PERSISTENT TAPETAL CELL1 Encodes a PHD-Finger Protein That Is Required for Tapetal Cell Death and Pollen Development in Rice[1][C][W][OA]

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In higher plants, timely degradation of tapetal cells, the innermost sporophytic cells of the anther wall layer, is a prerequisite for the development of viable pollen grains. However, relatively little is known about the mechanism underlying programmed tapetal cell development and degradation. Here, we report a key regulator in monocot rice (*Oryza sativa*), *PERSISTANT TAPETAL CELL1* (*PTC1*), which controls programmed tapetal development and functional pollen formation. The evolutionary significance of *PTC1* was revealed by partial genetic complementation of the homologous mutation *MALE STERILITY1* (*MS1*) in the dicot Arabidopsis (*Arabidopsis thaliana*). *PTC1* encodes a PHD-finger (for plant homeodomain) protein, which is expressed specifically in tapetal cells and microspores during anther development in stages 8 and 9, when the wild-type tapetal cells initiate a typical apoptosis-like cell death. Even though *ptc1* mutants show phenotypic similarity to *ms1* in a lack of tapetal DNA fragmentation, delayed tapetal degeneration, as well as abnormal pollen wall formation and aborted microspore development, the *ptc1* mutant displays a previously unreported phenotype of uncontrolled tapetal proliferation and subsequent commencement of necrosis-like tapetal death. Microarray analysis indicated that 2,417 tapetum- and microspore-expressed genes, which are principally associated with tapetal development, degeneration, and pollen wall formation, had changed expression in *ptc1* anthers. Moreover, the regulatory role of *PTC1* in anther development was revealed by comparison with *MS1* and other rice anther developmental regulators. These findings suggest a diversified and conserved switch of *PTC1/MS1* in regulating programmed male reproductive development in both dicots and monocots, which provides new insights in plant anther development.

Programmed cell death (PCD) has been defined as a sequence of genetically regulated events that lead to the elimination of specific cells, tissues, or whole organs (Lockshin and Zakeri, 2004). In multicellular organisms, PCD is essential for defense responses, to limit the spread of pathogens, and for development processes (Lam, 2004). Studies in animals have grouped PCD into three categories: apoptosis, necrosis, and autophagic cell death (Lockshin and Zakeri, 2004; Bras et al., 2005). Apoptosis is known to be promoted by the activation of a family of Cys proteases (caspases; Wolf and Green, 1999), leading to cell shrinkage, nuclear condensation and fragmentation, and "apoptotic body" formation (Adrain and Martin, 2001). Necrosis is associated with uncontrolled cell death, frequently due to overwhelming stress, when the cell is unable to enter the normal apoptotic pathway, and is characterized by morphological swelling rather than cell shrinkage (Bras et al., 2005). Autophagic cell death is characterized by the formation of autophagic vacuoles and dilation of the mitochondria and endoplasmic reticulum as well as enlargement of the Golgi (Bras et al., 2005).

PCD plays a fundamental role in developmental processes in plants, including leaf senescence, the removal of aleurone cells, root cap cells, and xylogenesis (Pennell and Lamb, 1997; Kuriyama and Fukuda, 2002; Rogers, 2005). PCD is also of special importance for plant reproduction, including embryonic devel-

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opment, tapetal degradation, anther dehiscence, pollen germination and pollen tube growth, pistil development, and pollen-pistil interactions (Wu and Cheung, 2000). Similar to the hallmarks of animal apoptosis, DNA fragmentation and mitochondrial changes are found in plant PCD (Danon et al., 2000), although it is rare to observe apoptotic bodies in plants (McCabe and Pennell, 1996). There are also no close sequence homologs of classical caspases in the plant genome, although caspase-like activities have been linked to plant PCD (Woltering, 2004; Reape et al., 2008). Currently, the signaling pathway and molecular mechanisms underlying plant PCD are largely unknown.

Male reproductive development in higher plants is a complex biological process involving the correlated differentiation of anther tissues and the generation of haploid microspores/pollen (Liu and Qu, 2008). The developed anther consists of the meiotic cells (also called microsporocytes) at the center, surrounded by the anther wall with four somatic layers: the epidermis, the endothecium, the middle layer, and the tapetum, from the surface to the interior (Goldberg et al., 1993). The tapetum, the innermost cell layer of the anther wall, plays a crucial role in regulating programmed anther development, microspore/pollen formation, and pollen wall formation (Li et al., 2006; Parish and Li, 2010). Tapetal cell development and differentiation are critical for the early events in male reproduction, including meiosis; however, during late pollen development, tapetal degeneration, triggered by an apoptosis-like process, is also vital for viable pollen formation (Papini et al., 1999; Varnier et al., 2005; Li et al., 2006; Aya et al., 2009). Currently, although several genes encoding putative transcription factors have been reported to be associated with tapetal function and degeneration, such as Arabidopsis (*Arabidopsis thaliana*) *MYB33/MYB65* (Millar and Gubler, 2005), *DYSFUNCTIONAL TAPETUM1* (*DYT1*; Zhang et al., 2006), *ABORTED MICROSPORE* (*AMS*; Sorensen et al., 2003; Xu et al., 2010), and *MALE STERILITY1* (*MS1*; Wilson et al., 2001; Ito and Shinozaki, 2002) and rice (*Oryza sativa*) *GAMYB* (Kaneko et al., 2004; Aya et al., 2009; Liu et al., 2010), *UNDEVELOPED TAPETUM1* (*UDT1*; Jung et al., 2005), *TAPETUM DEGENERATION RETARDATION* (*TDR*; Li et al., 2006), and *MADS3* (Hu et al., 2011), their detailed functional roles in regulating tapetal PCD during anther development are unclear. We have shown that the Arabidopsis *ms1* mutant displays altered tapetal development, with a lack of normal PCD and abnormal tapetal degeneration associated with large autophagic vacuoles and mitochondrial swelling (Vizcay-Barrena and Wilson, 2006). Previously, we also reported that *TDR*, the ortholog of the Arabidopsis *AMS* gene, plays a key role in tapetal PCD in rice; *tdr* shows delayed tapetal degeneration and nuclear DNA fragmentation as well as abortion of microspores after release from the tetrad. Moreover, TDR is able to directly bind the promoter of a Cys protease gene, *OsCP1*, in vivo and in vitro (Li et al., 2006). Recently, *GAMYB* has been shown to be regulated by GA, and the *gamyb* tapetal cells appear swollen and have defects in PCD (Aya et al., 2009; Liu et al., 2010).

Tapetal PCD is a physiological process in which timing is critical for pollen maturation and male fertility. To address the mechanism underlying tapetal PCD in the model crop rice, we have identified a key regulator, *PERSISTENT TAPETAL CELL1* (*PTC1*), which controls programmed tapetal development and degradation during rice anther development. Loss of function of *PTC1* results in uncontrolled tapetal cell proliferation and swelling, delayed DNA fragmentation, abnormal Ubisch bodies/orbicules, and pollen wall development, causing complete male sterility. *PTC1* encodes a putative PHD-finger (for plant homeodomain) protein that is transiently expressed in tapetal cells and microspores. Gene expression analysis revealed that *PTC1* affects expression associated with tapetal function and pollen exine formation. Furthermore, the conserved and crucial roles of *PTC1* and its Arabidopsis ortholog *MS1* were compared by genetic complementation and regulatory analysis. This work provides new insight into a molecular switch of programmed male reproductive development in both dicots and monocots.

RESULTS

Isolation of the *ptc1* Mutant

From our rice mutant library (Chu et al., 2005; Chen et al., 2006; Wang et al., 2006), the male-sterile mutant *ptc1* was identified. *ptc1* displayed complete male sterility and normal female development when tested by reciprocal cross analysis. All of the F1 progeny were fertile, with an approximate 3:1 ratio for phenotypic segregation in F2 plants (fertility:sterility = 189:55; χ^2 = 0.65), indicating that this mutation is due to a single recessive locus. Compared with wild-type plants, *ptc1* exhibited normal vegetative development (Fig. 1A). During the reproductive stage, *ptc1* plants developed normal panicles and floral organs (Fig. 1, B–D), but *ptc1* anthers were smaller, white, and lacked viable pollen grains (Fig. 1, D and E).

Defects of Anther Wall and Pollen Development in *ptc1*

Pollen development in the monocot rice can be characterized into similar stages to those observed in the dicot Arabidopsis (Zhang and Wilson, 2009). To further characterize the defects in *ptc1*, anther morphology was observed during the various developmental stages. Histological analysis indicated that early events in anther and pollen development progressed normally until after meiosis and microspore release. The epidermis, endothecium, middle layer, tapetum, and pollen mother cells formed normally in *ptc1* anthers at stage 6 before meiosis (Fig. 2, A and E). During meiosis (stage 8a), both the wild-type and *ptc1*

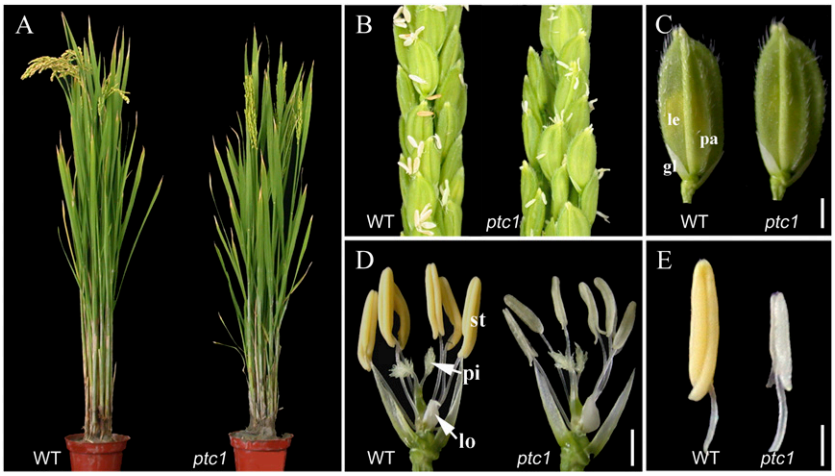

Figure 1. Phenotype comparison between the wild type (WT) and *ptc1*. A, Comparison between a wild-type plant (left) and the *ptc1* mutant plant (right) after heading. B, Comparison between a part of the wild-type panicle showing the dehisced anther (left) and a part of the *ptc1* mutant panicle (right) showing a smaller anther at the pollination stage. C, A wild-type flower (left) and a mutant flower (right) before anthesis. D, Comparison of wild-type (left) and *ptc1* (right) flower organs after removal of the palea and lemma. E, A wild-type yellow anther (left) and a mutant white and smaller anther (right) at stage 13. gl, Glume; le, lemma; lo, lodicule; pa, palea; pi, pistil; st, stamen. Bars = 2 mm.

pollen mother cells became associated with the tapetal layer and the tapetum appeared vacuolated (Fig. 2, B and F); subsequently (during stage 8b), tetrads with four haploid microspores formed and the middle cell layer became very thin and degenerated in both wild-type and *ptc1* cells (Fig. 2, C and G).

