The Rice Basic Helix-Loop-Helix Transcription Factor TDR INTERACTING PROTEIN2 Is a Central Switch in Early Anther Development

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INTRODUCTION

The formation of organized and functional organs requires the establishment of new cell lineages. Within the dominant diploid sporophytic generation, flowering plants generate male reproductive cells (microsporocytes), which differentiate into multicellular male gametes via meiosis and mitosis during the reduced haploid gametophytic generation (Walbot and Evans, 2003). In higher plants, male reproduction starts with the initiation of the anther primordium, which usually contains three layers, L1, L2, and L3. It is assumed that L1 differentiates into the epidermis, and L2 differentiates into sporogenous cells (microsporocytes) and three inner somatic cell layers: the epidermis, the middle layer, and the tapetum (from outside to the inside) (Ma, 2005; Wilson and Zhang, 2009; Zhang et al., 2011; Feng et al., 2013; Zhang and Yang, 2014). Previous studies hypothesized that a single hypodermal cell beneath the epidermis carries out sequential asymmetric cell divisions to generate three concentric rings of somatic layers and germ cells (Ma, 2005; Feng et al., 2013). However, recent investigations of maize (Zea mays) suggest that the peripheral L2-derived (L2-d) cells conduct asymmetric cell division to generate the two distinct cell types: the endothecium and secondary parietal cell (SPC), and then SPCs undergo subsequent symmetric cell divisions to produce the middle layer and the tapetum. The central L2-d cells produce enlarged sporogenous cells (Kelliher and Walbot, 2011).

Recent genetic and biochemical evidence suggests that redox status modulators, transcription factors, receptor-like protein kinases, and small peptides have roles as molecular switches in specifying the cell fate of somatic tissues and germ lines in plants (Zhang and Yang, 2014). Through modulating cellular redox, Arabidopsis thaliana CC-type glutaredoxins ROXY1 and ROXY2 (Murmu et al., 2010; S. Li et al., 2011), maize Male Sterile Converted Anther 1 (Chaubal et al., 2003), and rice (Oryza sativa) MICROSPORELESS1 (MIL1) (Hong et al., 2012a) can induce the centrally positioned anther precursor cells to gain the identity of archesporial cells, the precursors of male reproductive cells. Mutations in these genes cause the failure to differentiate into archesporial cells during early anther development. In Arabidopsis, SPOROCYTELESS (SPL)/NOZZLE is required for sporogenous cell formation, and BARELY ANY MERISTEM1 (BAM1) and BAM2 promote somatic cell fate by limiting the expression of SPL. In spl mutants, sporogenous cell generation is impaired, and bam1 bam2 mutants produce extra pollen mother cells at the expense of adjacent somatic cells (Schiefthaler et al., 1999; Yang et al., 1999; Hord et al., 2006; Liu et al., 2009).

Plasma membrane–localized leucine-rich repeat receptor-like kinases, including Arabidopsis EXCESS MICROSPOROCYTE1 (EMS1; also known as EXTRA SPOROGENOUS CELLS [EXS]) (Canales et al., 2002; Zhao et al., 2002), SOMATIC EMBRYOGENESIS RECEPTOR LIKE KINASE1 (SERK1) or SERK2 (Albrecht et al., 2005; Colcombet et al., 2005), and the small peptide ligand for EMS1 and SERK1/2, TAPETAL DETERMINANT1

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(TPD1) (Yang et al., 2003, 2005), determine the number of cells in microsporocytes and tapetal cell fate. TPD1-like small peptides are mainly expressed in microsporocytes and likely secreted into the interface between the tapetum and male reproductive cells to interact and form a receptor complex with the leucine-rich repeat receptor kinase, RECEPTOR-LIKE KINASE2 (RPK2) has been proposed to be needed for the cell fate specification of the middle layer. Mutation in RPK2 leads to loss of the middle layer and male sterility (Mizuno et al., 2007).

EMS1/EXS–TPD1–like signaling pathways play a conserved and diversified role in specifying the cell fate and number of anther wall cell layers and microsporocytes. Evidence came from the analysis of the mutants of TPD1 orthologs such as rice TPD1-LIKE1A/MIL2 (Hong et al., 2012b) and maize MULTIPLE ARCHESPORIAL CELLS1 (MAC1) (Wang et al., 2012), as well as the rice homolog of the Arabidopsis EMS1/EXS genes, MULTIPLE SPOROCYTE1 (MSP1) (Nonomura et al., 2003). MSP1 expresses not in the sporocytes, but in cells neighboring the male and female sporocytes, and msp1 has defects in anther and ovule development (Nonomura et al., 2003; Zhao et al., 2008). This is in contrast to the function of its Arabidopsis counterpart, ems1/exs, which shows no defect in female reproduction (Canales et al., 2002; Zhao et al., 2002). MIL2 is mainly expressed in inner parietal cells, and the mil2 mutant only has two anther wall layers, a phenotype which resembles that of msp1 but differs from that of the Arabidopsis ems/exs and tpd1 mutant (Nonomura et al., 2003; Hong et al., 2012b).

Unlike the role of Arabidopsis TPD1–like genes in restricting trans-differentiation of tapetal cells into meiocytes, maize MAC1 suppresses archesporial cell proliferation and promotes periclinal division of subepidermal cells; mac1 displays overproliferation of archesporial cells in both anther and ovule (Wang et al., 2012). These differences in mutant phenotypes imply that species-specific ligand-receptor coordination mechanisms function in regulating the formation of anther wall layers and reproductive cells.

