeats with three technique repeats.

For RNA in situ hybridizations, Specific fragments of the coding sequences were amplified by PCR using specific primers carried with T7 promoter (Supplemental Table S6). Antisense and sense probes were transcribed using the DIG RNA labeling kit (Roche). Freshly collected samples were fixed in FAA, dehydrated in a series of graded ethanol, infiltrated with Histoclear II, embedded in Paraplast Plus (Sigma-Aldrich), and sectioned into 6-μm-thick sections using a Leica RM2245 rotary microtome. RNA in situ hybridizations were performed as described by Li et al. (2006).

### ROS Staining Analysis with DAB

Freshly collected anthers were put in the fixative solution O.C.T. Compound (Sakura) and then frozen into liquid nitrogen. Embed fresh tissues were sectioned into 8-μm-thick sections using a Leica CM3050 S cryostat. Production of hydrogen peroxide was performed by incubating sectioned anthers in the DAB solution (Vector Laboratories) according to the manufacturer’s instructions and photographed with a Leica DM2500 microscope.

### RNA-Seq and GO Enrichment Analysis

Total RNAs were prepared from wild-type, *ostdl1a*, and *mspl-4* spikelets with three biological replicates. RNA-seq library preparation and sequencing were performed with Illumina sequencing technology (BGI-Shenzhen); these libraries and the mapping methods were described (Q Fei, L Yang, W Liang, D Zhang, B Meyers, unpublished data) Genes considered to be significantly differentially expressed if both the q-value < 0.01 and the fold change ≥ 2.0. GO analysis was performed by R package “topGO” (Alexa and Rahnenfuhrer, 2010).

### Accession Numbers

Sequence data from this article for the mRNA and genomic DNA can be found in the GenBank/EMBL data libraries under the following accession numbers: OsTDL1A (Os12g28750), MSP1 (Os01g58870), OsTGA10 (Os09g31390), OsTGA911 (Os11g05480), OsTGA912 (Os12g05680), OsGrx2 (Os01g17760), OsOshLHL006 (Os04g23550), OsOshLHL010 (Os01g50940), OsOshLHL063 (Os05g26210), OsOshLHL148 (Os06g53020), OsMAPKKK5S (Os01g50400), OsMAPKKK5S (Os01g50400), and OsMAPKKK63 (Os01g50570).

### Supplemental Data

The following supplemental materials are available.

**Supplemental Figure S1.** Map-based cloning of OsTDL1A and MSP1.

**Supplemental Figure S2.** Complementation of the *ostdl1a* mutant.

**Supplemental Figure S3.** DAPI-stained chromosome spreads of microsporocytes in the wild type, *ostdl1a*, and *mspl-4*.

**Supplemental Figure S4.** Expression pattern of OsTDL1A and MSP1.

**Supplemental Figure S5.** The LRR repeats of the MSP1 protein and sequence analysis of OsTDL1A.

**Supplemental Figure S6.** In vivo interaction between OsTDL1A and MSP1.

**Supplemental Figure S7.** Transcriptome comparison among rice (*ostdl1a* and *mspl-4*), maize (*macl*), and Arabidopsis (*em1*).

**Supplemental Figure S8.** Distribution of specific classes of transcription factors in the mutants.

**Supplemental Figure S9.** Expression pattern for individual members of TGA subfamily in another development.

**Supplemental Figure S10.** The expression analysis of OsMAPKKks in the mutants.

**Supplemental Table S1.** List of genes that were differentially expressed in mutants compared to the wild type in rice.

**Supplemental Table S2.** Genes differentially coexpressed among rice (*ostdl1a* and *mspl-4*), maize (*macl*), and Arabidopsis (*em1*).

**Supplemental Table S3.** Differentially expressed genes encoding transcription factors.
Supplemental Table S4. Differentially expressed genes associated with redox process.

Supplemental Table S5. Differentially expressed genes associated with signal transduction.

Supplemental Table S6. Lists of primers used in this study.

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LITERATURE CITED


OsTDLLA-MS1 Signal in Anther Cell Specification

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Song Q, Qi T, Huang H, Xie D (2013) Regulation of stamen development by coordinated actions of jasmonate, auxin, and gibberellin in Arabidopsis. Mol Plant 6: 1065–1073


Xing S, Zhang Q (2008) ROXY1 and ROXY2, two Arabidopsis glutaredoxin genes, are required for anther development. Plant J 53: 790–801