From stage 9 (free microspore released from the tetrad) to early stage 10 (vacuolated microspore), the wild-type tapetum became condensed, less vacuolated, and stained strongly (Fig. 2, D and I). In contrast, *ptc1* tapetal cells were more vacuolated and less stained (Fig. 2, H and M). From late stage 10 to stage 11 (mitosis I), the wild-type tapetum appeared to undergo degeneration, and the cytoplasmic constituents remained densely stained and clearly discernible (Fig. 2, J and K); however, the *ptc1* tapetum became abnormally enlarged, and cytosolic constituents seemed to be secreted into the anther locule (Fig. 2, N and O). Meanwhile, the wild-type microspores maintained a defined shape (Fig. 2, J and K), while the *ptc1* microspores degenerated (Fig. 2, N–P; Supplemental Fig. S1). At stage 13, when the mature pollen grains were formed, the wild-type tapetal layer and middle layer were completely degraded, and round, densely stained pollen grains were observed (Fig. 2L). However, at this stage in the *ptc1* anther, only cell debris of both tapetal cells and pollen grains remained (Fig. 2P).

Transmission electron microscopy was used to gain greater insight into the *ptc1* tapetal and microspore abnormalities. From stage 9 onward, the wild-type tapetal cytoplasm became condensed, with swollen and less defined organelles, which showed a loss of membrane integrity and a diffuse cellular organization, indicative of apoptotic degeneration (Fig. 3, A and B). Cytoplasmic condensation was reduced in the *ptc1* tapetum, with membrane and organellar integrity maintained (Fig. 3C). The *ptc1* tapetum had a high density of organelles, particularly mitochondria and endoplasmic reticulum, which appeared morphologically intact, giving the appearance of a highly active tissue without any signs of tapetal breakdown and apoptosis (Fig. 3, D, G, and H). Strikingly, extensive tapetal proliferation was also observed in *ptc1*, resulting in tapetal extrusions into the anther locule (Fig. 3, E–H). These extruded regions appeared to have a continuous outer membrane with the surrounding tapetal cells and contained an extremely high density of organelles, lipid bodies, and electron-dense materials (Fig. 3, G and H), implying that unregulated proliferation of the tapetum cellular contents was occurring. Ubisch bodies/orbicules containing electron-opaque materials were evident on the inner tapetal surface, facing the anther locule in both the wild type and *ptc1* (Figs. 3, B and C, and 4, A and E). Orbicules (i.e. Ubisch bodies) are sporopollenin particles seen in secretory tapeta, which are thought to be involved in transporting materials from the tapetum to microspores (Huysmans et al., 1998). Orbicule development was seen in the mutant but was decreased in size compared with the wild type, and by late stage 10 it showed a reduction of both the electron-opaque central region and the surrounding electron-dense sporopollenin (Fig. 4, A and E). Interestingly, orbicules were present on the membrane flanking the tapetal extrusions but were not seen on the extruded regions, suggesting that these extrusions may have been pushed out as a consequence of proliferation from specific regions of the tapetal cells (Fig. 3, F and G, arrow). The two layers of the pollen exine, nexine and sexine (Li and Zhang, 2010), were evident in both the wild type and *ptc1*, although the mutant exine was much reduced and the microspore plasma membrane appeared undulating (Fig. 4, B and F). Secreted pollen wall material was evident in the wild-type locule, and the orbicules were surrounded by densely stained materials, presumed to be sporopollenein precursors (Fig. 4C). The *ptc1* tapetum appeared to have less accumulation of pollen wall precursor materials (Fig. 4, A and E).

Consistent with this, the wild-type microspore developed a well-organized pollen wall, with extensive banding of electron-transparent material (Fig. 4, C and D), whereas *ptc1* developed a thin and abnormal pollen exine, with fewer electron-transparent channels across the pollen wall (Fig. 4, G and H). The *ptc1*

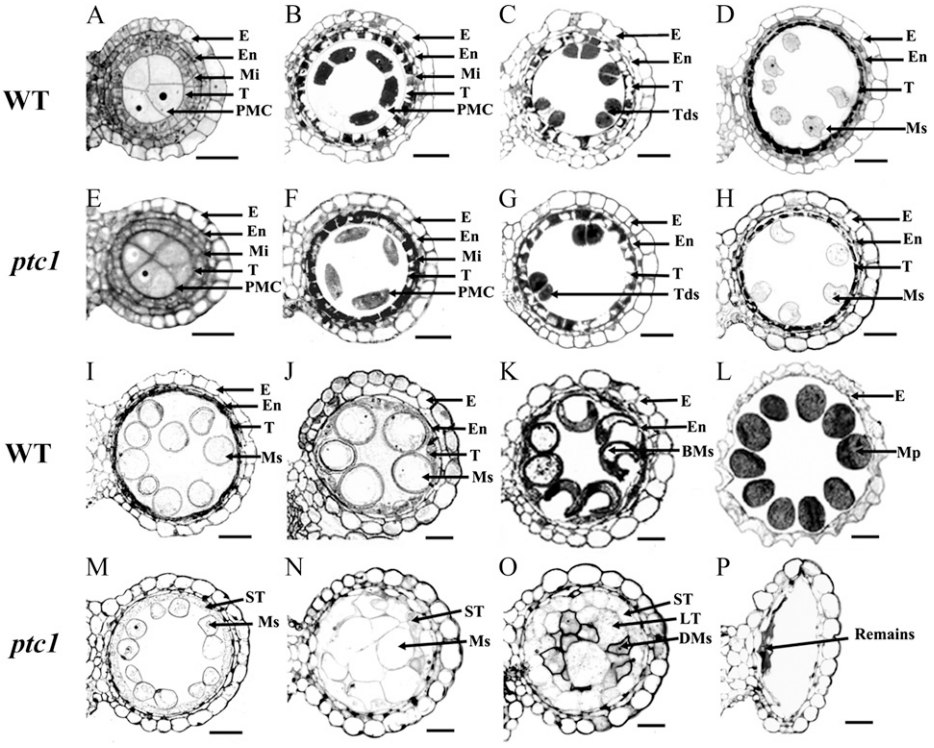

Figure 2. Histological features of anther development in the wild type (WT) and *ptc1*. Locules from the anther section of wild-type (A–D and I–L) and *ptc1* (E–H and M–P) plants from stage 6 to stage 13 are shown. A, and E, Stage 6. B and F, Stage 8a. C and J, Stage 8b. D and H, Stage 9. I and M, Early stage 10. Tapetal cells were swollen and the microspore development was abnormal in *ptc1* (M) compared with the wild type (I). J and N, Late stage 10. The tapetal cytoplasm became swollen and extruded into the locule and the tapetal layer was abnormal (N). K and O, Stage 11. Microspores underwent the second mitosis in the wild type, while *ptc1* tapetal constituents occupied most of the locule space and the microspores aborted. L and P, Stage 13. Only cellular debris was observed in the *ptc1* anther. BMs, Binuclear microspores; DMs, degenerated microspores; E, epidermis; En, endothecium; LT, leaking tapetal cytoplasm; Mi, middle layer; Mp, mature pollen; Ms, microspores; PMC, pollen mother cell; ST, swollen tapetal layer; T, tapetal layer; Tds, tetrads. Bars = 15 μm.

mutant appeared to have a reduced bacular structure, with no spine structures as well as an abnormal nexine, which was uneven on the internal microspore surface (Fig. 4, G and H). This was supported by scanning electron microscopy (SEM), which indicated that the surface of wild-type pollen grains exhibited a regular pattern (Fig. 4, I and K) with spine distribution on the exine (Fig. 4, C and D), whereas *ptc1* pollen showed a lack of the elaborate patterning, resulting in a smooth surface (Fig. 4L). Also, the *ptc1* pollen was about half the size of wild-type pollen at stage 11 (Fig. 4, I and J). These observations indicate that *ptc1* is unable to undergo normal tapetal PCD and pollen wall formation; instead, it undergoes tapetal proliferation and extrusion into the locule, followed by a subsequent rapid necrosis-like cell death during late anther development, leading to male sterility. Therefore, we named this mutant *ptc1*.

ptc1 Tapetal Cells Have Less DNA Fragmentation

Internucleosomal DNA cleavage, visualized as ladders, is considered a hallmark of apoptosis in animals (Danon et al., 2000), whereas necrotic cell death is associated with random DNA degradation resulting in a DNA smear (Schumer et al., 1992). Attempts to observe laddering in DNA isolated from stages 9 and 10 anthers of wild-type and *ptc1* plants failed due to technical difficulties, presumably since tapetal DNA was a minor component of the isolation. Therefore, DNA fragmentation was analyzed in situ using the Terminal Transferase dUTP Nick End Labeling (TUNEL) assay. Tapetal DNA fragmentation was not detected at stage 7 in both the wild type and *ptc1* (Fig. 5, A and C). However, at early stage 9, when the microspore was released from the tetrad, DNA fragmentation signal could be seen in the wild type (Fig. 5B), which is consistent with our previous data (Li et al., 2006), indicating the initiation of tapetal PCD. No TUNEL signal was detected in the *ptc1* mutant (Fig. 5, C and D), implying that regulated DNA fragmentation does not occur in the *ptc1* tapetal cells.