The four somatic helper tissues enclose microsporocytes and support gametogenesis for successful male reproduction in plants (Ma, 2005). They develop into cell layers with distinct cell morphology and function. However, in contrast to the progress on understanding the asymmetric and/or symmetric cell division, when differentiation of somatic cell layers is initiated and how this process is regulated remain largely unknown. Several transcriptional factors that regulate tapetal fate have been identified. An Arabidopsis basic helix-loop-helix (bHLH) transcription factor DYSFUNCTIONAL TAPETUM1 (DYT1) and its rice ortholog UNDEVELOPED TAPETUM1 (UDT1) are required for the differentiation of tapetum. In dyt1 and udt1 mutants, tapetal cells become abnormally vacuolated and hypertrophic later (Jung et al., 2005; Zhang et al., 2006; Feng et al., 2012). How DYT1 and UDT1 regulate tapetum differentiation is unknown. MYB33/65 (Millar and Gubler, 2005) and the rice protein GAMYB (Kaneko et al., 2004; Tsuji et al., 2006; Aya et al., 2009; Liu et al., 2010), together with TAPETUM DEGENERATION RETARDATION (TDR) (N. Li et al., 2006; Zhang et al., 2008), are positive regulators of tapetum programmed cell death (PCD). Mutation in these genes leads to a vacuolated and hypertrophic tapetum, similar to the phenotype observed in dyt1 and udt1 mutants. Whether these genes play a role in tapetum differentiation is unknown.

In this work, we demonstrate that rice TDR INTERACTING PROTEIN2 (TIP2), a bHLH transcription factor, controls cell differentiation and morphogenesis of the endothecium, the middle layer, and the tapetum and regulates normal meiosis and microspore release from the tetrad. Moreover, TIP2 directly activates the expression of TDR and ETERNAL TAPETUM1 (EAT1), two key regulators of tapetum development and degradation at later stages. This finding establishes TIP2 as an indispensable switch and reveals a regulatory cascade initiated by TIP2 in controlling anther development in rice.

RESULTS

Identification of the tip2 Mutant

To identify genes involved in male reproduction in rice, we isolated a male sterile mutant from our rice mutant library generated from an O. sativa ssp japonica cultivar, 9522 (Chen et al., 2006). The mutant was later named tip2 after the cloning of the gene and the finding that the gene product interacted with TDR (see below). Compared with the wild type, tip2 has normal vegetative growth, inflorescence and flower morphology (Figures 1A and 1B). However, tip2 has smaller anthers and does not produce mature pollen grains during reproductive stage (Figures 1C and 1D), suggesting that the TIP2 gene is specifically required for male reproduction in rice. When tip2 plants were backcrossed to wild-type plants, all F1 progenies were fertile, and F2 plants...
displayed a segregation of 192 wild-type and 68 mutant plants (3:1, $\chi^2 = 0.184 < \chi^2_{0.05, 1} = 3.84$), suggesting that tip2 is a single recessive mutation.

**TIP2 Promotes Cell Differentiation in the Three Inner Anther Wall Layers**

To further analyze the defect of tip2 anthers, we examined transverse sections of mutant anthers. At stage 6 of anther development, the wild-type anther formed four somatic layers (Figure 2A). At stage 7, each cell layer of the wild type showed characteristic cell morphology (Figure 2B): Cells in the epidermis and endothecium cell layers appeared a rectangular shape with transparent cytoplasm and globular nuclei; the middle layer became flattened; tapetal cells with densely staining cytoplasm displayed a square shape. At stage 8, both the endothecium and the middle layer became condensed (Figure 2C). However, the three inner somatic layers of tip2 anthers displayed no obvious difference in cell shape during stages 6 to 8 (Figures 2D to 2F). Moreover, after meiosis the wild-type middle layer and tapetum disintegrated (Figures 2C and 2G to 2I). However, tip2 had a vacuolated and expanded cell appearance of the three inner layers and degenerated microspore mother cells, which could not develop into viable pollen grains (Figures 2F, 2J to 2L).

To characterize tip2 defects in anther wall layer specification in more detail, we performed 4'-6-diamidino-2-phenylindole (DAPI) staining of cross-sectioned anthers. At stage 6, the three inner anther wall layers of the wild type and tip2 exhibited similar morphology, as revealed by staining of nuclei (Figures 3A and 3D). Starting from stage 7, the wild type displayed flattened nuclei in the middle layer (Figure 3B). At stage 8 in the wild type, bright DAPI signals can be observed in the two outer layers, but only weak DAPI staining signals can be detected in the tapetum, and

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**Figure 2.** Defects of Anther Wall and Pollen Development in tip2.

Semi-thin section analysis of anthers from stage 6 to stage 12 of anther development. The images are cross sections of a single locule. Wild-type anthers are shown in (A) to (C) and (G) to (I), and tip2 anthers are shown in (D) to (F) and (J) to (L). At stage 6, cell numbers of tip2 innermost three layers were 16.3 ± 1.6, 18.4 ± 2.0, and 22.0 ± 3.0, respectively; and 15.9 ± 1.6, 17.9 ± 2.0, and 21.4 ± 1.9 for the wild type. E, epidermis; En, endothecium; MI, middle layer; T, tapetum; Msp, microspore; MMC, meiocyte mother cell; Tds, tetrads; EnL, endothecium-like structure; MIL, middle layer–like structure; TL, tapetum-like structure; dMMC, degenerated MMCs. S6 to S12 refer to stages 6 to 12 of anther development. Bars =15 μm.
the middle layer appeared to have degenerated (Figure 3C). However, from stage 7 to stage 8, the three inner anther wall layers in tip2 remained undifferentiated, resembling cell morphology to that of wild-type cells at stage 6 (Figures 3A, 3E, and 3F).

