The rice (Oryza sativa) floral homeotic C-class gene, MADS3, was previously shown to be required for stamen identity determination during early flower development. Here, we describe a role for MADS3 in regulating late anther development and pollen formation. Consistent with this role, MADS3 is highly expressed in the tapetum and microspores during late anther development, and a newly identified MADS3 mutant allele, mads3-4, displays defective anther walls, aborted microspores, and complete male sterility. During late anther development, mads3-4 exhibits oxidative stress-related phenotypes. Microarray analysis revealed expression level changes in many genes in mads3-4 anthers. Some of these genes encode proteins involved in reactive oxygen species (ROS) homeostasis; among them is MT-1-4b, which encodes a type 1 small Cys-rich and metal binding protein. In vivo and in vitro assays showed that MADS3 is associated with the promoter of MT-1-4b, and recombinant MT-1-4b has superoxide anion and hydroxyl radical scavenging activity. Reducing the expression of MT-1-4b causes decreased pollen fertility and an increased level of superoxide anion in transgenic plants. Our findings suggest that MADS3 is a key transcriptional regulator that functions in rice male reproductive development, at least in part, by modulating ROS levels through MT-1-4b.

INTRODUCTION

In higher plants, the formation of the stamen, the male reproductive organ consisting of anther and filament, is a complex biological process that includes stamen specification and development of specialized anther tissues in which the haploid microspores/pollens are generated (McCormick, 1993). Genetic and functional analyses of homeotic mutants with changed floral organ identities in the model dicot plants Arabidopsis thaliana and Antirrhinum majus led to the ABC model, which explains how the stamen is specified by the combinatorial action of class B, C, and E genes (Coen and Meyerowitz, 1991; Ditta et al., 2004). Most of the A, B, C, and E genes encode MIKC-type MADS box transcription factors, which contain a 60–amino acid MADS box domain in the N-terminal region, a less conserved intervening region of ~30 amino acids, a moderately conserved ~70-amino acid keratin-like domain involved in protein–protein interaction, and a highly variable C-terminal region of variable length (Cho et al., 1999; Egea-Cortines et al., 1999; Yang et al., 2003).

Recent studies suggest that the ABC model can also partially explain how stamen determination is specified in the monocot plant rice (Oryza sativa; Nagasawa et al., 2003; Yamaguchi et al., 2006; Dreni et al., 2007; Ohmori et al., 2009; Zhang and Wilson, 2009; Li et al., 2010a). For example, a rice B-class gene, SUPERWOMEN1 (SPW1 or MADS16), which is orthologous to the Arabidopsis APETALA3 gene, has been shown to be crucial for stamen specification (Nagasawa et al., 2003). spw1 mutants show homeotic conversions of stamens to carpels and lodicules to palea/lemma-like structures. In Arabidopsis, the C-class gene AGAMOUS (AG) acts to specify stamen and carpel identities and floral meristem determinacy (Yanofsky et al., 1990; Bowman et al., 1991). Studies in rice identified two C-class MADS box genes, MADS3 and MADS58, that may have distinct functions in specifying stamen identity, with MADS3 playing a more important role (Yamaguchi et al., 2006).

Other MADS box genes, such as SPOROCYTELESS/NOZZLE (SPL/NZZ) (Yang et al., 1999; Schiefthaler et al., 1999) and AG (Ito et al., 2004, 2007) from Arabidopsis and MADS2 from maize (Zea mays; Schreiber et al., 2004), have been implicated in regulating anther development. SPL/NZZ regulates the formation of anther walls and pollen mother cells, as the primary sporogenous cells cannot form pollen mother cells in spl anthers, thereby blocking early cell differentiation (Yang et al., 1999). AG has been shown to activate the expression of SPL/NZZ, suggesting that this gene is necessary for early stamen development (Ito et al., 2004). During later developmental stages, AG continues its expression in the anther and regulates anther dehiscence by directly regulating the expression of the gene that encodes a jasmonic acid (JA)
synthetic enzyme, DEFECTIVE IN ANther DEhiscence1 (ito et al., 2007). Maize MADS2 is required for anther dehiscence and pollen maturation, and knockdown of MADS2 resulted in abortion of anthers and defective pollen development (Schreiber et al., 2004).

Reactive oxygen species (ROS) are generated in plants when molecular dioxygen (O₂) is used as a terminal electron acceptor, creating molecules such as superoxide anion (O₂⁻), hydroperoxide radicals (OH·), and hydrogen peroxide (H₂O₂). Most of these molecules are toxic by-products of aerobic metabolism in plants subjected to abiotic stresses, such as drought, salinity, flooding, heat, and cold (Miller and Mittler, 2006). Plants have evolved diverse protective mechanisms, including various enzymes and nonenzymatic systems, to adjust ROS levels (Mittler et al., 2004). The ROS-scavenging enzyme system contains superoxide dismutase, catalase, and peroxidase (Apel and Hirt, 2004). Superoxide dismutase is able to rapidly convert superoxide radicals to hydrogen peroxide and dioxygen, and the generated hydrogen peroxide is then converted to water and dioxygen by peroxidase and catalase (Gechev et al., 2006).

The so-called nonenzyme system includes low molecular mass antioxidants, such as ascorbate, glutathione, carotenoids, and metallothioneins (MTs), which are known to remove hydroxyl radicals and singlet oxygen (Gechev et al., 2006). MTs are low molecular weight, Cys-rich proteins; plant MTs are grouped into four types (Robinson et al., 1993; Cobbett and Goldsborough, 2002). Some rice MTs have been shown to be ROS scavengers. For instance, recombinant MT2b protein has superoxide- and hydroxyl radical-scavenging activities (Wong et al., 2004), and reduction in MT2b expression caused accumulation of ROS, which triggers epidermal cell death in rice roots (Steffens and Sauter, 2009).

ROS can also serve as important signaling molecules that participate in a diverse range of plant processes, such as root hair development and elongation, leaf expansion, apical dominance, tracheary element maturation, trichome development, senescence, and response to biotic and abiotic stress (Rodríguez et al., 2002; Foreman et al., 2003; Overmyer et al., 2003; Sagi et al., 2004; Gapper and Dolan, 2006; Gechev et al., 2006; Miller et al., 2008). Emerging evidence indicates that some ROS species, such as the superoxide anion radical and hydrogen peroxide, are central regulators of cell death in plants (Moeder et al., 2002; Overmyer et al., 2003; Bouchez et al., 2007). The cellular level of ROS is thus tightly regulated by an efficient and elaborate system, which modulates the production and scavenging of ROS. However, how plants regulate ROS levels according to cellular needs at different developmental stages and within different cell types and organs remains poorly understood.

In this study, we show that MADS3 has a critical role in regulating rice late anther development via modulating ROS homeostasis. MADS3 is expressed during late anther development, in the tapetum and microspores. The rice mads3-4 mutant is male sterile, contains aborted anther walls, and shows disrupted pollen development due to oxidative stress. The abnormal increase in ROS level, peroxisome-like organelles, superoxide dismutase, and peroxidase activities in the mads3-4 mutant during later anther development is likely the result of the altered expression of genes involved in maintaining ROS level. Moreover, using chromatin immunoprecipitation (ChIP) and the electrophoretic mobility shift assay (EMSA), we demonstrated that MADS3 is able to bind to the promoter region of a metallothionein gene, MT-1-4b, which encodes a small Cys-rich and metal binding protein in a ROS-scavenging pathway. These findings together provide insight into the role of MADS3 in regulating male reproductive development and show that it acts, at least in part, through regulating ROS homeostasis.

RESULTS

Isolation of a mads3 Allele with Complete Male Sterility

We identified a complete male sterile mutant from a rice mutant library, which had been generated by treating seeds of rice cultivar 9522 (Oryza sativa ssp Japonica) with 60Co γ-ray radiation (Chen et al., 2006; Li et al., 2006, 2010b). This mutant exhibits normal vegetative and panicle development (Figures 1A and 1B), yet its flowers have ectopic lodicules in whorl 2 and homeotic transformation of stamens into lodicule-like organs in whorl 3 (Figures 1C to 1F; see Supplemental Table 1 online). The mutant anthers seem to form a normal tetrad at stage 8 of anther development (see Supplemental Figures 1A and 1B online) and have a yellow appearance with bicellular pollen grains at stage 11, just like wild-type flowers (Figures 1C and 1E; see Supplemental Figures 1B and 1E online). However, the anthers start to turn brown at stage 12 (Figures 1D and 1F). At stage 12, the mutant contains degenerated pollen grains that are unable to enter or complete the second mitosis (Figures 1G and 1H; see Supplemental Figures 1C and 1F online). Transverse section analysis of the mutant anthers revealed a brown color that is caused by degenerated tapetum and pollen grains (Figures 1I and 1J). Although its flowers occasionally develop more carpels caused by degenerated tapetum and pollen grains (Figures 1I and 1J). Although its flowers occasionally develop more carpels, it is female fertile, as shown by reciprocal cross analysis. When the mutant was backcrossed with a wild-type plant, all F1 plants were fertile and F2 plants showed a phenotype segregation of 3:1 (fertility: sterility = 239:81), suggesting that this phenotype is most likely caused by a single recessive mutation.