Map-Based Cloning of *PTC1*

To identify *PTC1*, a map-based cloning approach was adopted. *PTC1* was mapped between two markers, Lh903-5 and Lh903-8, on chromosome 9 (Fig. 6A; Sup-

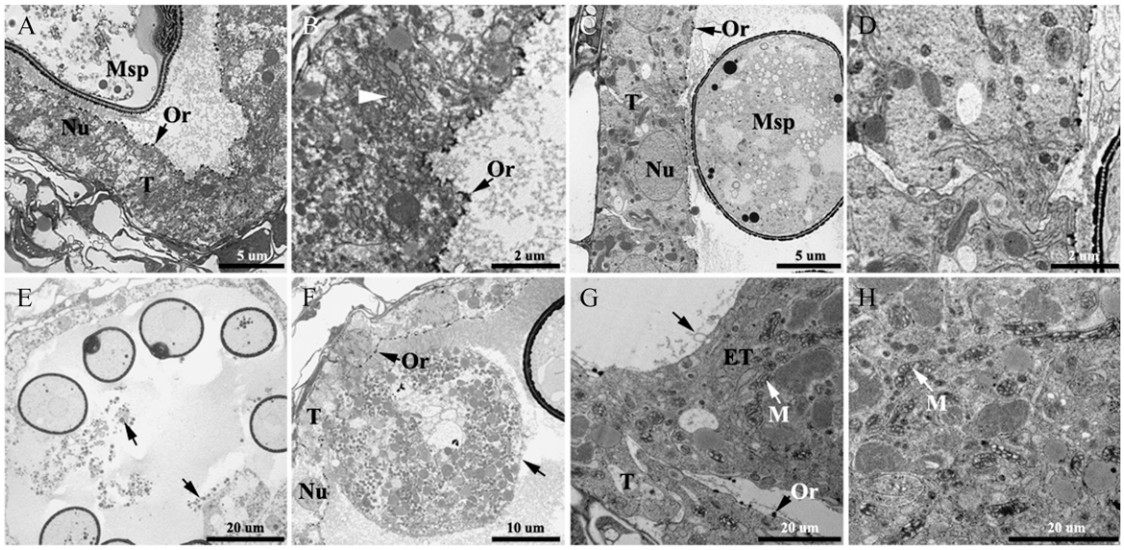

Figure 3. Transmission electron microscopy of wild-type and *ptc1* anthers. Tapetal cell and pollen development in the wild-type (A and B) and in the *ptc1* mutant (C–H) from stage 9 to late stage 10 are shown. A to D, The wild-type tapetum commences changes associated with early PCD (A and B), including cytoplasmic condensation, swelling of organelles (arrowhead), and nuclear membrane disintegration (B; enlargement of details in A), whereas the *ptc1* tapetum appears active, with prolific, prominent organelles, and the tapetal membrane is maintained (C and D [enlargement of details in C]). E to H, The *ptl1* mutant exhibits abnormal tapetal cytoplasmic proliferation and extrusion into the anther locule (F–H). These regions are contiguous with the tapetal cells, are surrounded by an intact membrane (G), and have a high density of organelles, which appear morphologically normal, lipids, and pollen wall materials (H). ET, Extruded region; M mitochondria; Msp, microspores; Nu, nucleus; Or, orbicule; T, tapetal layer. Bars = 5 μm (A and C), 2 μm (B and D), 10 μm (F), and 20 μm (E, G, and H).

plemental Table S1); this 63.6-kb region on bacterial artificial chromosome clone AP005308 contained seven putative genes (http://www.ncbi.nlm.nih.gov/). Six of these genes encoded hypothetical proteins, but one gene, Os09g0449000 from the National Center for Biotechnology Information (or Os09g027620 from The Institute for Genomic Research [TIGR]), encoded a PHD motif transcription factor. Sequencing this gene in *ptc1* revealed a single nucleotide T insertion in the putative second exon, causing a frame-shift mutation and altered reading frame after the 162nd amino acid, resulting in a lack of the PHD motif (amino acids 620–670; Fig. 6, B and C). To further verify that the male-sterile phenotype resulted from this mutation, calli induced from flowers of homozygous *ptc1* plants were transformed with *Agrobacterium tumefaciens* EHA105 containing a 5.255-kb genomic DNA fragment consisting of 3,043 bp upstream and the entire 2,212-bp genomic region of Os09g0449000. This fragment was able to rescue the male-sterile defect of *ptc1* in 35 plants (Fig. 6, D and E), demonstrating that the mutation in Os09g0449000 is responsible for the sterility in *ptc1*.

By comparing the cDNA sequence revealed by our 5′ end and 3′ end RACE-PCRs and the genomic sequence, the structure of *PTC1* was shown to comprise a 2,040-bp coding region with three exons and two introns (Fig. 6B). We also identified a 46-bp untranslated upstream region (UTR) and a 146-bp 3′ UTR (Supplemental Fig. S2); a nuclear localization signal (RRRKR) and a conserved PHD domain were predicted at the N and C termini, respectively (Fig. 6C).

PTC1 Is Preferentially Expressed in the Tapetum and Microspores from Stage 8 to Stage 9 during Anther Development

The main morphological defect of *ptc1* is male sterility, with no obvious vegetative phenotype suggesting anther-associated expression. According to the RiceGE (Gene Expression Atlas Data Sources), *PTC1* expression is highest in the 10- to 15-cm inflorescence (stages 7–10), gradually decreasing in the 15- to 20-cm inflorescence (stages 10–12; http://signal.salk.edu/cgi-bin/RiceGE). Recent expression profile data from laser microdissection coupled with microarray analysis also suggest that *PTC1* is expressed in both the tapetum and microspores at stage 8 (Hobo et al., 2008). Consistent with this, our reverse transcription (RT)-PCR and quantitative RT-PCR (RT-qPCR) results indicated that *PTC1* was preferentially expressed in the anther from stage 8 to stage 9 (Fig. 7, A and B). Strikingly, *ptc1* displayed an increased level of the mutant *PTC1* transcript (Fig. 7, A and B). This suggests either auto down-regulation of the *PTC1* transcript or that the mutant transcript may be more stable and accumulates more than the wild type.

In transgenic plants expressing the $PTC1_{pro}$:*GUS* fusion (driven by the 3,043-bp *PTC1* upstream region), GUS staining was specifically detected in the anther

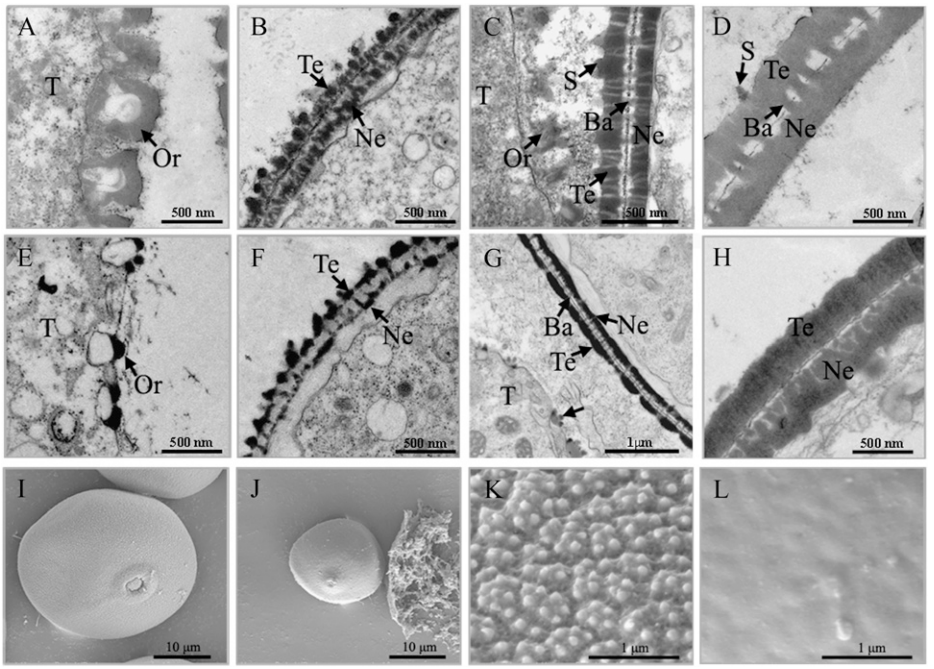

Figure 4. Transmission electron microscopy and SEM of the wild type and *ptc1*. A to H, Orbicule and pollen exine structure of wild-type (A–D) and *ptc1* (E–H) plants from stage 9 to stage 11. A and E show orbicule development at stage 10 in the wild type (A) and the *ptc1* mutant (E). I and J, The outer surface structure of wild-type (I) and *ptc1* (J) pollen at stage 10 by SEM analysis. K and L, Magnification of wild-type (K) and *ptc1* (L) pollen exine. Ba, Bacular; Ne, nexine; Or, orbicule; T, tapetal layer; Te, tectum. Bars = 1 μm (G, K, and L), 10 μm (I and J), and 500 nm (A–D, E, F, and H).

from stages 8 to 9 and at a low level in stage 10 (Fig. 7, C and D). No GUS expression was seen in the palea, lemma, pistil (Fig. 7, C and D) or other organs. Maximal GUS staining, albeit at a very low level, was seen in the tapetal tissues, but GUS was also apparent within the microspores (Fig. 7E), indicating that *PTC1* plays a specific function in tapetal cells during anther development.

Transcriptome Analysis of Wild-Type and *ptc1* Anthers

Transcript profiling of *ptc1* was performed on anthers at two developmental stages: (1) stage 8, during meiosis and tetrad formation, and (2) stage 9, during young microspore development, using Agilent 4*44K rice whole genome DNA microarrays. An empirical Bayes method was used to analyze the data (Smyth, 2004), a false discovery rate (FDR) cutoff of 0.5% was used for the initial filtering of candidate genes, and a secondary selection of greater than $\log_2$ fold difference was then applied. Putative functions of genes were obtained from TIGR (http://tigrblast.tigr.org/euk-blast). Furthermore, the microarray expression data were verified by RT-qPCR using 10 differentially expressed genes; the correlation (94%) of fold change between the RT-qPCR data was closely comparable to that obtained from microarray analyses (Supplemental Fig. S3).