Furthermore, DAPI staining showed that tip2 meiocytes have normal meiosis initiation. At early stage 7, chromosomes featured in meiosis can be observed in both wild-type and tip2 meiocytes. Meiosis in tip2 progressed normally from leptotene stage to metaphase I, but arrested at anaphase I and no cytokinesis happened at prophase II. Eventually, tip2 meiocytes degenerated and did not form tetrads and pollens (Supplemental Figure 1), suggesting that mutation in TIP2 does not directly affect meiotic initiation but causes the arrest of meiosis.

Transmission electron microscopy (TEM) analysis was conducted to observe in detail the cellular changes in the inner anther wall layers of tip2 mutants. At stage 6, cells in the three inner somatic layers appeared a similar shape and were occupied by a large nucleus (Supplemental Figures 2A to 2D). At stage 7 when meiosis initiated, wild-type anthers contained four somatic layers with unique features in each layer. Cells in both the middle-layer and tapetum had dense cytoplasm, abundant endoplasmic reticulum, and mitochondria (Figures 4B and 4C).

Tapetal cells also had numerous vesicles and small vacuoles scattered throughout the cytoplasm, indicating active metabolism (Figure 4B), the middle-layer cells became significantly compressed with cytoplasm occupied by large vacuoles (Figure 4C), and the endothecium had abundant organelles, such as chloroplasts that contained starch granules (Figure 4D). In tip2 anthers, although the three inner layers exhibited similar features to that of the wild type at stage 6 (Supplemental Figures 2A to 2H), at stage 7, these cells remained undifferentiated and contained a large number of plastid-like organelles (Figures 4E to 4H). These results supported the role of TIP2 in promoting cell differentiation of the three inner anther wall layers. Unlike the condensation and degeneration of wild-type tapetal cells at late stage 8 and stage 9, tip2 tapetal cells became significantly expanded (Figures 4I to 4L; Supplemental Figures 2I to 2X).

To further confirm the cell identity of inner anther wall layers in tip2, we detected the expression of a rice tapetum marker gene, LIPID TRANSFER PROTEIN45 (LTP45), in these cells. LTP45 is a homolog of Arabidopsis A9, a tapetum-specific marker for early anther developmental stages (Paul et al., 1992). LTP45 was preferentially expressed in tapetal cells at stage 7 and stage 8 in wild-type anthers, whereas in tip2 anthers its expression was not detectable (Figures 3G to 3N), suggesting that tip2 has defects in tapetal cell identity specification. We were unable to analyze the identity of the endothecium and middle layer due to lack of marker genes for these two layers in plants.

In addition, we observed extra periclinal division in tip2 tapetum-like layer at stage 8, with an average frequency of 3.68 ± 2.8 cells per locule (n = 71) (Supplemental Figure 3A; Figures 4J and 4L), which was not seen in wild-type anthers (n = 30). Consistent with this, the average cell number of tip2 tapetum-like layer had a significant increase (30.1 ± 4.4, n = 71) compared with the wild type (25.1 ± 1.9, n = 30) at stage 8 (Z = 14.3 > Z0.05 = 1.96), although at stage 6 the average cell numbers of tip2 innermost three anther wall layers had no significant difference from that of the wild-type plants. DAPI staining observation indicated that the periclinal dividing cells exhibit a mitosis-like pattern (Supplemental Figures 3B to 3D). These results suggested that tip2 anthers have prolonged periclinal cell division activity after the formation of the inner two anther wall layers via periclinal cell division from SPCs.

**Figure 3.** Defects of Anther Wall Differentiation in tip2.
(A) to (F) Cross section of rice anthers stained with DAPI. (G) to (N) In situ hybridization by the tapetum-specific probe LTP45. E, epidermis; En, endothecium; MI, middle layer; T, tapetum; Msp, microspore; MMC, meiocyte mother cell; Tds, tetrads. EnL, endothecium-like structure; MIL, middle-layer-like structure; TL, tapetum-like structure. S6 to S8 refer to stages 6 to 8 of anther development. Bars = 4 μm in (A) to (F) and 15 μm in (G) to (N).

**TIP2 Promotes Tapetal PCD and Degeneration of Callose Surrounding the Microspores**

Section analysis and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) DNA fragmentation assay was used to monitor PCD in tapetal cells. In the wild type, meiocytes formed tetrads after meiosis, tapetal cells became condensed and initiated PCD-promoted degeneration, and the middle layer also started to be degraded (Figures 2C and 5A) (N. Li et al., 2006; Niu et al., 2013). By contrast, cell morphology of the three inner anther wall layers of tip2 anthers remained similar, and no obvious tapetal PCD signals were detected (Figures 2F and 5A), suggesting that TIP2 is a positive regulator of tapetal PCD.

Tapetal cells secrete β-1,3-glucanase (callase), which can degenerate the callose wall outside tetrads to release young microspores (Arizumi and Toriyama, 2011). Cross section of anthers stained by aniline blue showed deposition of callose around the
meiocytes in both the wild type and tip2 at stages 7 and 8 of anther development (Figure 5B). However, in stark contrast to the degradation of callose enclosing wild-type microspores, tip2 displayed undegenerated callose around meiocytes, suggesting defective callose degeneration in the tip2 mutant.