A map-based cloning approach was used to clone the gene responsible for the mutant phenotype. First, we mapped the mutation roughly to a genetic distance between two markers OS103 (25.4 centimorgans) and ZH104 (28.4 centimorgans) on chromosome 1 (Figure 2A). Subsequently, by screening 1183 individuals of the F2 mapping population with six pairs of PCR primers (see Supplemental Table 2 online), the mutation was narrowed down to a region of 18 kb between two markers, FQ120 and FQ121 (Figure 2A). Through repeated sequencing of the 18-kb region in the mutant, we confirmed a deletion of two sequential nucleotides (TA) in the fifth exon of MADS3, which causes a frame shift and premature translational termination in the 137th amino acid within the K domain (Figure 2B). Expression of the full-length cDNA fragment of MADS3 under the control of the cauliflower mosaic virus (CaMV) 35S promoter restored fertility to the homozygous mutant plants (see Supplemental Figure 2 online), confirming that MADS3 is responsible for the
mutant phenotype. This mutant was thus termed mads3-4 because three alleles of MADS3 had been identified previously (Yamaguchi et al., 2006). Similar to the previously described strong allele mads3-3, mads3-4 has altered floral organ identities, but overall the defects seem weaker than those in mads3-3 (Yamaguchi et al., 2006).

MADS3 Is Expressed Strongly in the Tapetum during Late Anther Development

Yamaguchi et al. (2006) showed that the expression of MADS3 is detectable in stamen primordia, when the lemma and palea primordia initiate, but disappears soon after the appearance of the stamen primordia. However, mads3-4 seems to show strong defects in late anther development (Figures 1C to 1F). Thus, we performed RT-PCR analysis, a β-glucuronidase (GUS) assay, and in situ hybridization to test whether MADS3 is expressed during late anther development. RT-PCR detected no obvious expression of MADS3 in vegetative organs, nonreproductive floral organs (i.e., glumes, lemma, and palea) (Figure 3A). By contrast, an increase in MADS3 expression was detected in anthers starting from stage 9, when young microspores form. The expression of MADS3 peaked at stage 11, when the bicellular pollen forms, and decreased at stage 12 (Figure 3A). In addition, the MADS3 transcript was weakly detected in the pistill at the heading stage (Figure 3A). Consistent with the RT-PCR data, transgenic rice plants expressing the GUS gene driven by the MADS3 promoter (~3.1 kb) exhibited GUS activity from stage 9 to 12 during anther development (Figure 3B). GUS activity was also observed in the stigma of the pistil (Figure 3C). Furthermore, transverse anther sections showed GUS expression in tapetal cells and microspores (Figures 3D and 3E). Using in situ hybridization analysis, we confirmed the expression of MADS3 in the tapetum and microspores from stage 9 to stage 12, with stronger expression in the tapetum (Figures 3F to 3H). Only background-level signals were observed in the control (Figure 3I). Taken together, these results suggested that MADS3 likely plays a role in the late stages of anther development.

mads3-4 Has Defects in Late Anther Development

To further characterize the role of MADS3 in anther development, we performed a detailed anther morphological analysis. The mads3-4 mutant undergoes normal meiosis, as revealed by 4',6-diamidino-2-phenylindole (DAPI) staining (see Supplemental Figures 1A and 1D online). Semithin section analysis revealed no obvious phenotypic alterations in mads3-4 at stage 9 (Figures 4A and 4E). At stage 10, wild-type anthers develop condensed and deeply stained tapetal cells and form vacuolated microspores (Figure 4B). Although mads3-4 had vacuolated microspores, the tapetum seemed less condensed and weakly stained (Figure 4F). At stage 11, wild-type anthers formed falcate-shaped pollen with degenerated tapetal cells (Figure 4C), whereas the mads3-4 anther wall layers, including the tapetum, became disordered, enlarged, and broken, and microspores appeared degraded and irregularly shaped (Figure 4G). At stage 12, the wild-type anther epidermis was collapsed, and the middle layer and endothecium were mostly degraded (Figure 4D). The mature pollen grains were deeply stained, indicating that starch, lipids, and other nutrient materials were stored (Figure 4D). By contrast, the mads3-4 anther still had the anther wall layers, including the endothecium, degenerated tapetal cells, microspores,
and abundant granular objects in the microspores and at the edge of the anther locule (Figure 4H). These results suggested that MADS3 is required for the proper development of anthers.

mads3-4 Anthers Display Features of Cells Undergoing Oxidative Stress

Transmission electron microscopy (TEM) analysis detected no obvious differences between wild-type and mads3-4 anthers at stage 9. Both types of plants contained tapetal layers with band-like structures, abundant plastids, endoplasmic reticulum, and mitochondria, a number of Ubisch bodies at the interface between tapetal cells and the microspore surface, and a nearly completed pollen exine configuration (see Supplemental Figure 3 online). At stage 10, wild-type tapetal cells contained vacuoles and plastids and mitochondria (Figures 5A and 5E). By contrast, mads3-4 tapetal cells had a greatly increased number of single-membrane, round-shaped structures filled with an electron dense granular matrix, which are characteristic of peroxisomes (Antonenkov et al., 2010), whereas plastids and mitochondria were barely found (Figures 5B, 5F, and 5I). At stage 11, wild-type tapetal cells were more condensed, forming a band-like structure (Figures 5C and 5G), and microspores were full of cytoplasm and numerous plastid-like organelles and mitochondria (Figure 5I). By contrast, mads3-4 tapetal cells continued their abnormal development, containing dark and less condensed cytoplasm with peroxisome-like organelles (Figures 5D and 5H). Microspores also seemed to be damaged, with numerous round and deeply stained structures (Figure 5J). At stage 12 in wild-type anthers, tapetal cells were hardly visible (Figure 5K), pollen grains were deep stained with a lot of starch, and a well-organized intine configuration had developed (Figure 5M). By contrast, the mads3-4 pollen was shrunk, irregularly shaped, and contained abundant electron-dense compounds instead of starch grains (Figures 5L and 5N). These cellular defects seemed to be characteristic of cells subjected to oxidative stress.

Oxidative stress is frequently caused by higher levels of ROS, which cause oxidative damage to cellular structures and molecules (Gechev et al., 2006). ROS is believed to be a signal that promotes programmed cell death (PCD) in plants and animals (Wiseman, 2006; Doyle et al., 2010). Tapetal PCD is initiated from stage 8 of anther development in rice (Li et al., 2006), but whether ROS plays a role in tapetal development remains unknown. To examine whether changes in ROS levels are associated with the mutant phenotype in mads3-4, we measured ROS levels in both wild-type and mads3-4 anthers using 2',7'-dichlorofluorescin (DCFH), which can be oxidized by ROS to the highly fluorescent 2',7'-dichlorofluorescein (DCFH), which can be oxidized by ROS to the highly fluorescent 2',7'-dichlorofluorescein (Simontacchi et al., 1993; Schopfer et al., 2001). Low ROS levels were observed in both wild-type and mads3-4 anthers before stage 8, while fluorescent levels increased in wild-type and mads3-4 anthers at stage 8 (Figure 6A) and reached a higher level at stage 9. From stage 10 to stage 11, the ROS level decreased to a very low level in wild-type anthers but remained high in mads3-4 anthers. At stage 12, mads3-4 anthers still showed a relatively higher level of ROS compared with the wild type (Figure 6A).