Microarray analysis revealed 2,943 genes with a 2-fold or greater expression change over the two developmental stages in *ptc1* anthers compared with the wild type (570 down-regulated and 278 up-regulated at stage 8; 1,252 down-regulated and 843 up-regulated at stage 9; Supplemental Data Set S1). Since *PTC1* expression is specific to the tapetum and microspores, the *ptc1* data were compared with the laser microdissection-

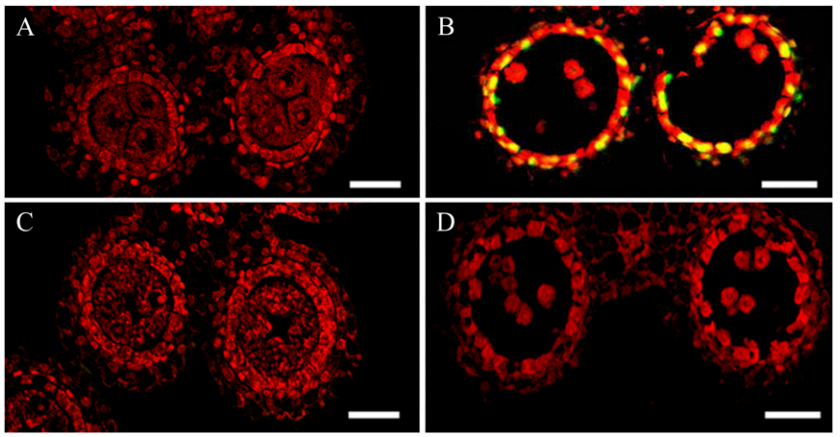

Figure 5. DNA fragmentation detection in wild-type and *ptc1* anthers. TUNEL signals of stage 7 and early stage 9 are shown in wild-type (A and B) and *ptc1* (C and D) anthers. There is no detectable DNA fragmentation signal at stage 7 in the wild type (A) and *ptc1* (C). Wild-type tapetal cells exhibit obvious DNA fragmentation signals at early stage 9 (B). No DNA fragmentation signal is seen in *ptc1* anthers (D) at early stage 9. Bars = 50 μm.

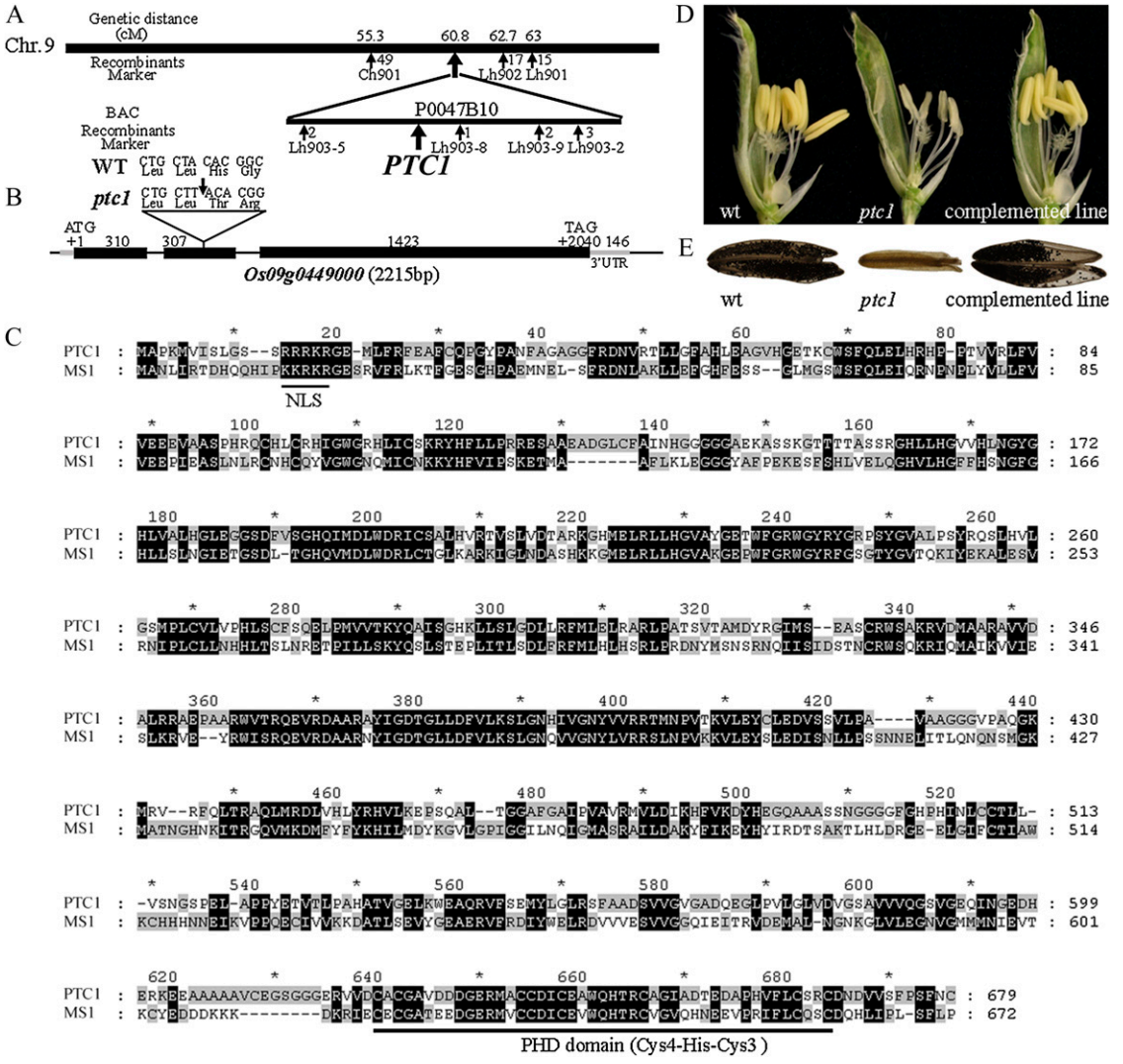

Figure 6. Cloning and analysis of *PTC1*. A, Fine mapping of *PTC1* on chromosome 9. Names and positions of the markers are noted. cM, Centimorgan. B, A schematic representation of three exons and two introns of *Os09g0449000*. +1 indicates the putative starting nucleotide of translation, and the stop codon (TGA) is +2,040. Black boxes indicate exons, and intervening lines indicate introns. The gray lines indicate the 3′ UTR and 5′ UTR. Numbers indicate the exon length (bp); the insertion site in *ptc1* is shown (arrow). BAC, Bacterial artificial chromosome; WT, wild type. C, Amino acid sequence alignment of PTC1 and its Arabidopsis ortholog MS1. The nuclear localization sequence and the putatively conserved PHD domain are underlined. D and E, Phenotypes of the flower (D) and pollen grain staining by I_2-KI (E) in the complemented line at stage 13. [See online article for color version of this figure.]

coupled microarray data of wild-type rice microspores/pollen and the tapetum (Hobo et al., 2008). We identified 1,569 down-regulated genes and 848 up-regulated genes (2,417 in total) with a change in expression of 2-fold or more in the tapetum and/or microspores at stages 8 and 9 between the wild type and *ptc1* (Supplemental Data Set S1). Some genes previously identified as critical for postmeiotic anther development were down-regulated in the *ptc1* microarray data (Table I), including a Cys protease (OsCP1; Lee et al., 2004), a fatty acyl-CoA reductase homologous to Arabidopsis MS2 (Aarts et al., 1997), lipid transfer proteins (Osc4, OsC6, YY1, and Os09g0525500; Tsuchiya et al., 1992; Hihara et al., 1996), a stilbene synthase (YY2; Hihara et al., 1996), BURP domain-containing proteins (RA8 and OsRAFTIN; Jeon et al., 1999), a P450 family member (CYP704B2; Li et al., 2010), a ribosome-inactivating protein (RA39; Jeon et al., 1999), and the putative homolog of AtMYB103 (Higginson et al., 2003; Zhang et al., 2007). Consistent with the RT-qPCR analysis, *PTC1* showed up-regulation in the *ptc1* mutant (2.97-fold at stage 8, 5.49-fold at stage 9), whereas genes involved in early tapetal and pollen regulation, such as *CYP703A3* (Aya et al., 2009), *TDR* (Li et al., 2006), *UDT1* (Jung et al., 2005), and *GAMYB* (Kaneko et al., 2004; Liu et al., 2010; Table I), showed no significant change.

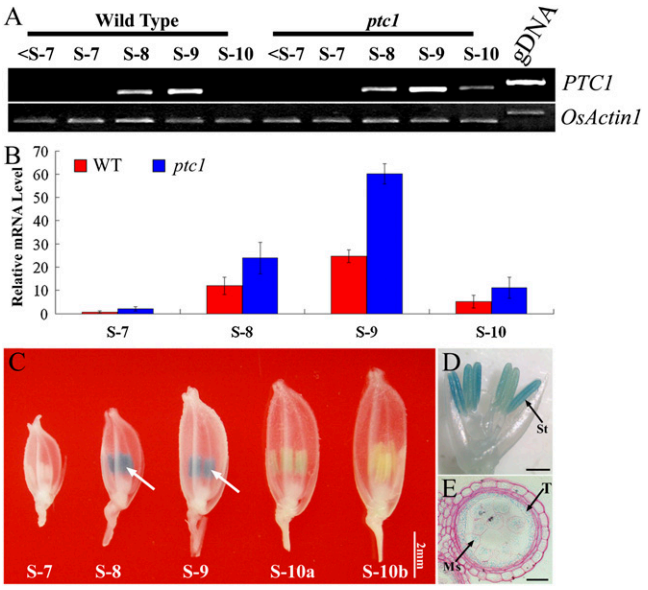

Figure 7. Expression pattern of *PTC1*. A, Maximum expression is seen by RT-PCR during late stage 8 and stage 9; increased expression of the nonfunctional *ptc1* mutant transcript is seen in the mutant at the stage 9, and its expression is prolonged to the early stage 10. <S-7, Flowers before stage 7; S-7, flowers at stage 7; S-8, anthers at stage 8, the tetrad stage; S-9, anthers at stage 9, free microspore; S-10, anthers at stage 10, vacuolated pollen stage. B, RT-qPCR analysis of *PTC1* in wild-type (WT) and *ptc1* anthers using TaqMan probe from stages 7 to 10. *PTC1* expression data were normalized against *OsACTIN1*. C, GUS staining is specifically detectable in the anther of the transgenic plants containing $PTC1_{pro}$:*GUS* from stages 8 to 9. S-7, Stage 7; S-8, stage 8; S-9, stage 9; S-10a, early stage 10; S-10b, late stage 10. Bar = 2 mm. D, GUS staining of the flower after removing the palea and lemma at stage 9. Bar = 20 μm. E, Expression of *PTC1* in tapetal cells and microspores at stage 9 by transverse section analysis. Bar = 15 μm.

To understand the regulatory role of *PTC1*, we grouped the 2,417 genes into four COG (for Cluster of Orthologous Groups of proteins) categories: I, information storage and processing (144 genes); II, cellular processes and signaling (363 genes); III, metabolism (551 genes); and IV, poorly characterized genes (1,359 genes; Fig. 8; Supplemental Table S2).