**TIP2 Encodes a bHLH Transcription Factor**

To identify the TIP2 gene, we employed a map-based cloning strategy. Using an F2 population of 340 individual mutant lines and six InDel markers, we located the TIP2 locus on chromosome 1 between Z6.1-5 and Z6.2-1, two markers that defined a 170-kb region (Figure 6A). By genomic sequencing of candidate genes within this region, we found a deletion of two continuous G nucleotides in the second exon of Os01g0293100, resulting in a frame shift of the protein (Figure 6B). Transgenic tip2 plants expressing TIP2 wild-type genomic DNA recovered male fertility (Supplemental Figure 4), demonstrating that Os01g0293100 is responsible for the male sterile phenotype in tip2.

The TIP2 cDNA encodes a putative bHLH transcription factor containing a conserved bHLH domain and a Domain Unknown Function domain, which shares high sequence similarity with EAT1 and is homologous to Arabidopsis AtbHLH010, AtbHLH089, and AtbHLH091 (X. Li et al., 2006; Niu et al., 2013). We observed the localization of TIP2 protein using transgenic rice plants expressing a TIP2cds-eGFP fusion driven by 35S promoter. Stably expressed TIP2 fused with GFP was observed in nuclei of root cells (Supplemental Figure 5).

**TIP2 Expresses Specifically in the Endothecium, Middle Layer, and Tapetum**

To further investigate the function of TIP2, we analyzed the expression pattern of TIP2 in different tissues using quantitative RT-PCR (qRT-PCR). TIP2 expression was specifically observed in anthers, from the meiosis stage to mitosis, with a maximum expression level at stages 7 and 8. This expression pattern is consistent with the defects shown in tip2. Interestingly, tip2 anthers displayed a lower level of TIP2 than the wild type from stage 6 to stage 8, but a higher level than the wild type after stage 9, when young microspores were released (Figure 7A), suggesting a possible feedback regulation of TIP2 transcription. Furthermore, in situ hybridization showed strong expression of TIP2 in the middle layer and tapetum and weak expression in the endothecium in the wild type (Figures 7B and 7C). By contrast, only background signal was detected in the same anther section with the sense probe (Figure 7D). We also detected expression of TIP2 in meiocytes at stages 7 and 8, when meiosis initiated (Supplemental Figure 6). These results supported the role of TIP2 in specifying the cell pattern of inner anther walls and functioning in meiosis progression.

Figure 4. TEM Analysis of tip2 Anthers.

(A) to (H) Transverse section of wild-type ([A] to [D]) and tip2 ([E] to [H]) anthers at stage 7. (B) to (D) and (F) to (H) are higher magnification of blank region in (A) and (E), showing tapetum ([B] and [F]), middle layer ([C] and [G]), and endothecium ([D] and [H]), respectively. (I) and (J) Transverse section of wild-type ([I]) and tip2 ([J]) anthers at stage 8. (K) and (L) Transverse section of wild-type ([K]) and tip2 ([L]) anthers at stage 9. E, epidermis; En, endothecium; MI, middle layer; T, tapetum; Msp, microspore; MMC, meiocyte mother cell; Tds, tetrads. EnL, endothecium-like structure; MIL, middle layer-like structure; TL, tapetum-like structure; Ne, nucleus; Cl, chloroplast; ER, endoplasmic reticulum. Arrowhead shows an extra layer of tapetum-like structures in tip2 mutant. Arrow shows proplastid-like structure in the innermost three layers in tip2 mutant. Bars = 5 μm in (A), (E), and (I) to (L) and 1 μm in (B) to (D) and (F) to (H).
TIP2 Interacts with TDR to Promote Tapetum Differentiation

It has been shown that bHLH transcriptional factors function as homodimers or heterodimers (Firulli et al., 2000). We previously reported that the bHLH protein ABORTED MICROSPORES (AMS), a key regulator of tapetum development and degradation, can interact with AtbHLH089 and AtbHLH091, two homologs of TIP2 in Arabidopsis (Sorensen et al., 2003; Xu et al., 2010; Ma et al., 2012). Consistent with this, TDR, the rice ortholog of AMS, interacts with EAT1, the paralog of TIP2 in vitro and in vivo (Niu et al., 2013). To understand whether TIP2 physically interacts with TDR or EAT1, yeast two-hybrid analysis was used. Yeast strains coexpressing TIP2 and TDR grew normally on medium lacking His and Ade in the presence of 10 mM 3-aminotriazole and displayed activation of the expression of the LacZ reporter genes, which confirmed the interaction between TIP2 and TDR (Figure 8A). In contrast, yeast strains coexpressing TIP2 and EAT1 failed to grow under the same selection conditions lacking His and Ade in the presence of 10 mM 3-aminotriazole (Supplemental Figure 7A), suggesting no interaction between TIP2 and EAT1. The interaction between TIP2 and TDR as well as no interaction between TIP2 and EAT1 were further confirmed using in vitro pull-down assay (Figure 8B; Supplemental Figure 7B).