Superoxide anion, hydrogen peroxide, and hydroxyl radicals are the major ROS species in plants, and superoxide anion and hydrogen peroxide are known to play central roles in plant cell death (Moeder et al., 2002; Overmyer et al., 2003; Bouchez et al., 2007). Given that hydroxyl radicals are unstable and difficult to be detected directly in biological samples (Babbs et al., 1989; Tiedemann, 1997), we performed an analysis of superoxide anion and hydrogen peroxide in wild-type and mads3-4 anthers. The presence of superoxide anion was quantified using WST, a water-soluble tetrazolium salt reagent, which can be efficiently reduced by superoxide to a stable water-soluble formazan dye with high molar absorptivity (Peskin and Winterbourn, 2000; Tan and Berridge, 2000; Schopfer et al., 2001). In both wild-type and mads3-4 anthers, the level of superoxide anion was relatively low before stage 8, notably increased from stage 8 to stage 9, and reached the highest level at stage 9 (Figure 6B). From stage 10 to
stage 11, superoxide anion content, like that of total ROS, decreased in the wild-type anthers but remained high in mads3-4 (Figure 6B). Furthermore, we analyzed the cellular localization of superoxide anion in rice anthers using nitroblue tetrazolium (NBT), which can be oxidized by superoxide anion to form a dark blue formazan precipitation (Liszkay et al., 2004). This approach obtained results consistent with WST measurements with respect to changes in superoxide anion levels in both wild-type and mads3-4 anthers (Figures 6C and 6D). Finally, transverse section analysis revealed the superoxide anion staining signals to be localized within the tapetal cells and microspores (Figures 6E and 6F). These results suggested that the dynamics of superoxide anion levels in rice anther development may play an essential role in proper microspore development and that the mads3-4 mutation may have disturbed the equilibrium between production and scavenging of superoxide molecules, thereby causing defective late anther development.

Measurement of hydrogen peroxide and staining of these molecules with 3,5,3’,5’-tetramethylbenzidine (TMB) showed no obvious differences in changes in the level of hydrogen peroxide between the wild type and mads3-4 (see Supplemental Figure 4 online), suggesting that the mads3-4 mutation has little effect on the hydrogen peroxide content in rice anther development.

Peroxisomes are subcellular organelles that not only participate in the production of ROS, leading to deleterious cellular effects, cell death, and diverse pathological conditions, but also house numerous ROS scavenging enzymes in both plants and animals (Lopez-Huertas et al., 2000; del Río et al., 2002). These organelles contain antioxidant molecules, such as ascorbate and glutathione, as well as a battery of antioxidant enzymes, including catalase, superoxide dismutase, peroxidase, glutathione...
reductase, and monohydroascorbate reductase (Schrader and Fahimi, 2006). Higher ROS levels usually induce increases in the number of peroxisomes (Schrader and Fahimi, 2006). Consistent with the occurrence of numerous peroxisome-like structures in mads3-4, higher activities of superoxide dismutase and peroxidase were also detected in the mutant anthers from stage 10 to stage 12 (see Supplemental Figure 5 online).

Transcriptome Analysis of Wild-Type and mads3-4 Anthers

To identify downstream targets of MADS3, we compared genome-wide mRNA levels in wild-type and mads3-4 anthers at stage 9, using microarray analysis with an Agilent 4 x 44K–oligonucleotide DNA chip. To assess the reproducibility of microarray signals, we performed two biological replicates for both the wild type and mads3-4. The empirical Bayes method was employed to analyze the data (Smyth, 2004). A false discovery (FDR) cutoff of 0.5% was used for the initial filtering of candidate genes, followed by a secondary selection of at least 2-fold changes in gene expression. The predicted functions of genes were obtained from the Institute for Genomic Research (http://tigrblast.tigr.org/euk-blast). A total of 1728 genes were found to show at least 2-fold expression changes in mads3-4 anthers compared with the wild type (see Supplemental Data Set 1 online). Among them, 1217 genes were previously found to be expressed in rice microspore/pollen and tapetum by laser microdissection (LM) and microarray analyses (Suwabe et al., 2008). Out of the 1217 genes, 630 were downregulated and 587 were upregulated (see Supplemental Data Set 2 online).

The 1728 genes with altered expression levels in mads3-4 were functionally grouped into four categories: (1) information storage and processing (53 down- and 62 upregulated), (2) cellular processes and signaling (166 down- and 176 upregulated), (3) metabolism (288 down- and 201 upregulated), and (4) unknown functions (388 down and 394 upregulated) (see Supplemental Table 3 online). To further characterize these genes, we employed the Web-based DAVID 6.7 program (http://david.abcc.ncifcrf.gov/) to search for differential distributions of gene ontology (GO) and putative biological functions of these genes with altered expression in the mads3-4 mutant (Huang et al., 2009). A high proportion of the GO terms were associated with the polysaccharide metabolic/catabolic process, oxidation reduction/stress response, lipid transport/localization, sexual reproduction, cell wall organization/modification/macromolecule metabolic process, and carbohydrate catabolic process (see Supplemental Table 4 online).

Besides MADS3, which was downregulated 5.66-fold, 95 genes that encode putative transcriptional factors also showed altered gene expression. These include MADS box proteins MADS47, MADS68, and MADS98, a basic helix-loop-helix protein (Os04g0599300), and MYB proteins Os05g0490600, Os01g0709000, Os08g0433400, and Os04g0470600 (see Supplemental Table 5 online). Os01g0709000, which was downregulated 2.87-fold in the mutant, is a close homolog to the Pinus taeda gene, MYB1, which encodes a putative transcription factor involved in the regulation of phenylpropanoid metabolism and secondary cell wall biogenesis (Bomal et al., 2008).

Phytohormones, such as gibberellic acid (GA), jasmonic acid, and auxin, play crucial roles in many development processes, including anther and pollen development (Ishiguro et al., 2001; Feng et al., 2006; Aya et al., 2009). In mads3-4 anthers, a group of genes involved in phytohormone signaling and metabolism exhibited altered expression. Among these were genes putatively encoding the gibberellin receptor GID1L2, gibberellin 2-oxidase, gibberellin 3-β-dioxygenase 2-2, auxin-induced protein 5NG4, auxin response factor (Os04g0644400), and an auxin efflux carrier component (Os01g0643300) (see Supplemental Table 6 online).

The expression of genes known to be associated with early rice anther development, such as MULTIPLE SPOROCYTE1 (MSP1) (Nonomura et al., 2003; Wang et al., 2006), HOMOLOGOUS PAIRING ABERRATION IN RICE MEIOSIS2 (PAIR2)
MADS3 Regulates Anther Development

The wild-type tapetum at stage 10 with abundant peroxisomes. (A) The wild-type tapetum at stage 10. (B) The mads3-4 tapetum at stage 10 with abundant peroxisomes. (C) is a magnified picture of a peroxisome-like structure from the mutant. (D) and (H) The wild-type (G) and mads3-4 (H) tapetum at stage 11. (I) and (J) The wild-type (I) and mads3-4 (J) microspore at stage 11. (K) and (L) Stage 12 anthers from wild-type (K) and mads3-4 (L) plants. (M) The wild-type mature pollen at stage 12, showing lots of starch granules. (N) The mads3-4 microspore at stage 12, showing the accumulation of dark, electron-dense compounds.

E, epidermis; En, endothecium; Ex, exine; In, intine; Mp, Mature pollen, Msp, microspore; Mt, mitochondrion; Pe, peroxisome; Pl, plastid; St, starch; T, tapetum; V, vacuole; Ub, UBisch body. Bars = 2 μm in (A) to (D), 1 μm in (E) to (H) and (K) to (N), 10 μm in (I) and (J), and 0.5 μm in (F).

Figure 5. Transmission Electron Micrographs of Wild-Type and mads3-4 Anthers from Stages 10 to 12.

(A) to (D) Anthers were obtained from stage 10 (A and B) and 11 (C) and (D) plants. (A) and (C) are the wild-type, and (B) and (D) are from the mutant.

(E) The wild-type tapetum at stage 10.

(F) The mads3-4 tapetum at stage 10 with abundant peroxisomes. (I) is a magnified picture of a peroxisome-like structure from the mutant.

(G) and (H) The wild-type (G) and mads3-4 (H) tapetum at stage 11.

(I) and (J) The wild-type (I) and mads3-4 (J) microspore at stage 11.

(K) and (L) Stage 12 anthers from wild-type (K) and mads3-4 (L) plants.

(M) The wild-type mature pollen at stage 12, showing lots of starch granules.

(N) The mads3-4 microspore at stage 12, showing the accumulation of dark, electron-dense compounds.