Postmeiotic tapetal PCD is thought to play an important role in pollen development through providing materials for pollen wall formation (Li et al., 2006). Endopeptidases have been implicated as executors of PCD and are grouped into Ser, Cys, aspartic, metallopeptidases, and Thr endopeptidases (Rawlings and Barrett, 1999). Two genes encoding putative aspartic proteinases and a Cys protease had decreased expression in *ptc1* (Supplemental Table S3). Several genes putatively involved in protein turnover, including SKP1 (for S-phase kinase-associated protein) family proteins, ubiquitin family protein, and RING-H2-finger protein, were down-regulated in *ptc1* (Supplemental Table S3), and expression of three senescence-related, putative subtilisin homologs was greatly down-regulated in *ptc1* (Supplemental Table S3). Reduced expression was also seen in *ptc1* for cell wall biogenesis-related genes, encoding pectinesterases and polygalacturonases (Supplemental Table S3).

In agreement with the observed pollen exine defect, 175 genes putatively related to lipid transport and metabolism and secondary metabolite biosynthesis, transport, and catabolism had expression changes in *ptc1* (Supplemental Table S3).

Transcription factors such as MYB and basic helix-loop-helix (bHLH) families have been shown to play key roles in modulating anther development; many of these involved in the early stages in pollen development were unchanged in *ptc1* (Table I). However, 15 mid- to late-stage transcription factors, including homologs of AtMYB99 and other MYB family members, bHLH domain protein, ACTIVATOR PROTEIN2 domain-containing protein, and zinc-finger proteins, showed changed regulation in *ptc1* (Supplemental Table S3).

Functional Conservation of *PTC1* in Plants

To gain additional insights into the phylogenetic relationship between PTC1 and its close homologs, BLASTP searches were conducted with the full-length amino acid sequence of PTC1. An unrooted tree of PTC1 and its 25 homologs from moss to flowering plants was constructed (Fig. 9; Supplemental Data Set S2). PTC1 was located in the same clade with Arabidopsis MS1 (Wilson et al., 2001; Ito and Shinozaki, 2002; Ariizumi et al., 2005). In this clade, PTC1 homologs were encoded by a single subfamily member in rice, *Sorghum bicolor*, Arabidopsis, grape (*Vitis vinifera*), poplar (*Populus trichocarpa*), *Ricinus communis*, and *Physcomitrella patens* (Fig. 8). No putative PTC1 close homolog was identified in green algae. In addition, another homolog of PTC1, Arabidopsis MALE MEIOCYTE DEATH1/DUET, previously reported as involved in male gametophyte meiosis (Yang et al., 2003), was located in a separate clade. Subsequently, we used the full-length PTC1 protein to search for closest relatives in EST databases. Among those ESTs with E values higher than 2.00E-12, we found that most of the PTC1 homologous ESTs were from reproductive organs in monocot species (Supplemental Table S4). Together, these observations imply an evolutionarily conserved PTC1-related pathway in reproductive development ranging from moss to higher plants.

The phylogenetic analysis indicates that PTC1 and Arabidopsis MS1 are orthologs with 43.76% overall identity (Fig. 6C), suggesting that they may have similar functions. To confirm the evolutionary relatedness of these two genes, we conducted functional complementation of the *ms1* mutant with *PTC1*. Arabidopsis *ms1* shows aberrant tapetal PCD after microspore release, abnormal sporopollenin deposition leading to pollen breakdown (Vizcay-Barrena and Wilson, 2006). A binary plasmid carrying the Arabidopsis *MS1* promoter (2.9 kb) and *PTC1* coding region fused with the synthetic GFP was introduced into

Table I. *Expression in the ptc1 mutant of genes previously reported as altered in expression by TDR, GAMYB, and UDT1 and involved in anther development*

The number 1 indicates no changed expression, and "Down" indicates reduced expression in the mutant. Other numbers indicate the fold change in *ptc1*.

Gene Locus	Gene Name in Rice	Arabidopsis Ortholog	Gene Annotation	Fold Change (Log[Mutant/Wild Type)]				
				ptc1		*tdr*	*gamyb-2*	*udt1*
				Stage 8	Stage 9	Stage 8	Stage 9	Stage 9
Os03g0167600	*OsMS2*	*MS2*	Fatty acyl-CoA reductase	−3.0	−5.4	Down	Down	Down
Os04g0470600	*OsMYB103*	*AtMYB103*	MYB family transcription factor	1	2.7	1	1	Down
Os07g0556800	*RA39*		Ribosome-inactivating protein	−5.7	−4.2	1	Down	1
Os08g0131100	*CYP703A3*	*CYP703A2*	Cytochrome P450	1	1	1	Down	1
Os08g0496800	*OsRAFTIN*		BURP domain-containing protein	1	−2.2	Down	Down	1
Os08g0546300	*Osc4*		LTPL44 lipid transfer protein precursor	−2.5	−11.5	Down	Down	Down
Os09g0449000	*PTC1*	*MS1*	PHD-finger domain-containing protein	1.6	2.5	1	Down	1
Os09g0525500	*YY1*		LTPL45 lipid transfer protein precursor	1	−5.5	Down	1	1
Os10g0484800	*YY2*		Stilbene synthase	1	−1.3	Down	Down	1
Os07g0549600	*UDT1*	*DYT1*	bHLH domain transcription factor	1	1	1	1	1
Os02g0120500	*TDR*	*AMS*	bHLH domain transcription factor	1	1	Down	1	1
Os04g0670500	*OsCP1*		Cys protease	−1.4	−4.9	Down	1	Down
Os09g0480900	*RA8*		BURP domain-containing protein	1	−1.4	Down	1	1
Os11g0582500	*OsC6*		LTPL68 -LTP family protein precursor	1	−2.46	Down	Down	v
Os03g0168600	*CYP704B2*	*CYP704B2*	Cytochrome P450	1	−1.2	Down	Down	1
Os01g0812000	*GAMYB*	*MYB33/65*	MYB family transcription factor	1	1	1	Down	1

MS1/ms1 heterozygous plants. Three lines were identified that carried the *PTC1* gene in the homozygous *ms1/ms1* background (Supplemental Fig. S4); all showed rescue of fertility, although silique length and seed set were slightly reduced compared with the wild type (Fig. 10, A–C). SEM analysis also confirmed that the characteristic ridged pollen exine pattern was restored by *PTC1* (Fig. 10D) as compared with the immature *ms1* pollen, which appeared very small with irregular exine patterning (Fig. 10E). These results indicate conservation of PTC1/MS1 function in regulating anther and pollen development of dicots and monocots, possibly from moss to higher plants.

To further explore the conserved regulatory role, comparison of microarray data between *ptc1* and *ms1* was conducted (Yang et al., 2007). BLASTP analysis using the 260 genes changing in *ms1* identified 130 genes and their close homologs changing in *ptc1*, representing 50% of the genes changing in *ms1*; 30% (79 of 260) of these genes had similar changed expression in the two mutants (Supplemental Data Set S3). Nine of these down-regulated genes were related to lipid and secondary metabolism for pollen exine development, including APG proteins (anther-specific Pro-rich-like protein), lipid transfer protein, β-ketoacyl-CoA synthase, caleosin-related proteins, pollen-specific protein, and trans-

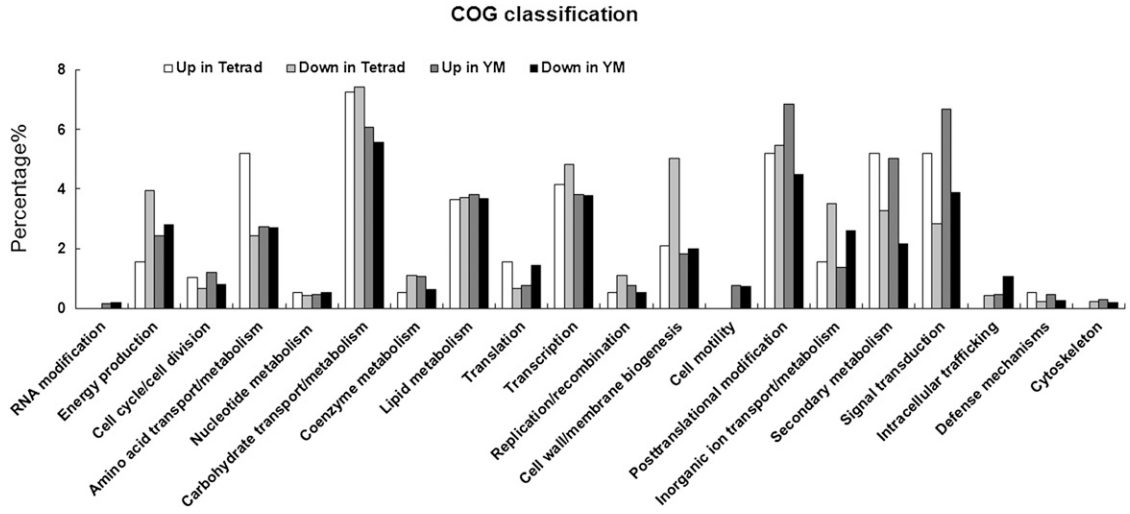

Figure 8. Functional classification by COG analysis of genes that were up-regulated and down-regulated at least 2-fold in *ptc1*.