Our previous study showed that TDR is preferentially expressed in tapetum at stages 7 to 9 and mutation in TDR affects the tapetum PCD (N. Li et al., 2006). The interaction of TDR with TIP2 suggests that TDR may play a role at earlier developmental stages. We reexamined the phenotypes of tdr at stages 7 and 8 using TEM and DAPI staining. tdr displayed normally characteristic endothecium and middle layer but less differentiated tapetal cells at stage 7 (Supplemental Figures 8A to 8F and Supplemental Figure 9A), and innermost three layers became vacuolated and expanded at stage 8 (Supplemental Figures 8G to 8L). Also, we observed a substantial decrease in transcript levels of the rice tapetum-specific gene LTPL45 in tdr, as we...
Observations in tip2 anthers. In contrast, no significant expression change of LTP45 was detected in eat1 at stage 7, but LTP45 transcript levels increased at stage 8 (Supplemental Figure 9B).

Those similar properties of defective tapetum in both tip2 and tdr indicated that TIP2 may cooperate with TDR in regulating early tapetal cell differentiation.

**TIP2 Regulates TDR and EAT1**

To further investigate the regulatory function of TIP2, we performed qRT-PCR to analyze the expression level of genes involved in tapetum development and degradation. The expression level of transcription factors TDR, EAT1, and PERSISTENT TAPETAL CELL1 (PTC1) (H. Li et al., 2011) decreased significantly in tip2 compared with that in wild-type plants (Figure 9A; Supplemental Figure 10). By contrast, UDT1 (Jung et al., 2005), another key regulatory bHLH transcription factor, was upregulated in tip2. GAMYB showed little difference in expression level at meiosis stage and slightly increase after meiosis (Supplemental Figure 10).

These results suggested that TIP2 may positively regulate the expression of TDR, EAT1, and PTC1 in tapetum. UDT1 and GAMYB could be involved in parallel regulatory pathways.

bHLH transcription factors regulate gene transcription by binding to the E-box (CANNTG) in the promoter region of target genes (Xu et al., 2010). Therefore, we next used quantitative chromatin immunoprecipitation (qChIP)-PCR to determine whether TIP2 directly regulates these genes. We produced TIP2 polyclonal antibodies against a peptide that corresponds to amino acids 1 to 102 (see Methods) and determined the specificity of the antibody using protein gel blot assay (Supplemental Figure 11). Our qChIP-PCR results showed that TIP2 could directly bind to the E-box-containing promoter regions of TDR and EAT1 (Figure 9C). The binding of TIP2 to the promoter regions of TDR and EAT1 was confirmed by electrophoretic mobility shift assay (EMSA) using qChIP-PCR enriched promoter regions (Figure 9B).

The tdr mutant plants display normally characteristic endothecium and middle layer but expanded tapetum and retarded tapetal PCD (N. Li et al., 2006). The eat1 mutants exhibit well developed innermost three anther wall layers but delay in tapetal PCD (Niu et al., 2013). Moreover, tdr eat1 double mutants show defects similar to tdr single mutant, and EAT1 is downregulated in tdr (Niu et al., 2013). To determine the genetic interaction among TDR, EAT1, and TIP2, we constructed double mutants tdr tip2 and tip2 eat1. Histological analysis showed that both double mutants displayed aborted meiocytes, abnormal development of the three inner layers, and expanded tapetum-like cells in anthers, which was similar to the phenotype of the tip2 single mutant (Figure 9D; Supplemental Figure 12). Therefore, our results supported the idea that TIP2 acts upstream of TDR and EAT1.

Consistent with the downregulation of TDR and EAT1, we also detected the decreased expression of a cysteine protease-encoding gene, CP1, and two aspartic protease-encoding genes, AP25.
and AP37 (Supplemental Figure 13). CP1 is required for anther development, and its expression is directly regulated by TDR (Lee et al., 2004; N. Li et al., 2006), and AP25 and AP37 are directly regulated by EAT1 (Niu et al., 2013).

In agreement with the tip2 defect in callose degradation, we detected reduced expression of three glucanase-encoding genes putatively related to callose degradation, including G1 (Wan et al., 2011), and two homologs of Arabidopsis A6 (Hird et al., 1993) in rice, Os09g32550 and Os08g41410 (Supplemental Figure 13). In addition, other genes involved in tapetum late function, including those related to lipid metabolism and transport, such as CYP703A3 (Aya et al., 2009), CYP704B2 (Li et al., 2010), C6 (Zhang et al., 2010), and DEFECTIVE POLLEN WALL (DPW) (Shi et al., 2011), also exhibited significantly decreased transcription in tip2 (Supplemental Figure 13). These results suggested the presence of a TIP2-mediated transcription cascade that regulates tapetum development and degeneration in rice.

**DISCUSSION**

**TIP2 Plays a Key Role in Specifying Cell Pattern of Three Inner Anther Somatic Layers**

Timely cell differentiation is of critical importance for the patterning of plant organs. In this study, we demonstrate that TIP2 plays a key role in controlling cell differentiation of inner anther wall layers during early anther development. Even though tip2 anthers seem to be able to undergo normal cell division in generating four anther wall layers from the precursor cells, incomplete differentiation of the three inner anther wall layers and
even extra tapetal cell division occur in the mutant (Figures 2 to 4). Therefore, our findings demonstrate that TIP2 is a key regulator in suppressing the proliferation of meristemoid cells and promoting acquisition of cell identity in the three inner anther wall layers during early anther development.