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(Nonomura et al., 2004), GAMYB (Kaneko et al., 2004; Aya et al., 2009; Liu et al., 2010), Wax-deficient anther1 (Wda1) (Jung et al., 2006), CP1 (Lee et al., 2004), and RAFITIN (Wang et al., 2003), was not significantly changed in mads3-4 (see Supplemental Table 7 online), suggesting that these genes may act earlier than MADS3 in regulating anther development. Conversely, several genes related to late anther development, such as MST8, which encodes a monosaccharide transporter crucial for the sugar unloading pathway, and INV4, which encodes a cell wall invertase required for hexose production and starch synthesis for pollen maturation (Oliver et al., 2005, 2007), were significantly downregulated (8.88- and 9.99-fold, respectively; see Supplemental Table 7 online). A reduction in the expression of MST8 and INV4 was shown to be related to male sterility in rice (Oliver et al., 2005, 2007). In mads3-4 anthers, we also observed a 2.36-fold upregulation of Tapetum Degeneration Retardation (TDR), which is required for tapetal development and degeneration in rice, with its mutants showing delayed tapetal PCD (Li et al., 2006). Moreover, the expression of C6, which encodes a small mobile lipid transfer protein required for postmeiotic anther development in rice (Zhang et al., 2010a), was upregulated 2.36-fold in mads3-4 (see Supplemental Table 7 online). The NADPH-dependent HC-toxin reductase gene, Os07g0602000, showed a 17.9-fold reduction in expression. This protein likely plays a role in increasing the level of the NAD(P)H pool and preventing the host from induced cell death (Hayashi et al., 2005). Furthermore, the expression of Os08g0515900, which encodes a putative dihydroflavonol 4-reductase essential for male fertility in Arabidopsis (Tang et al., 2009), was upregulated 21.93-fold in mads3-4. Finally, four male sterility-related genes also had changed expression, including, Os04g0354600 (7.47-fold downregulated), Os04g0353600 (5.16-fold downregulated), Os09g0567500 (3.29-fold upregulated), and Os03g0167600 (4.46-fold upregulated). These results together support the view that MADS3 plays a key role in late anther development.

Plants have a large number of P450 proteins with diverse functions in biosynthetic and detoxication pathways (Schuler and Werck-Reichhart, 2003). For example, many P450s are involved in the synthesis of lignins, UV protectants, pigments, defense compounds, fatty acids, hormones, and signaling molecules, as well as in the breakdown of compounds, including those that are toxic (Schuler and Werck-Reichhart, 2003). In mads3-4 anthers, we detected changes in gene expression for 42 genes encoding P450s: 29 were downregulated and 13 were upregulated (see Supplemental Table 8 online). Among them, CYP704B2, a gene essential to rice postmeiotic anther development, was upregulated 4.29-fold. The CYP704B2 gene is expressed in the tapetum and microspores, its loss-of-function mutant, cyp704B2, is male sterile, and recombinant CYP704B2 expressed in yeast is able to catalyze the production of ω-hydroxylated fatty acids with 16 and 18 carbon chains (Li et al., 2010b). Similarly, another rice male sterile gene, CYP703A3 (Aya et al., 2009), was upregulated 9.58-fold in mads3-4. Furthermore, Os03g0371000, which encodes a putative cytochrome P450 with the ability to cleave the oxidative ring to convert loganin into secolagin (Irmler et al., 2000), was downregulated 7.41-fold.

We verified the microarray expression data by quantitative RT-PCR (qRT-PCR), using independently generated RNA samples and focusing on nine differentially expressed genes. Genes subjected to validation were MADS3, MT-like protein (Os12g0568500), two peroxidases (Os05g0499300 and Os11g012400), three transcription factors (Os03g0186600, Os03g0167600, and Os01g0972800), two cytochrome P450s (Os02g0596300 and Os04g0469800) (see Supplemental Figure 6 online). Proteins encoded by most of these genes have
been shown or predicted to be involved in ROS homeostasis. Close correlations were found between changes detected by the microarray analysis and those obtained with qRT-PCR, confirming the reliability of the microarray results.

**Expression Changes in Genes Involved in ROS Homeostasis in mads3-4 Anthers**

In agreement with the abnormally high levels of ROS in mads3-4 anthers, we observed changes in the expression of a number of genes related to ROS homeostasis in the mutant. In plant cells, ROS are mainly produced by enhancing the enzymatic activity of plasma membrane–bound NADPH oxidases, pH-dependent cell wall–bound peroxidases, and amine oxidases in the apoplast (Mittler et al., 2004). Genes involved in the ROS-producing pathway (e.g., Os01g0360200, Os12g0541300, Os01g0835500, Os09g0438000, Os01g0734200, and Os05g0528000), which encode putative respiratory burst oxidases (Mittler et al., 2004; Gechev et al., 2006), had minimal expression changes in mads3-4 anthers. On the other hand, genes encoding putative peroxidases (20 upregulated; seven downregulated) and amine oxidases (downregulated, such as Os09g0368500; upregulated, such as Os04g0671300) showed at least a 2-fold change in expression in mads3-4 anthers (Table 1). Because no detailed annotation of peroxidases is available in the rice genome, we could not distinguish cell wall–bound peroxidases involved in ROS production from other peroxidases required for ROS removal.

The balance between the activity of superoxide dismutase, catalase, and/or peroxidase and sequestering of metal ions by metal binding proteins is thought to be crucial in preventing the formation of the highly toxic ROS (Mittler et al., 2004). Notably, a total of 49 genes (27 upregulated; 22 downregulated) among the 1728 genes with altered expression levels in mads3-4 were annotated as being involved in ROS scavenging. The gene products include glutathione S-transferase (four downregulated and five upregulated), metallothionein (five downregulated), monodehydroascorbate reductase (two downregulated and one upregulated), glutaredoxin (Os02g0512400, upregulated; Os01g0235900, downregulated), peroxiredoxin (Os07g0638300, downregulated), and thioredoxin (Os01g0168200, downregulated) (Table 1). These data suggested that the mads3-4 mutation affects the expression of genes whose protein products may be components in the network of ROS homeostasis during anther development.

**MADS3 Is Associated with the Promoter of a ROS-Scavenging Gene**

MADS box proteins have been shown to regulate gene expression by binding to a consensus core element called the CArG-box \[5'-CC(A/T)4NNGG-3'\] (Riechmann et al., 1996). To further reveal the regulatory role of MADS3 during rice anther development, putative MADS binding sequences (CArG-box) were identified using the tools described in plant CARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/; Thijs et al., 2002). A total of 189 of the 895 downregulated genes and 169 of the 833 upregulated genes (see Supplemental Data Set 1 online) were found to contain at least one CArG-box within the 1-kb upstream region (see Supplemental Data Sets 3 and 4 online), suggesting that MADS3 may directly regulate a number of target genes during late anther development in rice. These genes encode factors with putative roles in signal transduction, hormone metabolism, and stress response; notably, four of the 189 genes (two MTs and two peroxidases), and 10 of the 169 genes (six peroxidases, three glutathione S-transferases, and one thioredoxin) are related to the ROS-scavenging system (Table 1). To confirm the binding ability of MADS3 to these promoters, we selected three genes that are downregulated in mads3-4: Os12g0571100 (encoding a metallothionein, MT-1-4b, 7.37-fold downregulation), Os12g0150200 (encoding P450, 7.37-fold downregulation), and Os12g0734200 (encoding a peroxidase, 7.37-fold downregulation).