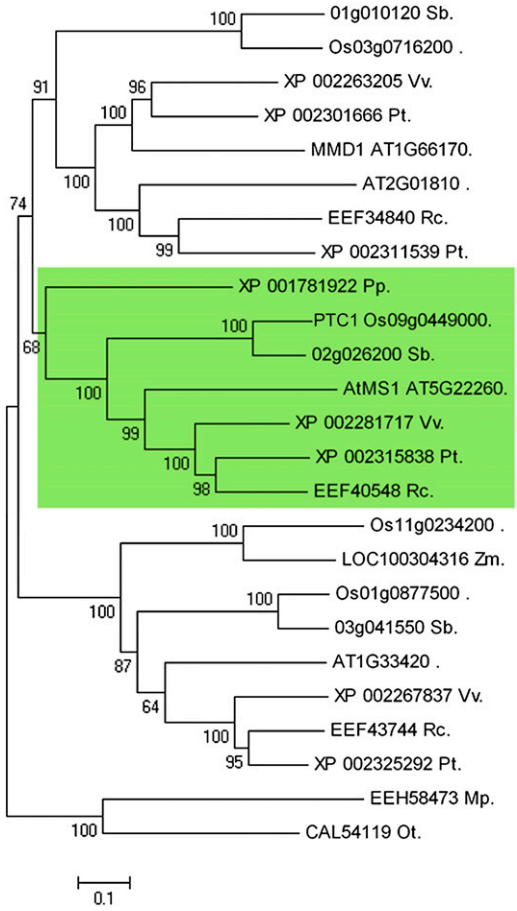

Figure 9. Phylogenetic analysis of PTC1 and its related proteins. An unrooted maximum likelihood tree was created by MEGA 3.1 using the *PTC1*-related sequences from rice (Os), *Physcomitrella patens* (Pp), *Zea mays* (Zm), Arabidopsis (At), *Vitis vinifera* (Vv), *Populus trichocarpa* (Pt), *Ricinus communis* (Rc), and *Sorghum bicolor* (Sb). The EEH58473 protein of *Micromonas pusilla* and CAL54119 of *Ostreococcus tauri* were used as an outgroup. Bootstrap values are shown as percentages. Black dots indicate *PTC1* subfamily members. [See online article for color version of this figure.]

caffeoyl-CoA 3-*O*-methyltransferase. In addition, two genes encoding chalcone synthases and ATP-binding cassette transporter were up-regulated (Supplemental Data Set S3) and eight genes putatively required for pectin modification and degradation were down-regulated (Supplemental Data Set S3).

Comparison of the *PTC1* Regulatory Role with Those of *GAMYB*, *UDT1*, and *TDR* in Anther Development

To aid characterization of the regulatory role of *PTC1*, the expression profiles of the *gamyb-2*, *udt1*, and *tdr* mutants were compared (Jung et al., 2005; Zhang et al., 2008; Aya et al., 2009). We observed that 40.3% (93 of 231) of the genes changing in *tdr*, 10.5% (128 of 1,225) in *udt1*, and 24.2% (210 of 870) in *gamyb-2* had expression changes in *ptc1* (Supplemental Data Set S3). However, transcription factors involved in regulating early tapetal differentiation or sporopollenin synthesis, including *GAMYB* (Kaneko et al., 2004; Aya et al., 2009; Liu et al., 2010), *UDT1* (Jung et al., 2005), *TDR* (Li et al., 2006), and *CYP703A3* (Aya et al., 2009), showed no obvious change (Table I), suggesting that they act upstream of *PTC1* (Fig. 11). Conversely, *PTC1* was shown to have reduced expression in *gamyb-2* mutants (Aya et al., 2009) but no obvious expression change in *udt1* and *tdr* (Jung et al., 2005; Zhang et al., 2008).

Twelve genes showed expression changes (11 down-regulated and one up-regulated) in all four mutants (*gamyb-2*, *udt1*, *tdr*, and *ptc1*; Supplemental Data Set S3); seven are putatively related to lipid metabolism and transport, including 3-oxoacyl reductase, β-ketoacyl-CoA synthase, fatty acyl-CoA reductase (OsMS2), GDSL-like lipase, and LTP family proteins (LTPL44, OsC6, LTPL2), indicating the importance of lipid metabolism for tapetal and pollen development. Three genes are putatively involved in facilitating the transport of water and small neutral molecules (aquaporin protein) and cell wall/membrane/envelope biogenesis (pectinesterase and subtilisin homolog OsSub12), respectively. One gene putatively encodes a plant thionin family protein precursor, THION18, and another one has unknown function (Os08g0216900). This suggests that these genes are essential for tapetum and

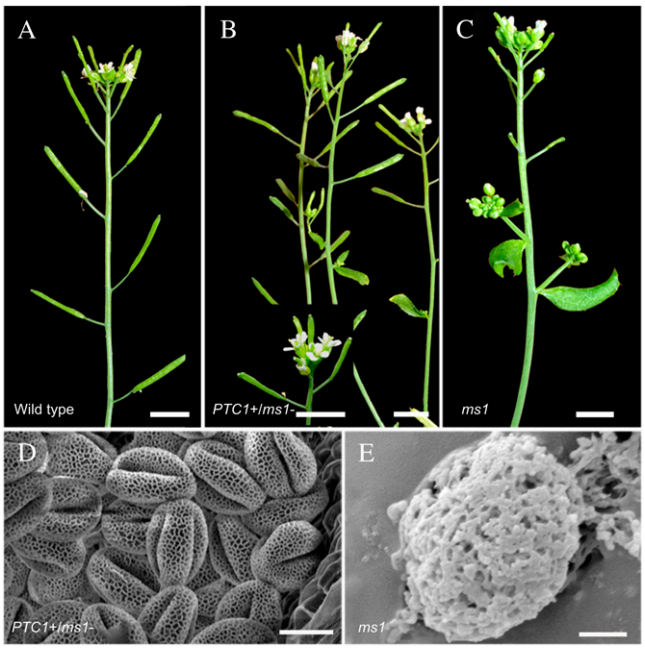

Figure 10. Analysis of the conserved role of *PTC1* and *MS1*. A, Wild-type Arabidopsis siliques. B, Transgenic lines of $MS1_{pro}$:*PTC1* cDNA in the Arabidopsis *MS1*−/− mutant, displaying rescue of *ms1* fertility, although the siliques were slightly shorter than in the wild type. C, Arabidopsis *MS1*−/−. Bars = 5 mm for A to C. D, The observation of pollen surfaces of transgenic lines containing *PTC1*+/*ms1*− by SEM. Bar = 10 μm. E, The pollen of Arabidopsis *ms1* mutant (*MS1*−/−). Bar = 500 nm.

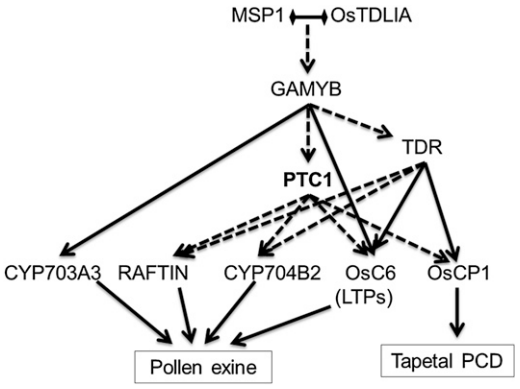

Figure 11. Regulatory role of PTC1 in tapetal PCD and pollen exine formation in rice. MSP1 binds OsTDLIA to determine tapetal cell differentiation and formation during the early stage (Nonomura et al., 2003; Wang et al., 2006; Zhao et al., 2008). Down-regulation of *GAMYB* was seen in *msp1-4* anthers, suggesting that GAMYB acts downstream of MSP1-OsTDLIA (Wang et al., 2006). PTC1 functions downstream of GAMYB (Aya et al., 2009) and in parallel with TDR (Li et al., 2006) in regulating programmed anther development and pollen formation. As direct target genes of TDR, *OsCP1* and *OsC6*, encoding a Cys proteinase and a lipid transfer protein (LTP), respectively (Lee et al., 2004; Zhang et al., 2010), are also directly or indirectly regulated by GAMYB and PTC1. Meanwhile, pollen exine-forming genes *CYP704B2*, *CYP703A3*, *RAFTIN*, and *OsC6* (LTPs; Aarts et al., 1997; Wang et al., 2003; Aya et al., 2009; Li et al., 2010; Liu et al., 2010; Zhang et al., 2010), involved in lipid metabolism and transport, are affected by GAMYB, PTC1, and TDR. Furthermore, *TDR* may act downstream of *GAMYB* (Liu et al., 2010).

pollen exine development and are probably directly or indirectly regulated by genes acting upstream of *PTC1*, possibly by *GAMYB*, *UDT1*, and *TDR*. Interestingly, *OsC6* is regulated by both TDR and GAMYB in vivo and in vitro (Li et al., 2006; Aya et al., 2009; Fig. 11), suggesting that regulation may involve multiple members of the transcriptional regulatory network.

DISCUSSION

Rice is the major staple food feeding the global population; however, maintaining and increasing yields are significant challenges for future food security. Manipulation of pollen fertility is of particular importance to increase the grain yield in highly inbreeding species, including rice. Currently, much of the pollen fertility information has been obtained from model plant systems such as Arabidopsis (Wilson and Zhang, 2009). In this study, we report the characterization of *PTC1*, an essential gene for programmed tapetal cell fate and pollen formation in the monocot crop rice. Its evolutionary relevance has been demonstrated by the restoration of fertility in the Arabidopsis *ms1* mutant. Moreover, by comparing expression data, we propose that *PTC1/MS1* plays a key regulatory role in anther development. These findings suggest that PTC1/MS1 controls an evolutionary switch essential for programmed male reproductive development, greatly extending our understanding of plant anther development.

PTC1 Determines a Conserved and Diversified Switch of Apoptosis and Necrosis in Tapetal Cells

Timely initiation of tapetal PCD is essential for the regulated release of wall materials, including carbohydrate, lipidic molecules, and other nutrients, from the tapetum to the developing microspore. At stage 8, the wild-type rice anther initiates an apoptosis-like cell death in the tapetum, displaying typical apoptotic hallmarks including cell shrinkage and fragmentation of chromatin DNA (Papini et al., 1999; Li et al., 2006). However, chromatin condensation, as indicated by TUNEL analysis, does not occur in the *ptc1* tapetum (Fig. 5, C and D). Instead, the mutant tapetum appears highly metabolically active, with increased numbers of mitochondria and endoplasmic reticulum and the apparent proliferation of cellular constituents (Fig. 3, F–H). Normal tangential tapetal cell wall breakdown does not appear to occur in *ptc1*; therefore, instead of materials being secreted out into the locule, they are retained within the mutant tapetum. Tapetal PCD fails to occur in the *pcl1* tapetum, which appears to go through overproliferation, resulting in the extrusion of cytoplasmic contents, which are constrained by the persistent plasma membrane, into the locule (Fig. 3). This phenotype is distinct from other tapetal mutants that have been described, such as Arabidopsis *ams* (Xu et al., 2010) and rice *tdr* (Li et al., 2006) mutants, which exhibit tapetal defects involving swelling of the tapetum as a consequence of vacuole increase rather than cytoplasmic proliferation. Subsequent to this proliferation, very rapid degeneration of the tapetum and microspores occurs in *ptc1* via necrosis-like breakdown. Therefore, in *ptc1*, there seems to have been a departure from the standard PCD pathway to one of uncontrolled proliferation, altered organelle organization, and subsequent necrotic death. In *ms1*, the Arabidopsis ortholog, although a lack of DNA fragmentation and tapetal PCD is also seen, and the cytoplasm of the tapetum cells and microspores becomes granular and abnormally vacuolated (Vizcay-Barrena and Wilson, 2006), the changes in tapetal and organelle proliferation are not observed. This may reflect the differences in tapetal development in monocots and dicots. Rice and Arabidopsis both have secretory tapeta where the cells remain intact until their disintegration (Huysmans et al., 1998; Furness and Rudall, 2001). However, the tapeta of rice and other cereals exhibit characteristic orbicules/Ubisch bodies, which are thought to export tapetum-produced sporopollenin precursors across the hydrophilic cell wall to the locule. Orbicules have not been observed in the Brassicaceae, including Arabidopsis, which contain unique secretory tapeta with specialized organelles such as tapetosomes. Whether the observed tapetal proliferation in *ptc1* compared with *Atms1* is a consequence of these differences remains to be determined.