The precursor cells (primary parietal cells [PPCs]) of the inner three somatic cell layers are formed by asymmetric cell division of L2-d cells or alternatively adopting somatic fate by the instruction of centrally localized L2-d cells (Zhang and Yang, 2014). PPCs undergo two more rounds of periclinal cell division: The first round of division generates two secondary parietal cell layers, and the inner SPC layer subsequently divides into two somatic cell layers. Our detailed observations revealed that in rice, the newly formed cell layers display similar morphological and cellular features at stage 6 (Supplemental Figures 2A to 2D) and establish distinct characteristics at stage 7 when meiocytes enter meiosis (Figures 4A to 4D). These observations indicate that differentiation of L2 derived somatic cells starts nearly simultaneously and an early endothecium cell-like feature might be the default status of these cells. In tip2, three inner anther wall layers exhibited no difference compared with those of the wild type till stage 6 (Supplemental Figures 2E to 2H), suggesting that TIP2 is not required for the periclinal divisions and early cell fate decision. But development of these cell layers is arrested and deviated from that of wild type after stage 6 (Figures 4E to 4H), implicating the essential role of TIP2 in cell patterning. Consistently, TIP2 transcripts can be detected in the newly formed endothecium, middle layer, and tapetum at stage 6, but not in the PPCs and SPCs (Figures 7B to 7D).

Several of the signaling components involved in rice early anther cell fate determination display a similar spatial expression pattern before the meiosis stage. MSP1 and MIL2 are first expressed in archesporial cells then preferentially in PPCs and SPCs, later gradually confined to the middle layer and the tapetum. This spatial distribution pattern suggests that MSP1 and MIL2 may regulate both the generation and differentiation of the three inner anther wall layers (Nonomura et al., 2003; Hong et al., 2012b). MIL1 is also expressed in the precursor cells and restricted to the middle layer and tapetum at later developmental stages. In mil1 mutants, the inner secondary parietal layer can normally divide into two layers, but these two layers fail to differentiate into middle layer and tapetum, indicating that MIL1 is involved in specifying the cell pattern of these two layers in addition to regulating meiosis initiation (Hong et al., 2012a). TIP2 expresses later than these genes and may act downstream of these regulators. Consistent with this hypothesis, the expression level of MSP1 and MIL2 may regulate both the generation and differentiation of the three inner anther wall layers (Nonomura et al., 2003; Hong et al., 2012b). MIL1 is also expressed in the precursor cells and restricted to the middle layer and tapetum at later developmental stages. In mil1 mutants, the inner secondary parietal layer can normally divide into two layers, but these two layers fail to differentiate into middle layer and tapetum, indicating that MIL1 is involved in specifying the cell pattern of these two layers in addition to regulating meiosis initiation (Hong et al., 2012a). TIP2 expresses later than these genes and may act downstream of these regulators. Consistent with this hypothesis, the expression level of MSP1 and MIL2 did not show significant changes in tip2 (Supplemental Figure 10). UDT1 is another early regulator of rice anther development and has an overlapping transcript distribution with TIP2. Mutation in UDT1 results in defective differentiation of inner somatic cell layers (Jung et al., 2005; Hong et al., 2012b). UDT1 is upregulated in the tip2 mutant (Supplemental Figure 10), suggesting that they may act in parallel regulatory pathway and the upregulation of UDT1 may be induced by a feedback signal.

The division of PPCs generates two layers of SPCs, and the inner SPCs obtain meristemoid identity and retain periclinal cell division activity. In wild-type anthers, the two layers derived from the division of inner SPC layer can only divide in an anticlinal manner. By contrast, in tip2 mutants, extra periclinal cell division can be observed in some cells of the innermost cell layer (Figures 4I to 4L; Supplemental Figure 3A). Similarly, more than four cell layers can be observed in some regions of the mil1 parietal tissues (Hong et al., 2012a). It seems that after each round of parietal cell division, the inner descendants retain the meristemoid characteristics and the ability to carry on periclinal division. Cell differentiation may be required to halt this cell division activity. However, not all inner cells in tip2 and mil1 mutants undergo periclinal division, indicating that cell division and differentiation of the innermost cell layer may be controlled by overlapping regulatory pathways.

**TIP2 Regulates Tapetal PCD**

Because the innermost anther wall layer is next to the male reproductive cells, the tapetum provides nutritive support for microspore development and pollen wall formation by undergoing a PCD-triggered degradation after meiosis (N. Li et al., 2006; Niu et al., 2013). Premature or retarded tapetal degeneration frequently causes male sterility (N. Li et al., 2006; Li et al., 2007; Zhang et al., 2007; Shi et al., 2009; Phan et al., 2011). Genetic investigations discovered several transcription factors involved in rice tapetal degeneration, such as GAMYB (Kaneko et al., 2004; Liu et al., 2010), TDR (N. Li et al., 2006), EAT1 (Niu et al., 2013), and Os-API5 (X. Li et al., 2011; Zhang et al., 2011). The tip2 mutant displays uncontrolled tapetum-like cell enlargement and delayed PCD (Figures 2A to 2L and 5A), which is similar to the phenotypes of gamyb (Kaneko et al., 2004) and tdr mutants (N. Li et al., 2006), but differs from eat1 (Niu et al., 2013), ptc1 (H. Li et al., 2011), and osapi5 (X. Li et al., 2011), in which no obvious cell enlargement was observed. These findings suggest that TIP2,
GAMYB, and TDR may be required for controlling tapetal cell size and promoting tapetal PCD during rice anther development. Moreover, the major function for EAT1, PTC1, and Os-API5 may be the regulation of tapetal PCD.

