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DTC1 Controls Tapetal PCD by Binding to OsMT2b

Corresponding author
Gynheung An
Crop Biotech Institute, Kyung Hee University, Yongin 446-701, Korea
+82-31-201-3470
genean@khu.ac.kr

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Defective Tapetum Cell Death 1 (DTC1) Regulates ROS Levels by Binding to Metallothionein during Tapetum Degeneration

Jakyung Yi, Sunok Moon, Yang-Seok Lee, Lu Zhu, Wanqi Liang, Dabing Zhang, Ki-Hong Jung, and Gynheung An*

Department of Genetic Engineering and Crop Biotech Institute, Kyung Hee University, Yongin 446-701, Korea (J.Y., S.M., Y.S.L., K.-H.J., G.A.); State Key Laboratory of Hybrid Rice, Shanghai Jiao Tong University–University of Adelaide Joint Centre for Agriculture and Health, School of Life Sciences and Biotechnology, Shanghai Jiao Tong University, Shanghai 200240, China (L.Z, W.L., D.Z.)

DTC1 induces programmed cell death in the tapetum by binding to OsMT2b and modulating temporal ROS.

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2These authors contributed equally to the article.

S.M. conceived the original research plan; J.Y. and S.M. designed the experiments and analyzed the data; J.Y. performed the transcriptomics experiments and TUNEL assay; S.M. performed histological analysis and crossing; Y.S.L. performed ROS level analysis; L.Z and W.L. performed the transmission electron microscope analyses; J.Y. wrote the article with contributions of D.Z, K.-H.J.,
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*Corresponding author: email genean@khu.ac.kr.
ABSTRACT

After meiosis, tapetal cells in the innermost anther wall layer undergo program cell death (PCD)-
triggered degradation. This step is essential for microspore development and pollen wall maturation.
We identified a key gene, Defective Tapetum Cell Death 1 (DTC1), that controls this degeneration by
modulating the dynamics of reactive oxygen species (ROS) during rice male reproduction. Mutants
defective in DTC1 exhibit phenotypes of an enlarged tapetum and middle layer with delayed
degeneration, causing male sterility. The gene is preferentially expressed in the tapetal cells during
early anther development. In dct1 anthers, expression of genes encoding secretory proteases or lipid
transporters is significantly reduced, while transcripts of PCD regulatory genes, e.g., UDT1, TDR1,
and EAT1/DTD, are not altered. Moreover, levels of DTC1 transcripts are diminished in udt1, tdr, and
eat1 anthers. These results suggest that DTC1 functions downstream of those transcription factor
genes and upstream of the genes encoding secretory proteins. DTC1 protein interacts with OsMT2b, a
ROS-scavenger. Whereas wild-type plants accumulate large amounts of ROS in their anthers at Stage
9 of development, those levels remain low during all stages of development in dct1 anthers. These
findings indicate that DTC1 is a key regulator for tapetum PCD by inhibiting ROS-scavenging
activity.
INTRODUCTION

The developing anther is composed of microsporocytes within a locule that is surrounded by four anther wall layers: tapetum, middle layer, endothecium, and epidermis. As the innermost sporophytic layer, the tapetum plays major roles in microspore formation. The tapetum originates from primary parietal cells through a series of divisions between Stage 3 (ST3) and ST5 (Zhang et al. 2011). After the sporogenous cells generate microspore mother cells and meiocytes in sequence at ST6 and ST7, they form dyads (ST8a) and tetrads (ST8b) of haploid microspores through meiosis. Beginning at ST9, free microspores are released from the tetrads and then vacuolated (ST10). Following two mitosis events, bi-nuclear pollen (ST11) and tri-nuclear pollen (ST12) are produced. During microspore development and maturation, the tapetum provides nutrients for growth and enzymes for the release of microspores from their special callose wall at the tetrad stage (Bedinger, 1992). Functioning as polar secretory cells, the tapetum undergoes cellular degradation starting from ST8. That process of programmed cell death (PCD) involves cytoplasmic shrinkage, breakdown of the nuclear membrane, oligonucleosomal cleavage of DNA, vacuole rupture, and swelling of the endoplasmic reticulum (ER) (Papini et al., 1999).