![Figure 6. Analyses of ROS and Superoxide Anion levels in Wild-Type and mads3-4 Anthers.](image-url)
| Locus                | Annotation                                      | Fold Change | Direction | Position (Relation to TSS) | Box Sequence     |
|---------------------|------------------------------------------------|-------------|-----------|----------------------------|------------------|
| **ROS-producing (2)** |                                               |             |           |                            |                  |
| Os09g0368500       | Amine oxidase                                  | 2.09        |           |                            |                  |
| Os04g0671300       | Amine oxidase                                  | 2.04        |           |                            |                  |
| **ROS-scavenging (23)** |                                              |             |           |                            |                  |
| **Downregulated (15)** |                                             |             |           |                            |                  |
| Os03g0288000       | MT, putative, expressed                        | −8.83       | −         | −138 to 129                | CCAATTCTGG       |
| Os03g0288000       | MT, putative, expressed                        | −8.83       | −         | −425 to 416                | CCAATTCTGG       |
| Os12g0568500       | MT, putative, expressed                        | −4.86       |           |                            |                  |
| Os12g0571100       | MT, putative, expressed                        | −7.37       | +         | −879 to 870                | CCAATTGGGG       |
| Os12g0571000       | MT, putative, expressed                        | −4.58       |           |                            |                  |
| Os09g0454500       | Monodehydroascorbate reductase                 | −4.33       |           |                            |                  |
| Os07g0638300       | Peroxiredoxin, putative                       | −3.19       |           |                            |                  |
| Os08g0423500       | Monodehydroascorbate reductase                 | −3.04       |           |                            |                  |
| Os01g0353400       | Glutathione S-transferase, putative, expressed | 2.46        |           |                            |                  |
| Os10g0525500       | Glutathione S-transferase, N-terminal          | −2.41       |           |                            |                  |
| Os12g0567800       | MT, putative, expressed                        | −2.34       |           |                            |                  |
| Os01g0370200       | Glutathione S-transferase, putative, expressed | 2.25        |           |                            |                  |
| Os01g0168200       | Thioredoxin, putative                          | 2.36        | +         | −653 to 644                | CCAATTGGAGG      |
| Os10g0395400       | Glutathione S-transferase, putative, expressed | 2.42        |           |                            |                  |
| Os02g0512400       | Glutaredoxin subgroup III                     | 2.49        |           |                            |                  |
| Os03g0134900       | Glutathione S-transferase, putative, expressed | 2.86        |           |                            |                  |
| Os08g0550500       | Glutathione S-transferase, C-terminal          | 3.58        | −         | −456 to 447                | CCAATAGGG        |
| **Upregulated (8)** |                                               |             |           |                            |                  |
| Os02g0533300       | Monodehydroascorbate reductase                 | 2.14        |           |                            |                  |
| Os01g0370200       | Glutathione S-transferase, putative, expressed | 2.25        |           |                            |                  |
| Os01g0168200       | Thioredoxin, putative                          | 2.36        | +         | −653 to 644                | CCAATTGGAGG      |
| Os10g0395400       | Glutathione S-transferase, putative, expressed | 2.42        |           |                            |                  |
| Os02g0512400       | Glutaredoxin subgroup III                     | 2.49        |           |                            |                  |
| Os03g0134900       | Glutathione S-transferase, putative, expressed | 2.86        |           |                            |                  |
| Os08g0543800       | Glutathione S-transferase, putative, expressed | 3.01        | +         | −519 to 510                | CCAATACGGG       |
| Os08g0550500       | Glutathione S-transferase, C-terminal          | 3.58        | −         | −456 to 447                | CCAATAGGG        |
| **Peroxidase (ROS-producing and ROS-scavenging) (26)** |                               |             |           |                            |                  |
| **Downregulated (7)** |                                             |             |           |                            |                  |
| Os05g0499300       | Peroxidase precursor, putative, expressed      | −15.45      |           |                            |                  |
| Os12g0112000       | Peroxidase precursor, putative, expressed      | −12.54      | −         | −336 to 327                | CCAATTGGG        |
| Os11g0112400       | Peroxidase precursor, putative, expressed      | −6.41       |           |                            |                  |
| Os07g0639400       | Peroxidase precursor, putative, expressed      | −2.97       |           |                            |                  |
| Os06g0681600       | Peroxidase precursor, putative, expressed      | −2.56       |           |                            |                  |
| Os01g0327400       | Peroxidase precursor, putative, expressed      | −2.13       | +         | −621 to 612                | CCAATTAGG        |
| Os01g0327400       | Peroxidase precursor, putative, expressed      | −2.13       | −         | −622 to 613                | CCAATAGG         |
| **Upregulated (19)** |                                             |             |           |                            |                  |
| Os03g0368000       | Peroxidase precursor, putative, expressed      | 2.02        |           |                            |                  |
| Os03g0368300       | Peroxidase precursor, putative, expressed      | 2.03        | +         | −814 to 805                | CCAATTAAGG       |
| Os02g0236600       | Peroxidase precursor, putative, expressed      | 2.35        |           |                            |                  |
| Os03g0235000       | Peroxidase precursor, putative, expressed      | 2.37        | −         | −186 to 177                | CCAATACGGG       |
| Os10g0536700       | Peroxidase precursor, putative, expressed      | 2.38        | −         | −13-4                      | CCAATTGGG        |
| Os10g0536700       | Peroxidase precursor, putative, expressed      | 2.38        | −         | −172 to 163                | CCAATTGGG        |
| Os03g0368900       | Peroxidase precursor, putative, expressed      | 2.44        | +         | −239 to 230                | CCAATATAGG       |
| Os01g0205900       | Peroxidase precursor, putative, expressed      | 2.66        |           |                            |                  |
| Os01g0326900       | Peroxidase precursor, putative, expressed      | 2.67        |           |                            |                  |
| Os04g0465100       | Peroxidase precursor, putative, expressed      | 2.73        | −         |                            |                  |
| Os11g0210100       | Peroxidase precursor, putative, expressed      | 2.82        |           |                            |                  |
| Os12g0191500       | Peroxidase precursor, putative, expressed      | 2.96        |           |                            |                  |
| Os04g0698000       | Peroxidase precursor, putative, expressed      | 3.05        |           |                            |                  |

(Continued)
downregulation), and Os01g0327100 (encoding a peroxidase, 12.54-fold downregulation), for ChIP experiments. First, we developed rabbit polyclonal antibodies against a bacterially expressed recombinant protein that contains a unique region at the C terminus of rice MADS3 (see Methods). Specificity of the antibody was confirmed using immunoblot analysis. We detected a band of ~35 kD, which is slightly larger than the expected size, in protein isolated from the wild type, implying possible protein modification of MADS3 in vivo. By contrast, no obvious signal was observed in the mads3-4 mutant because the C-terminal region, against which the antibody was raised, was absent in the truncated MADS3 protein in the mutant (see Supplemental Figure 7 online). ChIP-qPCR analysis showed specific enrichment of the affinity-purified MADS3 antibody on the promoter region of MT-1-4b, which contains the CARG-box, but not on the promoters of Os12g0150200 and Os01g0327100, both of which also contain the predicted CARG-box (Figure 7A).

To further confirm the ChIP-qPCR data, we performed EMSA using the recombinant MADS3 protein and a DNA fragment containing the MT-1-4b promoter region (~980 to ~837 bp upstream of ATG). A supershifted band was observed when the MT-1-4b promoter region was incubated with MADS3 (Figure 7B, lane 2), while no supershifted signal was observed in the control sample containing purified bacterial protein expressed from the empty pET-32a plasmid (Figure 7B, lane 1). Moreover, we observed decreased signals when a 25-, 50-, or 100-fold molar excess of unlabeled MT-1-4b probe was added to the EMSA reaction as competitors (Figure 7B, lanes 3 to 5). This result suggested that MADS3 directly regulates MT-1-4b expression. Consistent with the ChIP-qPCR and mobility shift results, qRT-PCR analysis revealed strong expression of MT-1-4b in wild-type anthers from stage 9 to stage 12 (Figure 7C), and MT-1-4b transcripts were previously found in rice microspore/pollen and tapetum using LM samples and microarray analysis (Suwabe et al., 2008). In addition, MT-1-4b is highly expressed in roots (see Supplemental Figure 8 online), which is in agreement with the previous report by Zhou et al. (2006).

A previous study demonstrated superoxide- and hydroxyl radical-scavenging activities for the recombinant rice MT2b protein, a homolog of MT-1-4b (Wong et al., 2004). To test whether MT-1-4b has similar activities, we determined the hydroxyl radical-scavenging activity of recombinant MT-1-4b in vitro. In this method, a reaction between salicylate and hydroxyl radicals leads to the formation of dihydroxybenzoic acid, which can be suppressed by a scavenger in a dose-dependent manner (Smirnoff and Cumbes, 1989). Addition of recombinant MT-1-4b at concentrations of 10 to 50 μM effectively protected salicylate from attack by hydroxyl radicals (Figure 7D), suggesting that MT-1-4b is a potent scavenger of hydroxyl radicals. In addition, recombinant MT-1-4b was shown to have superoxide scavenging activity, whereby it was able to scavenge superoxide anion by inhibiting the oxidation of WST in vitro (Figure 7E).