Delayed PCD in tip2 may be an indirect result of the deficiency in cell differentiation. Our analysis revealed that TIP2 can directly activate the expression of TDR and EAT1, indicating a direct role of TIP2 in promoting PCD. Supportively, our expression analysis revealed that in tip2, CP1, AP25, and AP37 are downregulated (Supplemental Figure 13).

**Consecutive Functions of Three bHLH Transcription Factors in Anther Development**

bHLH proteins often function in transcriptional cascades in cell identity specification and differentiation in plants and animals (Weintraub et al., 1991; Pillitteri et al., 2007; Pillitteri and Torii, 2012). Homologous bHLH proteins in animals, such as the four mammalian myogenic regulators, MyoD, Myf-5, myogenin, and MRF4, as well as the achaete-scute complex in Drosophila melanogaster, control sequential processes in a hierarchical fashion (Olson, 1990; Campuzano and Modekell, 1992; Weintraub, 1993). In Arabidopsis, three closely related bHLH proteins, SPCH, MUTE, and FAMA, are key switches for three key consecutive steps of stomatal formation (Ohashi-Ito and Bergmann, 2006; MacAlister et al., 2007; Pillitteri et al., 2007; Lampard, 2009; Hachez et al., 2011; Triviño et al., 2013).

Our previous work shows that, phylogenetically, TIP2, EAT1, and TDR are closely grouped (X. Li et al., 2006), and they might be generated by gene duplication during evolution. This work and our previous discovery reveal that these three bHLH proteins display successive expression patterns and have overlapping and divergent functions during anther development (Figure 10) (N. Li et al., 2006; Niu et al., 2013). TIP2 expresses in the endothecium, the middle layer, and the tapetum from stage 6 to stage 10, and these three inner anther wall layers are undifferentiated in tip2 mutants, suggesting the role of TIP2 in specifying cell pattern of the endothecium, the middle layer, and the tapetum in early anther development. In addition, TIP2 is required for the activation of late function genes in tapetum. TDR highly expresses from stage 7 to stage 9 and specifically in the tapetum and plays dual role in promoting tapetum differentiation and degradation. EAT1 highly expresses at stage 8 to stage 10, mainly in tapetum, and eat1 mutants show three well-developed inner cell layers but delayed tapetal PCD, suggesting that EAT1 may play a major role in triggering tapetal PCD in anthers (Figure 10).

Our previous expression and genetic analyses show that TDR acts upstream of EAT1. EAT1 expression is reduced in tdr, but TDR expression is not changed in eat1. Moreover, the tdr eat1 double mutant exhibits the defects similar to tdr, suggesting that the function of EAT1 relies on a functional TDR protein (Niu et al., 2013). In this study, we show that TIP2 directly activate the expression of TDR and EAT1 and physically interact with TDR. Therefore, we propose that the three closely related bHLH proteins, TIP2, TDR, and EAT1, might have evolved by gene duplication and their expression and function in anther pattern formation underwent diversification during evolution. TIP2 can promote cell differentiation of three inner somatic cells. TIP2 initiates the expression of TDR and EAT1 and interacts with TDR. TDR regulates tapetal differentiation and promotes tapetal PCD. Finally, TDR interacts with EAT1, and EAT1 triggers cell death in the tapetum. Together, TIP2, TDR, and EAT1 are components of a sequential regulatory cascade for anther wall layer development (Figure 10).

In summary, we demonstrate TIP2 as a new regulator of cell patterning of three inner somatic anther wall layers and the microspore development in rice. Three bHLH proteins, TIP2, TDR, and EAT1, form a sequential regulatory cascade in regulating consecutive steps including cell differentiation, cell morphogenesis, and cell death during anther development. This finding provides new insights into the role of bHLH proteins in plant reproduction.

**METHODS**

**Plant Materials, Growth Conditions, and Molecular Cloning of TIP2**

Rice (Oryza sativa) plants used in this study are in the 9522 background (O. sativa ssp. japonica) and were grown in the paddy field of Shanghai JiaoTong University. F2 progenies for mapping were generated by a cross between wild-type GuangLuAi species (indica) and the tip2 mutant (japonica). Male sterile plants in the F2 population were selected for mapping. To map the TIP2 locus, bulked segregates analysis was used and 24 pairs of InDel molecular markers were designed based on the sequence difference between japonica and indica. Further fine-mapping of TIP2 was performed using the previously published method (Chu et al., 2006).

**Characterization of Mutant Plant Phenotypes**

DAPI staining of microspores was performed as reported (Wang et al., 2010). TEM, callose staining, TUNEL assay, and semi-thin section analysis were performed according to a previous study (Tan et al., 2012). Anthers from different developmental stages, as defined by Zhang et al. (2011), were collected based on the comparison and analysis of wild-type and tip2 plants on glume length and morphology (Supplemental Figure 14), and the developmental stages of wild-type anthers were further confirmed by semi-thin section.

**Complementation of tip2**

A 4.8-kb genomic sequence of TIP2 including the entire TIP2 coding region, 3.1-kb upstream sequence, and 0.5-kb downstream sequence was amplified using a BAC that contained TIP2 as the template. The amplified fragment was cloned into the binary vector pCAMBIA1301, which contains a hygromycin resistance gene. Calli induced from young panicles of homozygous tip2 mutant plants were used for transformation with Agrobacterium tumefaciens (EHA105) carrying pCAMBIA1301-TIP2 plasmid or the control plasmid pCAMBIA1301. Over 50 positive transgenic plants were obtained and confirmed by PCR (primers used are listed in Supplemental Table 1).