In animals, necrosis is thought to be an uncontrolled and passive form of cell death caused by severe nonphysiological and/or physical/chemical factors (Galluzzi et al., 2007). However, recent evidence suggests that necrosis can be genetically regulated in a similar manner to apoptosis (Vande Velde et al., 2000; Zong and Thompson, 2006; Hitomi et al., 2008). To date, the molecular control of necrosis in both plants and animals remains largely unknown. In plants, a group of proteins such as Nep1 (for necrosis and ethylene-inducing peptide)-like proteins found in many plant pathogens have the ability to trigger a necrotic response with the leakage of cell metabolites (Bae et al., 2006). In addition, cell lines in suspension culture under higher temperature conditions displayed necrotic responses (McCabe and Leaver, 2000; Burbridge et al., 2007; Reape et al., 2008). As far as we know, there are no reports of nuclear gene mutations displaying proliferation and subsequent severe necrosis-like cell death in plants. Therefore, our work provides a system to investigate necrosis-like cell death in plants and to investigate the role of membrane breakdown and tapetal proliferation in triggering the regulation of PCD in the anther.

Cys proteases are thought to be involved in the process of plant cell death (Solomon et al., 1999), although currently no caspase homologs have been identified in plants, but emerging evidence indicates that Cys/Ser proteases may function as caspase-like executors (Beers et al., 2000; Woltering, 2004). In agreement with the role of *PTC1* in regulating tapetal PCD, cell death-associated genes encoding Cys proteases show altered expression in *ptc1*. *OsCP1*, encoding a Cys protease, is regulated by TDR at the transcriptional level and is expressed in the tapetum (Li et al., 2006), and reduction of *OsCP1* expression causes reduced viability of pollen grains (Lee et al., 2004). *OsCP1* expression is also down-regulated in *ptc1* (Table I). Another gene (Os03g0689300) encoding a plasma membrane ATPase was down-regulated and is thought to be responsible for maintaining ATP levels that can regulate a switch between apoptosis and necrosis (Kroemer et al., 1998; Proskuryakov et al., 2003; Supplemental Data Set S3).

The data indicate that PTC1 plays a critical role in the regulation of tapetal PCD, although it is currently unclear how this occurs. PTC1 may act, alongside other transcription factors, for example *TDR*, by directly regulating executor proteases and other PCD initiators. Alternatively and possibly more likely, PTC1 may act in an indirect way by regulating overall tapetal function and pollen wall biosynthesis. If abnormal, this then causes prolonged activity of the tapetum and the avoidance of PCD induction, which then causes the induction of death by necrosis.

PTC1 Controls Pollen Wall Formation

PTC1 encodes a PHD-finger protein that is specifically expressed in tapetal cells and microspores. The PHD domain comprises about 50 to 80 amino acids, contains a conserved Cys_4-His-Cys_3 motif, and is found mainly in proteins involved in eukaryotic transcription regulation (Aasland et al., 1995). Consistent with the defective pollen wall in *ptc1*, gene expression profiling analysis indicates that *PTC1* regulates genes related to lipid synthesis, metabolism, and transport for anther development and pollen wall formation.

Lipidic pollen exine is made of sporopollenin, an extremely resilient material derived from the polymerization of fatty acid metabolites and phenolic acid (Piffanelli et al., 1998; Morant et al., 2007). Even though the pollen grains of rice and Arabidopsis have similar pollen exine layers, rice has a distinct pollen wall ontology compared with Arabidopsis. The outer surface of Arabidopsis pollen grains has elegant reticulate cavities with abundant pollen coat (tryphine) deposited inside the pollen exine. Whereas rice pollen have a smooth and particulate exine patterning that has more interlayer space between the nexine (foot layer) and sexine compared with Arabidopsis (Li et al., 2010; Li and Zhang, 2010). This is likely to be a consequence of different pollination strategies; Arabidopsis belongs to insect-pollinated (entomophilous) plants, in contrast to rice, which is wind pollinated (anemophilous; Ariizumi and Toriyama, 2011).

The expression of genes involved in flavonoid and lipid biosynthesis, including fatty acyl-CoA reductase (homologous to MS2), chalcone synthase, and P450 proteins, is down-regulated in *ptc1* (Table I; Supplemental Data Set S1). In Arabidopsis, *MS2* encodes a fatty acyl reductase that is thought to convert fatty acids into fatty alcohols for sporopollenin production and exine formation (Aarts et al., 1997). We recently reported that CYP704B2 is expressed in the tapetum and microspores and catalyzes the production of ω-hydroxylated fatty acids with 16- and 18-carbon chains, which are essential for the formation of both anther cuticle and pollen exine during rice male reproductive development (Li et al., 2010).

Tapetal cells are able to actively synthesize lipidic precursors that are transported in rice by orbicules onto the microspore surface. *RAFTIN* has been shown to be present in pro-orbicules, to accumulate in orbicules, and then to be targeted to the microspore exine (Wang et al., 2003). In *ptc1*, orbicules were smaller in size with less sporopollenin material surrounding the reduced electron-transparent central region (Fig. 4, A and E) and had reduced expression of *RAFTIN* (Table I). This leads to abnormal assembly of the mutant pollen wall, with a reduced amount of channels within the wall and a smooth surface (Fig. 4, F–H, K, and L). In addition, reduced expression of some LTP family members (i.e. *OsC6*) was observed in the *ptc1* mutant (Table I), implying associated alterations in lipid movement with the mutant tapetum. *OsC6* encodes a small fatty acid-binding protein that is required for postmeiotic anther development in rice; silencing of *OsC6* causes defective development of orbicules and pollen exine (Zhang et al., 2010).

PTC1 Plays a Conserved Role in Plant Anther Development

PTC1 shares homology with a number of proteins containing the PHD-finger motif in animals, yeast, and higher plants (Halbach et al., 2000). Phylogenetic analysis indicates that Arabidopsis *MS1*, poplar *PtMS1*, and rice *PTC1* form a separate group within the entire family (Ito et al., 2007), suggesting that PTC1 and its close homologs may have a conserved role in plant reproductive development. Consistent with this hypothesis, *PTC1* driven by the *MS1* promoter is able to rescue pollen wall development and pollen fertility of the homozygous *ms1* mutant, suggesting a conserved role regulating programmed anther development in monocot and dicot species (Fig. 10).

Moreover, *PTC1* and *MS1* show similar tapetal expression patterns from late tetrad to microspore release (Yang et al., 2007). Also, neither *ms1* (Ito et al., 2007) nor *ptc1* could be rescued using the equivalent wild-type gene driven by the cauliflower mosaic virus 35S promoter, suggesting that the precise control of *MS1/PTC1* expression is critical for pollen development. *MS1* expression in the *ms1* mutant shows increased expression (Wilson et al., 2001; Yang et al., 2007), suggesting that *MS1* is able to negatively regulate its own expression. Similarly, we also observed an elevated and prolonged expression level of *PTC1* in the *ptc1* mutant. This implies that a conserved mechanism regulates the transcription of *MS1* and *PTC1* in both rice and Arabidopsis, with functional *MS1/PTC1* down-regulating their own expression in the wild-type plants. Furthermore, genes involved in RNA processing and modification are down-regulated in both *ptc1* (*OsPAB184*) and *ms1* (*PAB3*; Supplemental Data Set S2). *OsPAB184* encodes a poly(A)-binding protein that is the homolog of Arabidopsis *PAB3*. *PAB3* has been shown to be specifically expressed in the tapetal cell and pollen at the young microspore stage (Belostotsky, 2003) and can promote mRNA degradation (Chekanova and Belostotsky, 2003). Therefore, we speculate that down-regulation of both *OsPAB184* and *PAB3* may result in the transcript accumulation of *PTC1/MS1* in the mutants.

Microarray analysis also revealed that PTC1 and MS1 may have partly conserved and common regulatory pathways for pollen wall development and tapetal cell death. For example, a gene (Os02g0672500) with reduced expression in both *ptc1* and *ms1*, encoding a mitochondrial import inner membrane translocase, is predicted to mediate the protein import pathway in mitochondria (Hong et al., 2006).

Regulatory Network of Tapetal PCD

Several transcription factors have been shown to regulate tapetum development and degeneration by analyzing the tapetum-defective mutants, including rice *gamyb*, *udt1*, and *tdr* (Kaneko et al., 2004; Jung et al., 2005; Li et al., 2006; Liu et al., 2010). However, *ptc1* has distinct morphological changes from these mutants. *UDT1* and *TDR* are bHLH transcription factors, and *GAMYB* encodes a MYB domain transcription factor. Tapetal cells in *gamyb*, *tdr*, and *udt1* display delayed degeneration and abnormal enlargement, occupying the locule space and causing microspore abortion and defective pollen exine (Kaneko et al., 2004; Jung et al., 2005; Li et al., 2006; Zhang et al., 2008; Liu et al., 2010). Furthermore, no expression alteration of *PTC1* was observed in *tdr* and *udt1* (Jung et al., 2005; Zhang et al., 2008), while significant reduction of *PTC1* was seen in *gamyb-2* (Aya et al., 2009), suggesting that PTC1 may act downstream of GAMYB in rice anther development and, consistent with this, that *PTC1* is expressed after *GAMYB* (Fig. 11).

In summary, we have identified a PHD-finger protein, PTC1, as a conserved key regulator for tapetal PCD in plant pollen development. The mutation of *PTC1* caused tapetal proliferation, lack of PCD-based tapetal degeneration, and subsequent necrosis-like cell death in tapetal cells, resulting in abnormal pollen wall development, aborted pollen grains, and male sterility.