### Silencing of MT-1-4b Causes Defective Pollen Development

To understand the biological role of MT-1-4b in rice anther development, we adopted an artificial microRNA (amiRNA) approach to reduce the expression of the MT-1-4b gene. Two 21-nucleotide amiRNA sequences, which target a MT-1-4b-specific region, were designed by the customized version of the Web MicroRNA Designer platform and used to replace the sequence of endogenous microRNA and microRNA* of osa-MIR528 (Schwab et al., 2006; Ossowski et al., 2008; Warthmann et al., 2008). The final construct was transformed into calli induced from wild-type rice mature seeds, and a total of 11 T0 transformant lines expressing MT-1-4b-amiRNA were obtained. None of the transgenic plants displayed any obvious growth phenotypes during vegetative or floral organ development. However, using I2-KI staining, seven lines were found to contain reduced mature pollen grain formation, suggesting defective anther development and pollen formation in these transgenic plants. Three of the seven lines (lines 1, 3, and 4; called MT-1-4b-amiRNA-S lines) showed a relatively strong phenotype by having a greatly reduced number of deeply stained pollen grains (Figures 8A and 8B), whereas the other four lines (called MT-1-4b-amiRNA-W lines) showed relatively weak phenotypes (Figures 8A and 8C). In addition, qRT-PCR analysis of stage 9 anthers revealed an overall correlation between the level of MT-1-4b expression and the severity of the phenotype in pollen maturation (Figure 8D).

To further determine whether MT-1-4b functions as a ROS scavenger in vivo, we examined ROS levels in the MT-1-4b-amiRNA lines. At stages 10 and 11, the MT-1-4b-amiRNA-S lines displayed relatively high levels of ROS compared with the wild-type anthers, whereas at stage 12, the wild-type and amiRNA anthers showed equally high levels of ROS, suggesting that MT-1-4b may be crucial for ROS homeostasis (Figure 8E). Moreover, abnormal accumulation of superoxide anion was observed in the anthers of MT-1-4b-amiRNA-S lines at stages 10 and 11 (Figures 8F and 8G). Additional cytological analysis showed the abnormal
accumulation of superoxide anion signals in the tapetal layer and microspores of MT-1-4b-amiRNA-S lines (Figures 8H and 8I).

DISCUSSION

Rice MADS3 Regulates Late Anther Development and Pollen Formation

Previous investigations showed that the rice floral homeotic C-class gene, MADS3, is expressed in stamen and carpel primordia during early floral development, and that the protein it encodes is crucial for stamen specification (Kang et al., 1998; Yamaguchi et al., 2006). In this study, we characterized a mutant allele of MADS3 (mads3-4), further analyzed the expression pattern of this gene, and revealed an important role for MADS3 in regulating postmeiotic anther development in rice.

Of the previously reported MADS3 alleles, both mads3-1 and mads3-2 are in the Nipponbare (a japonica cultivar) background and contain Tos17 retrotransposon insertions in the gene. mads3-1 is a weak allele carrying an insertion in the C-terminal region of the gene and showing no defective floral organs, whereas mads3-2 is an intermediate allele with a mutation at the C terminus of the K-domain and reduced expression of the gene, which resulted in mild transformation of stamens into lodicules (Yamaguchi et al., 2006). mads3-3 is a strong allele in the Dongjin (a japonica cultivar) background. It contains a T-DNA insertion in the second intron of the gene, which leads to no detectable MADS3 transcript and homeotic transformation of nearly all stamens in whorl 3 into lodicule-like organs (Yamaguchi et al., 2006). mads3-4, which results in a mutation in the middle region of MADS3, seems to be another intermediate mutant with reduced expression of MADS3 (see Supplemental Figure 6 online). In contrast with mads3-3, the allele introduced in this study is in the 9522 (a japonica cultivar) background and is male sterile. These phenotypic differences between the mads3 alleles may be caused by differences in genetic background and/or the nature of the mutations in MADS3. Moreover, we cannot...
exclude the possibility that mads3-2 also has defective anther development because in the previous report on mads3-2, the authors did not perform a detailed observation of anther development (Yamaguchi et al., 2006).

The finding that MADS3 is important for postmeiotic anther development in rice is consistent with the expression pattern of MADS3 in the tapetum and microspores from stage 9 to stage 12 (Figure 9). Furthermore, transcriptome analyses demonstrated that MADS3 affects the expression of 1728 genes, many of which are associated with cellular processes and signaling, carbohydrate transport and metabolism, secondary metabolite biosynthesis, detoxification, and transcriptional regulation. Among these 1728 genes, some genes, such as TDR (Li et al., 2006; Zhang et al., 2008), CYP703A3 (Aya et al., 2009), CYP704B2 (Li et al., 2010b), C6 (Zhang et al., 2010a), MST8, and INV4 (Oliver et al., 2005, 2007), have been shown to be involved in anther development, suggesting that MADS3 may regulate anther development by affecting the expression of these genes. Unlike MADS3, the Arabidopsis C-class gene AG is expressed from anther initiation to anther maturation in sporogenous cells, the connective, anther walls and the filament, and no AG expression was detected in pollen grains (Bowman et al., 1991). The distinct spatial and temporal expression patterns of AG and MADS3 suggest that these two proteins have diversified functions in anther development. AG plays a key role in regulating early anther development by activating the expression of the MADS box gene SPL/NZZ (Ito et al., 2004) and in late anther development and anther dehiscence by regulating JA synthesis (Ito et al., 2007) (Figure 9). Besides MADS3 and AG, there are other MADS box genes reported to be expressed in anther development in rice (Hobo et al., 2008), maize (Schreiber et al., 2004), Arabidopsis (Alvarez-Buylla et al., 2000), and Antirrhinum (Zachgo et al., 1997), indicating important roles for MADS box genes in plant anther development and pollen formation.

**MADS3 Regulates ROS Homeostasis during Anther Development**

Cell death is involved in various plant development events, from seed setting to gametophyte development. The tapetum, the innermost sporophytic layer within the anther wall, undergoes cell degeneration to supply nutrients for pollen development.

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**Figure 8. Analyses of the MT-1-4b-amiRNA Lines.**

(A) to (C) I2-KI–stained pollen grains of the wild type (WT) and MT-1-4b-amiRNA lines at stage 12. The percentage of deeply stained pollen grains among 400 pollen grains from the wild type was 98.8% ± 2.44%, that for the three MT-1-4b-amiRNA-S lines (MT-1-4b-amiRNA-1, 3, and 4) was 51.52, 57.81, and 51.23%, respectively (average percentage = 53.50% ± 3.73%), and that from the four MT-1-4b-amiRNA-W lines (lines 2, 5, 6, and 7) was 72.5, 77.8, 67.03, and 71.4%, respectively (average percentage = 72.18% ± 4.43%) (n = 3). Pollen grains from the wild type (A), line 1 (B), and line 2 (C) are shown.

(D) qRT-PCR analysis of MT-1-4b transcripts in MT-1-4b-amiRNA lines (lines 1 to 7, indicated by 1 to 7, respectively) at stage 9 of anther development. Data are given as means ± SE (n = 3).

(E) and (F) Comparison of ROS (E) and O2⁻ (F) levels in wild-type and MT-1-4b-amiRNA lines. WST could be reduced by O2⁻ to a stable water-soluble formazan with high absorbance at 450 nm. Data are means ± SE (n = 3 for the wild type and MT-1-4b-amiRNA-S; n = 4 for MT-1-4b-amiRNA-W).

(G) Comparison of O2⁻ production in wild-type and MT-1-4b-amiRNA anthers from stage 9 to 12 using NBT staining.

(H) and (I) O2⁻ signals detected in the tapetum and microspores in stage 10 wild-type and MT-1-4b-amiRNA anthers. T, tapetum; Msp, microspore; BS8, anthers before stage 8; S8-S12, anthers at stage 8 to 12. Bars = 20 μm in (A) to (C), 50 μm in (G), and 10 μm in (H) and (I).
stress to Arabidopsis causes the induction of cytosolic but not chloroplastic defense enzymes (Karpinski et al., 1997, 1999; Pnueli et al., 2003). Moreover, to date, there are at least three different enzymes of plant ROS-removal pathways found to be dual targeted to chloroplasts and mitochondria, implying the close coordination of these two types of organelles in defense responses (Creissen et al., 1995; Obara et al., 2002; Chew et al., 2003).