**qRT-PCR and in Situ Hybridization**

Total RNA was isolated using TRI reagent from rice tissues. One microgram of RNA per sample was used to synthesize cDNA using the Primescript RT reagent kit with genomic DNA eraser (Takara). qRT-PCR was performed on a cycler apparatus (Bio-Rad) using SYBR Premix Ex Taq GC (Takara) according to the manufacturer’s instructions. Amplification was conducted following this protocol: 95°C for 2 min, 40 cycles of 95°C for 5 s, and 60°C for 35 s. ACTIN (Supplemental Table 1) was used as an internal control, and a relative quantitation method (Δ cycle threshold) was used to quantify the
relative expression level of target genes. Three biological repeats with three technique repeats each were included in producing statistical analysis and error range analysis. In situ hybridization was performed as described by Tan et al. (2012). Two TIP2 cDNA fragments generated by PCR were used for preparing antisense and sense probes (Supplemental Table 1).

Yeast Two-Hybrid Assays

Yeast two-hybrid assays were performed according to instructions of the Matchmaker GAL4 two-hybrid system (Clontech). Truncated TDR fragment containing the N-terminal bHLH domain (TDRΔ, amino acids 1 to 344) was from Niu et al. (2013). His and Ade selection and X-Gal filter assay were performed according to the manufacturer’s instructions.

Pull-Down Assay

TIP2 fused with maltose binding protein (MBP) and TDR fused with glutathione S-transferase (GST) were expressed in BL21(DE3), Escherichia coli cells. Proteins were purified using amylose and GST resin, respectively. The eluent was analyzed using 10% SDS-PAGE, according to the method published by Bai et al. (2012). Monoclonal antibodies against MBP (NEB) and GST (Beyotime) were used.

qChIP-PCR and EMSA Analysis

A TIP2-specific DNA fragment, which encodes a 102-amino acid peptide (1 to 102), was synthesized in vitro using optimized codons for E. coli. Two repeats of these fragments were linked together, fused with the His tag in the pET32a vector, and expressed in E. coli (BL21(DE3)). Full-length TIP2 fused with GST tag in pGEX4T-1 vector was expressed and purified for antibody purification. The specificity of the TIP2 antibody was examined according to the method described by Zhang et al. (2010). The fold enrichment of TIP2 target promoter regions was compared with the input sample, and IgG antibody was added as negative control. Ten biological repeats with three technique repeats each were included in producing statistical analysis.

Full-length TIP2 coding region fused with His-tag was expressed in E. coli (BL21(DE3)). DNA fragments of TDR and EAT promoter regions, which were positively enriched in qChIP-PCR, were amplified using dig-dNTP (Yinuojin). Experimental procedures of qChIP-PCR and EMSA were performed following the descriptions by Xu et al. (2010).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: EAT1 (Os04g0599300), TDR (Os02g0120500), GAMYB (Os01g0812000), CP1 (Os04g0675000), AP25 (Os03g0186900), AP37 (Os14g0448500), CYP704B2 (Os03g0188600), CYP703A3 (Os08g0131100), Os-C6 (Os11g0582500), UDT1 (Os07g0549600), LTPL45 (Os09g0525500), RA68 (Os02g0230300), DPW (Os03g0167600), MSP1 (Os01g0917500), UGPase1 (Os09g0553200), UGPase2 (Os02g0117700), G1 (Os01g0947700), GT1 (Os01g0262000), PDA1 (Os06g0607700), PTC1 (Os09g0449000), MIL2 (Os12g0152500), and MADS3 (Os01g0201700).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Analysis of Meiosis in tip2 by DAPI Staining.

Supplemental Figure 2. TEM Analysis of Anther Wall Cell Differentiation.

Supplemental Figure 3. Tapetum-Like Layer Exhibits Extra Periclinal Cell Division in tip2.

Supplemental Figure 4. Complementation of tip2 by TIP2 Genomic DNA.

Supplemental Figure 5. Subcellular Localization of TIP2.

Supplemental Figure 6. In Situ Hybridization of TIP2.

Supplemental Figure 7. Analysis of the Interaction between TIP2 and EAT1.

Supplemental Figure 8. TEM Analysis of Undifferentiated Tapetum in tdr.

Supplemental Figure 9. Defects of Tapetum Differentiation in tdr.

Supplemental Figure 10. Expression Analysis of Reported Genes Involved in Tapetal Development in tip2 Using qRT-PCR.

Supplemental Figure 11. Specificity Analysis of TIP2 Antibody.

Supplemental Figure 12. Transverse Section Analysis of eat1 tip2 and tdr tip2 Anthers.

Supplemental Figure 13. Expression Analysis of Reported Genes Involved in Tapetal PCD, Callose Dissociation and Pollen Development in tip2 Using qRT-PCR.

Supplemental Figure 14. Size of Flowers and Anthers of Wild Type and tip2 at Various Development Stages.

Supplemental Table 1. Size of Flowers and Anthers of Wild Type and tip2 at Various Development Stages.

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Author Contributions

Z.F. carried out major parts of all experiments. J.Y. performed the EMSA analysis. X.C., prepared sections for DAPI staining and in situ hybridization. X.Z. and Z.L. performed DAPI analysis of anther meicocytes. J.X. helped in qChIP-PCR analysis. M.C. screened the tip2 mutant and generated F2 population for mapping. D.Z. and W.L. designed the study and prepared the article.

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