MATERIALS AND METHODS

Mutant Materials and Growth Conditions

All rice (*Oryza sativa*) plants were grown in the paddy field of Shanghai Jiao Tong University. The F2 mapping population was generated from a cross between the *ptc1* mutant (*japonica*) and Guan Lu Ai (*indica*; Chu et al., 2005; Liu et al., 2005; Chen et al., 2006; Wang et al., 2006). In the F2 population, male-sterile plants were selected for genetic mapping.

Characterization of the Mutant Phenotype

Plants and flowers were photographed with a Nikon E995 digital camera. *ptc1* tetrads and microspores were stained by 0.2% 4,6-diamino-2-phenylindole dihydrochloride *n*-hydrate to observe microspore development. Observation of anther development by semithin sections and transmission electron microscopy were performed as described by Li et al. (2006). SEM was performed as described by Zhang et al. (2008).

Isolation of *PTC1* Full-Length cDNA

To obtain the full-length cDNA of *PTC1*, RACE-PCR was performed with the GeneRacer kit (Invitrogen) as described before (Yuan et al., 2009). The *PTC1* cDNA was amplified using 2 μL of RACE product from rice anther RNAs (stage 9) with Phusion hot-start high-fidelity polymerase (New England Biolabs). *PTC1*-specific primers PTC1 Race 5R1 and PTC1 Race 5R2 were used for 5′ RACE, and primers PTC1 Race 3R1 and PTC1 Race 3R2 were used for 3′ RACE. The cDNA of *PTC1* was amplified using primers PTC1 FL-cDNA F and PTC1 FL-cDNA R.

Cloning of *PTC1* and Complementation

A 5.255-kb wild-type genomic fragment of *PTC1* with a 3,043-bp 5′ upstream region and a 2,212-bp genic region (including introns) was amplified using the primer pair PTC1 com F and PTC1 com R. Then, the fragment was cloned into the entry vector pENTR/D-TOPO and destination vector pGWB7 to produce plasmid *pGWB7:PTC1*. The *pGWB7:PTC1* plasmid was transferred into *Agrobacterium tumefaciens* EHA105 and introduced into the *ptc1* mutant plants by *A. tumefaciens*-mediated transformation (Zeng et al., 2007). Pollen grains of transgenic lines were assayed by I_2-KI staining. Transgenic lines were identified by PCR amplification using primers B7-com

screen F and PGWB7 vector primer B7-com screen R. The segregation of fertility plants and sterility plants was approximately 3:1 for line 1 (23 fertility:7 sterility) and line 2 (16 fertility:6 sterility) of the T1 generation.

Arabidopsis (*Arabidopsis thaliana*) *MS1* gene promoter (2,981 bp) was amplified by primers AtMS1 Pro F and AtMS1 Pro R, and the *PTC1* cDNA was amplified by primers PTC1 FL-cDNA F and PTC1 FL-cDNA R. The two fragments were ligated together by *Kpn*I and *Eco*RI restriction enzymes in the pBluescript SK+ vector (the plasmid was designated pMOG1). Then, the GFP fragment including the *MS1* terminator (GFPt, including the GFP fragment) was amplified by primers GFPt F and GFPt R, and the GFPt fragment, digested with *Bam*HI and *Sac*II, was ligated to pMOG1 by *Bgl*II and *Sac*II restriction enzyme (the plasmid was designated pMOG2). Then, the whole sequence, including the *MS1* promoter, *PTC1* cDNA, *GFP*, and *MS1* terminator, was digested with *Kpn*I and *Xba*I and inserted into the pBIN19 vector (the plasmid was designated pMOG3). Plasmid pMOG3 was then transferred into *A. tumefaciens* GV3101 and Arabidopsis heterozygous *ms1* plants. The transformants were screened for the presence of transgene on kanamycin medium. RNA was isolated from buds of the T1 lines (Invitrogen), and 0.3 μg of total RNA was used to synthesize cDNA using the Rever Tra Ace-α First Strand cDNA synthesis kit (Fermentas). One microliter of the RT product was subsequently used as the template for RT-PCR. Then, the transgenic Arabidopsis lines with exogenous *PTC1* expression were analyzed. The genetic background of transgenic lines was identified by RT-PCR using the primers PTC RT-PCR F and PTC RT-PCR R. Control RT-PCR analyses were conducted using Arabidopsis *ACTIN* primers AtActin F and AtActin R.

RT-PCR and Promoter Fusions

Total RNA was isolated using Trizol reagent (Generay), as described by the manufacturer, from rice tissues: the root, shoot, leaf, lemma/palea, pistil, and anther at different stages. The stages of anthers were classified according to spikelet length (Feng et al., 2001; Zhang and Wilson, 2009). After treatment with DNase (Promega), 0.3 μg of RNA was used to synthesize the oligo(dT)-primed first-strand cDNA using the ReverTra Ace-a-First Strand cDNA synthesis kit (Fermentas). One microliter of the RT products was subsequently used as the template for RT-PCR.

A 3,043-bp upstream region of the *PTC1* gene was amplified from rice leaf genomic DNA and subcloned into the entry vector pENTR/D-TOPO and destination vector pGWB3. The pGWB3:$PTC1_{pro}$:GUS construct was introduced into calli of the wild-type rice by *A. tumefaciens* transformation (Zeng et al., 2007). GUS activity was visualized by staining the root, stem, leaf, and different stage flowers from heterozygous spikelets of transgenic lines overnight in 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid solution (Willemsen et al., 1998), and the tissues were then cleared in 75% (v/v) ethanol. The cleared anthers were fixed in formalin-acetic-alcohol solution and embedded in Technovit 7100 resin. Sections (10 μm) were then stained for 20 min in ruthenium red (0.05% [w/v], pH 9), mounted, and photographed using a Leica DM2500 microscope.

TUNEL Detection

TUNEL assay was performed using the TUNEL apoptosis detection kit (DeadEnd Fluorometric TUNEL System; Promega) on paraffin sections as described by Li et al. (2006). Samples were analyzed with a fluorescence confocal scanner microscope (Zeiss LSM 510).

Phylogenetic Analysis

The full-length amino acid sequence of PTC1 and most similar sequences identified via BLAST search were aligned with the MUSCLE tool (Edgar, 2004) using the default parameters. The EEH58473 protein of *Micromonas pusilla* was added for rooting purposes. The alignment (Supplemental Data Set S2) was used to construct a maximum likelihood tree with MEGA 3.1 (Kumar et al., 2004) using the following parameters: Poisson correction, pairwise deletion, and 1,000 bootstrap replicates.

Microarray Experiments and Analyses

Agilent 4*44K rice oligoarrays (Agilent Technologies) containing 44,000 features were used for two-color oligoarrays in this study. Two biological replicates of total RNA were prepared from *ptc1* and wild-type anthers at stages 8 and 9 using TRIzol reagent (Invitrogen), and the mRNA was isolated from total RNA using the RNAeasy Mini Kit (Qiagen). Developing anthers were classified according to callose staining of the tetrad and spikelet length (Feng et al., 2001; Zhang and Wilson, 2009). Microarray hybridization was performed in a hybridization chamber (Agilent G2534A) according to procedures provided by the manufacturer. The microarrays were scanned with an Agilent instrument (G2565BA), and the quality of the chip data was analyzed with R statistical language and the limma package of the Bioconductor project (http://www.bioconductor.org/; Smyth, 2005). Acquired signals were normalized internally and across all arrays as described by Smyth and Speed (2003), and the average signals of replica were used for analysis. We obtained the ratio of *ptc1* to wild-type fluorescence, and the change fold was the value of the $\log_2$-normalized signal ratio. Meanwhile, linear models and empirical Bayes methods were applied to find the differentially expressed genes (Smyth, 2004). Excel was used to sort and find the differently expressed genes by 2-fold cutoff, FDR $>$ 0.05%, and B $>$ 1.5 (Smyth, 2004). B stands for the B-statistic (lods or B), which is the log-odds that the gene is differentially expressed. The annotations of genes were obtained from TIGR (http://rice.plantbiology.msu.edu/).

Sequence data from this article for the cDNA and genomic DNA of PTC1 can be found in the GenBank/EMBL data libraries under accession numbers NM_001069854, GU597363, and AC005308. Accession numbers for the sequences used in the phylogenetic analysis on the tree in Figure 8 are as follows: OsCP1 (Os04g0670500), OsMS2 (Os03g0167600), Osc4 (Os08g0546300), OsC6 (Os11g0582500), YY1 (Os09g0525500), YY2 (Os10g0484800), RA8 (Os09g0480900), OsRAFTIN (Os08g0496800), CYP704B2 (Os03g0168600), RA39 (Os07g0556800), OsMYB103 (Os04g0470600), CYP703A3 (Os08g0131100), TDR (Os02g0120500), UDT1 (Os07g0549600), GAMYB (Os01g0812000), OsPAB184 (Os06g0589700), and PAB3 (AT1G22760).

Supplemental Data

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

Supplemental Figure S1. 4,6-Diamino-2-phenylindole dihydrochloride *n*-hydrate staining of microspores of wild-type and *ptc1* plants from stage 8 to stage 11.

Supplemental Figure S2. Nucleotide and amino acid sequences of *PTC1*.

Supplemental Figure S3. RT-PCR analyses of gene expression levels in *ptc1*.

Supplemental Figure S4. RT-PCR analysis of *AtMS1* and *PTC1* expression in the transgenic lines containing $MS1_{pro}$:*PTC1* cDNA in Arabidopsis.

Supplemental Table S1. Markers used for mapping the *PTC1* gene.

Supplemental Table S2. Functional classification by COG analysis of genes that were up-regulated or down-regulated in *ptc1* as identified by Bayes analysis (FDR $<$ 0.5%) and showing a greater than $\log_2$ fold change in expression.

Supplemental Table S3. List of genes that were up-regulated or down-regulated at least 2-fold in the *ptc1* tapetum and microspores.

Supplemental Table S4. ESTs of *PTC1* homologs in monocot species.

Supplemental Table S5. Primer sequences used in this study.

Supplemental Data Set S1. Genes changed in the data of *ptc1* microarray.

Supplemental Data Set S2. Text file of the protein alignment among rice PTC1 and other members on the phylogenetic tree.

Supplemental Data Set S3. Comparison of the *ptc1* microarray data with those of *gamyb*, *udt1*, and *tdr*.

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