In rice, however, functional aspects of ROS-related genes remain less understood. We observed expression changes in 51 genes with putative functions in the ROS network in mads3-4 anthers (Table 1), suggesting that MADS3 may play a role in adjusting ROS homeostasis during anther development. Consistent with the gene expression profile analysis, mads3-4 has high ROS accumulation in the anther, starting from stage 9, when young microspores form. Furthermore, mads3-4 tapetal cells at stages 10 to 12 display features characteristic of those associated with oxidative stress, including less cytoplasmic condensation, fewer mitochondria, and more peroxisome-like organelles. Increased ROS might be associated with the decreased expression of ROS-scavenging genes, including those encoding MTs and peroxidase. The increased number of peroxisomes in mads3-4 tapetal cells and elevated peroxisome-associated enzyme activities, such as superoxide dismutase and peroxidase, may in part result from higher ROS levels during late anther development. Our in vivo and in vitro analyses suggested that MADS3 may directly regulate a ROS-removal gene, MT-1-4b. Consistent with these results, an LM-mediated microarray analysis revealed the expression of MT-1-4b in the tapetum and microspores during late anther development (Hobo et al., 2008), an expression pattern that is similar to that of MADS3 in rice anther development. The biological importance of the regulation of MT-1-4b by MADS3 is further supported by the biochemical activity of the recombinant MT-1-4b protein in removing the superoxide anion and hydroxyl radical (Figure 7). Moreover, knockdown of MT-1-4b led to reduced pollen fertility and abnormal accumulation of superoxide anion in the transgenic lines, which is similar to the phenotypes in mads3-4 during late anther development. Lastly, we found changes in the expression of a few other genes encoding proteins putatively involved in ROS removal in mads3-4, suggesting that these genes may also encode components of a ROS-scavenging network that modulates late anther development.

In conclusion, we have demonstrated a role of the floral homeotic C-class gene MADS3 in regulating late anther development and pollen formation. We have shown that this protein functions, at least in part, by regulating ROS homeostasis in anthers through the ROS-removal protein MT-1-4b. Although it remains to be seen whether MADS3 functions in late anther development through other cellular targets, our study sheds light on the molecular mechanisms underlying the genetic control of postmeiotic tapetum degeneration and development.

METHODS

Mutant Material and Growth Conditions
The F2 mapping population was generated from a cross between mads3-4 (japonica) and GuangLuAi4 (indica) rice (Oryza sativa). In the
F2 population, male sterile plants were selected for gene mapping. All plants were grown in the paddy field of Shanghai Jiao Tong University.

**Characterization of Mutant Phenotypes**

Plants were photographed with a Nikon E995 digital camera and a Motic K400 dissecting microscope. Observation of anther development by semithin sections and TEM was performed as described by Li et al. (2006). DAPI staining was performed according to Han et al. (2006).

**Histochemical GUS Assays**

Histochemical GUS staining was performed as described by Jefferson et al. (1987) and Dai et al. (1996). Freshly collected samples from transgenic plants expressing the promoter-GUS fusion were put in staining solution under vacuum conditions for 15 min, followed by 70% ethanol for 1 h, before incubation at 37°C overnight. After staining, samples were rinsed in 70% ethanol for 1 h before a photo was taken with a Nikon E995 digital camera. Staining solution contained 0.02 M 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.1 M NaH₂PO₄, 0.25 M EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1.0% (v/v) Triton X-100, pH 7.0.

**RT-PCR and qRT-PCR**

Total RNA was isolated from rice tissues (e.g., root, culm, leaf, and lemma/palea) at stage 9 of anther development as well as anthers at different stages from the wild type and mads3-4. The stages of anthers were classified according to spikelet length (Feng et al., 2001; Zhang and Wilson, 2009). First-strand cDNA was synthesized with the ReverTra Ace-a-First Strand cDNA synthesis kit (Ferment). RT-PCR analysis was performed using SYBR Premix EX Taq (Takara) on a Rotor-Gene G3000A detection system (Corbett Research). Observation of anther development by a Motic 528 digital camera. Staining solution was prepared in 0.02 M 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, 0.1 M NaH₂PO₄, 0.25 M EDTA, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1.0% (v/v) Triton X-100, pH 7.0.

**Complementation of the mads3-4 Mutant**

For functional complementation of the rice mads3-4 mutant, the MADS3 fragment was amplified with primers MADS3-F and MADS3-R from the cDNA clone (RGRC-NIAS, Japan; http://www.rgrc.dna.affrc.go.jp/stock.html) (see Supplemental Table 2 online) and inserted into a binary vector pHB (kindly provided by Hongquan Yang), which contains a double CaMV 35S promoter and a hygroycin resistance marker, to make pHB-MADS3. Calli induced from homogenous mads3-4 young panicles were used for transformation with Agrobacterium tumefaciens EHA105 carrying the pHB-MADS3 plasmid, as described by Hiei et al. (1997).

**Map-Based Cloning**

Bulked segregant analysis used to identify markers linked to mads3-4, and fine mapping was performed as described by Li et al. (2006) using mapping markers listed in Supplemental Table 2 online.

**Microarray Analysis**

Agilent 4’4K rice oligoarrays (Agilent Technologies) containing 44,000 features were employed for two-color oligoarrays in this study. Two biological replicates of total RNA were prepared from mads3-4 and wild-type anthers at stage 9 using TRIzol reagent (Invitrogen), and the mRNA was isolated from total RNAs using the RNAeasy mini kit (Qiagen). Developing anthers were classified according to microscopy analysis and spikelet length (Feng et al., 2001; Zhang and Wilson, 2009). Microarray hybridization was carried out in a Hybridization Chamber (Agilent, G2534A) according to procedures provided by the company. 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. Meanwhile, linear models and empirical Bayes methods were applied to find the differentially expressed genes (Smyth, 2004).

**In Situ Hybridization**

A gene-specific region at the 3’ end untranslated region of MADS3 (796 to 1209 bp), which shares little sequence similarity with other genes according to the results of a BLASTn analysis, was amplified by PCR from the cDNA clone, digested with BamHI and HindIII, and transcribed in vitro under T7 and SP6 promoters with RNA polymerase, using the DIG RNA labeling kit (Roche). In situ hybridization was performed according to Kouchi and Hata (1993) and Li et al. (2006).

**Rice MADS3 Polyclonal Antibody Preparation and Specificity Analysis**

For production of the MADS3-specific polyclonal antibody, a MADS3-specific fragment (643 to 828) was amplified from the cDNA clone and with the primer pair MADS3-anti-F1 and MADS3-anti-R (see Supplemental Table 2 online). The PCR product, called MADS3-1, was cloned into the BamHI and SalI sites of pET-32a (Novagen) to produce p32-MADS3. Then, the primer pair MADS3-anti-F2 and MADS3-anti-R (see Supplemental Table 2 online) was used to amplify the same MADS3-specific fragment (643 to 828) for insertion at the 5’ end of MADS3-1 at HindIII and XhoI sites in p32-MADS3-1 to obtain p32-MADS3. The fused protein tagged by 6×His at the N terminus was expressed and purified according to Novagen instructions for pET-32a, and the antibody was prepared as described by Huang et al. (2003). Nuclear extracts used for immunoblot analysis to test the specificity of the MADS3 antibody were isolated following the protocol used for ChIP experiments, except that the tissue was not fixed (Zhang et al., 2010b).

**ChIP-qPCR Analysis**

Rice spikeleates at stage 10 were treated and sonicated with a Ultrasonic Crusher Noise Isolating Chamber (Scientz). The procedure for ChIP of MADS3-DNA complexes in wild-type and mads3-4 anthers was modified from Haring et al. (2007).

For qPCR analysis, primers are listed in Supplemental Table 2 online, and reactions were performed on a Rotor-Gene RG3000A detection system (Corbett Research) using SYBR Green I. For PCR reactions, 0.5 μL of recovered DNA from ChIP or controls, or 1 μL of 50-fold diluted input DNA, was added as template, and each reaction was repeated three times. The normalized mean cycle threshold (Ct) of each gene was calculated and used for fold change calculations using the method described by Rotor-Gene version 6.0 (Build 38) software and Zhang et al. (2010b). The difference between the Ct of the wild type and mads3-4 was calculated to obtain the relative enrichment of the upstream fragment.

**Determination of the Levels of ROS, Superoxide Anion, and Hydrogen Peroxide, and Activities of Superoxide Dismutase and Peroxidases**

Measurement of ROS and superoxide anions was performed as described by Schopfer et al. (2001), with DCFH-diacetate from Boehringer Mannheim and 50 mg freshly detached anthers in each reaction. For superoxide anion measurement, 50 mg freshly detached anthers were incubated in 1 mL K-phosphate buffer (20 mm, pH 6.0) containing 500 μM WST (Dojindo) for 8 h at 25°C in the darkness on a shaker. Blanks without
plant material were run in parallel and used as the control. Hydrogen peroxide was measured with a commercial kit (Biyantian Laboratories), in which ferrous ions (Fe$^{2+}$) are oxidized to ferric ions (Fe$^{3+}$) by hydrogen peroxide, and the Fe$^{3+}$ then forms a complex with an indicator dye, xylene orange ($\text{3,3-bis(N,N-di(carboxymethyl)-aminomethyl)-o-cresol-sulfone-phthahlein}$ (Deiana et al., 1999). Protein extractions used for superoxide dismutase and peroxidase activity assays were prepared as described by Chen et al. (2004). Superoxide dismutase activities were measured with a Superoxide Dismutase Assay Kit-WST (Dojindo Laboratories).

**Histochemical Assays of the Production of Superoxide Anion and Hydrogen Peroxide**

In vivo hydrogen peroxide staining was performed as described by Barcelo´ (1998), using TMB. Freshly collected anthers were put in the staining solution (0.1 mg mL$^{-1}$ TMB solution in Tris-acetate, pH 5.0) under vacuum conditions for 15 min and then incubated at 25°C until a blue color appeared. Production of superoxide anion was visualized by incubating intact anthers in 10 mM K-citrate buffer, pH 6.0, containing 0.5 mM NBT.

**MT-1-4b-amirNA Construct**

The online tool WMD2 (http://wmwd2.weigelworld.org) was used to select the suitable MT-1-4b-amirNA sequences (Schwab et al., 2006; Ossowski et al., 2008). The recommended 5'-TATGGATTACGTCGACCTCG-3' as MT-1-4b-amirNA sequence and 5'-GTGAGACGCTCTGAATCCTA-3' as MT-1-4b-amirNA* sequence were employed to replace the natural osa-mir528 precursor as described by Warthmann et al. (2008). The osa-mir528-MT-1-4b-amirNA sequence synthesized by the Jie Rui Biotech Company (Shanghai) was then inserted into the BamHI and XbaI sites of the binary vector pHB, which has the double CaMV 35S promoter, for rice transformation.

**EMSA**

The MADS3 fragment, which contains 702 bp of the putative full-length coding region, was amplified with MADS3-PF and MADS3-PR (see Supplemental Table 2 online) and cloned into pET32a at BamHI and XhoI sites before the construct was transformed into BL21 (DE3) pLYsS. The transformed cells were cultured at 37°C until the OD$_{600}$ was 0.6 and then induced with 1 mM isopropyl $\beta$-D-thiogalactopyranoside for 3 h at 15°C. For purification of the fusion protein, the cells were lysed using a high-pressure cell crusher, and theHis-tagged fusion proteins were purified with Ni-NTA resin (Qiagen) according to the manufacturer’s instructions. The P-MT-1-4b DNA fragment containing the CCGTTTGGGG box was amplified using primers MT-1-4b-EMSA-F and MT-1-4b-EMSA-R (see Supplemental Table 2 online). The DNA fragments were cloned into pMD18-T (TaKaRa) for sequence confirmation and then labeled with the DIG labeling kit (DDLK-010). The DNA binding reactions were performed according to Wang et al. (2002).

**Production of Recombinant MT-1-4b and Assays to Test Its Activity**

A fragment containing the entire open reading frame (237 bp) of MT-1-4b was amplified using primers MT-1-4b-F and MT-1-4b-R (see Supplemental Table 2 online), cloned into the EcoRI and XhoI sites of pET-32a (Novagen) to produce a pET-32a-MT-1-4b expression vector, and then transformed into BL21 (DE3) pLYsS. The transformed cells were cultured at 37°C until the OD$_{600}$ reached 0.6 and then induced with 1 mM isopropyl $\beta$-D-thiogalactopyranoside for 4 h at 37°C. Purification of the native ET-32a-MT-1-4b fusion protein (32 kD) was performed following the protocol used for MADS3 recombinant protein purification in the EMSA experiment (see above).

For hydroxyl radical scavenging assays, antioxidant-mediated competitive inhibition of the salicylate hydroxylation by hydroxyl radicals was performed as described previously (Smirnoff and Cumbes, 1989) using protein expressed from pET-32a as a control. The measurement was repeated three times. For superoxide anion scavenging assays, the inhibition of WST reduction by an antioxidant was performed as described by Wong et al. (2004).

**Accession Numbers**

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: MADS3 (Os01g0201700), cDNA of MADS3 (AK108568), MT2b (Os05g0111300), MADS47 (Os03g0186600), MADS68 (Os11g0658700), MADS98 (Os01g0913900), GID1L2 (Os07g0643000), gibberellin 2-oxidase (Os05g0158600), gibberellin 3-β-dioxygenase 2-2 (Os05g0178100), auxin-induced protein 5NG4 (Os02g0103600), MSP1 (Os01g0917500), PAIR2 (Os09g0568000), GAMYB (Os01g0812000), Wda1 (Os10g0471100), CP1 (Os04g0670500), RAPFITTIN (Os08g0496800), MSTM1 (Os01g0567500), INIV4 (Os04g0413200), TDR (Os02g0120500), C6 (Os11g0582500), CYP704B2 (Os03g0168600), CYP703A3 (Os08g0131100), and UDT1 (Os07g0549600).

**Supplemental Data**

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

**Supplemental Figure 1.** DAPI Staining Analysis of Wild-Type and mads3-4 Microspores.

**Supplemental Figure 2.** Complementation of the mads3-4 Mutant.

**Supplemental Figure 3.** Transmission Electron Micrographs of Anthers in Wild-Type and mads3-4 Plants at Stage 9.

**Supplemental Figure 4.** Higher Superoxide Dismutase and Peroxidase Activities in mads3-4 Anthers from Stage 10 to Stage 12.

**Supplemental Figure 5.** Analysis of Hydrogen Peroxide in Wild-Type and mads3-4 Anthers.

**Supplemental Figure 6.** qRT-PCR Analysis of Selected Genes Showing Changed Expression in mads3-4 Anthers.

**Supplemental Figure 7.** Analysis of the Specificity of Polyclonal Antibodies against MADS3.

**Supplemental Figure 8.** Analysis of Spatial and Temporal Expression of MT-1-4b by Qualitative and Quantitative RT-PCR.

**Supplemental Table 1.** Summary of Floral Organ Defects in mads3-4.

**Supplemental Table 2.** Primers Used in This Study.

**Supplemental Table 3.** Functional Classification by Cluster of Orthologous Groups of Proteins (COG) Analysis of Genes That Are Upregulated or Downregulated (at Least 2-Fold Change in Expression) in Anthers, as Identified by Bayes Analysis (<0.5% FDR).

**Supplemental Table 4.** GO Term Enrichments (P Value < 0.05) for Genes with Expression Changes of at Least 2-Fold (mads3-4/Wild Type), as Identified by Bayes Analysis (<0.5% FDR).

**Supplemental Table 5.** Genes Encoding Transcriptional Factors with Expression Changes of at Least 2-Fold (mads3-4/Wild Type) in mads3-4 Anthers.

**Supplemental Table 6.** Genes with Putative Functions in Phytohormone Biosynthesis, Metabolism and Signaling That Have Expression Changes of at Least 2-Fold (mads3-4/Wild Type) in mads3-4 Anthers.
Supplemental Table 7. Changes in Expression of Genes Required for Rice Anther Development in mads3-4.

Supplemental Table 8. Forty-Two Cytochrome P450 Genes That Have Expression Changes of at Least 2-Fold (mads3-4/Wild Type) in mads3-4 Anthers and Putative CArG-Boxes within the 1-kb Upstream Region in These Genes.

Supplemental Data Set 1. Genes Whose Expression Differs in Stage-9 mads3-4 Anthers from the Wild Type (by at Least 2-Fold), as Identified by Limma Analysis (FDR > 0.05%).

Supplemental Data Set 2. Genes with an Expression Change of at Least 2-Fold in mads3-4 and Found to Be Expressed in Tapetal Cells and/or Microspores.

Supplemental Data Set 3. The 189 Downregulated Genes with Expression Changes of at Least 2-Fold in mads3-4 Anthers and Putative CArG-Boxes within the 1-kb Upstream Regions of These Genes.

Supplemental Data Set 4. The 169 Upregulated Genes with Expression Changes of at Least 2-Fold in mads3-4 Anthers and Putative CArG-Boxes within the 1-kb Upstream Region of These Genes.

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