Reactive oxygen species (ROS) induce PCD during tapetum degeneration, aleurone degradation, formation of aerenchyma tissue, and the development of tracheary elements (Fath et al., 2001; Obara et al., 2001; Li et al., 2006; Muhlenbock et al., 2007; Hu et al 2011; Qu et al., 2014; Yoo et al., 2015). High levels of ROS indiscriminately attack cellular constituents, leading to membrane leakage and cell lysis (Van Breusegem and Dat, 2006). Such damage can be avoided through a critical balance between ROS production and scavenging that regulates subcellular levels of ROS. Superoxide radicals are converted to hydrogen peroxide ($\text{H}_2\text{O}_2$) by superoxide dismutase. This $\text{H}_2\text{O}_2$ is then converted to water and dioxygen by peroxidase and catalase (Gechev et al., 2006). All three enzymes are ROS-scavengers (Apel and Hirt, 2004; Koffler et al., 2015).

Low-molecular-mass antioxidants, such as metallothioneins (MTs), ascorbate, glutathione, and
carotenoids, remove hydroxyl radicals and singlet oxygen (Gechev et al., 2006). The MTs are
cysteine-rich proteins that bind to metals via thiol groups (SH) of cysteine residues (Coyle et al.,
2002). Because of their SH activity in molecules, MTs can interact with many substances, including
other proteins. Metallothioneins have two domains – α and β – that differ in their ability to bind
metals. These proteins form dimers via their domains (Mackay et al., 1993) and are widely distributed
in animals, plants, fungi, and cyanobacteria (Guo et al., 2003). For example, OsMT2b and OsMT-I-4b
proteins in rice (Oryza sativa) scavenge superoxides and hydroxyl radicals (Wong et al., 2004; Hu et
al., 2011). A reduction of OsMT2b expression causes epidermal cell death in rice roots due to ROS
accumulations (Steffens and Sauter, 2009). Decreased MT-I-4b expression results in less pollen
fertility (Hu et al., 2011).

Disturbances in tapetum development and degeneration are associated with male sterility; those
two events occur sequentially during anther development (Wilson and Zhang, 2009; Wang et al., 2013;
Zhang and Yang, 2014). In rice, tapetal cell identification begins after specification of the anther wall
by the LRR RLK receptor kinase MULTIPLE SPOROCYTES 1 (MSP1) and TDR INTERACTING
PROTEIN2 (TIP2), a helix-loop-helix (bHLH) transcription factor (Nonomura et al., 2003; Fu et al.,
2014). TIP2 plays a crucial role in regulating the meristemoid transition and cell differentiation during
early anther development. Mutations in TIP2 are associated with a lack of differentiation of the inner
three anther wall layers as well as defective tapetal PCD. An ER-membrane protein, DEFECTIVE
TAPETUM AND MEIOCYTES 1 (DTM1), controls early tapetum development (Yi et al., 2012).
Undeveloped Tapetum 1 (UDT1) encodes a bHLH protein and regulates tapetum development (Jung
et al., 2005). Defects in UDT1 generate an undifferentiated and vacuolated tapetum. TAPETUM
DEGENERATION RETARDATION (TDR) enhances tapetal PCD. Located downstream of UDT1, the
bHLH protein directly associates with the promoter regions of Oryza sativa Cysteine Proteases 1
(OsCP1) and OsC6 (Li et al., 2006). The former, encoding a putative cysteine protease, is involved in
the release of microspores from the tetrads (Lee et al., 2004). Belonging to a group of lipid transfer
proteins, OsC6 shows lipid-binding activity (Zhang et al., 2010) and has a crucial role in the
development of lipidic orbicules and pollen exine. A pollen exine-forming gene, \textit{CYP704B2}, catalyzes the production of \(\omega\)-hydroxylated fatty acids with 16- and 18- carbon chains, which are essential for production of the anther cuticle as well as the exine (Li et al., 2010; Li and Zhang, 2010). Expression levels of these genes are reduced in \textit{tdr} mutants (Li et al., 2011). Mutants in \textit{ETERNAL TAPETUM 1} (\textit{EAT1}), encoding a bHLH transcription factor, have reduced transcript levels of \textit{AP25} and \textit{AP37}, which encode aspartic proteases. Overexpression of those genes induces cell death in both yeast and plants (Niu et al., 2013). Furthermore, PCD is delayed in the tapetum degeneration mutant \textit{eat1}. This delayed PCD phenotype is also observed in \textit{delayed tapetum degeneration (did)} mutants, alleric to \textit{eat1} (Ji et al., 2013).

RESULTS

Male-Sterile Rice Mutant is Defective in Anther Development

A male-sterile rice mutant, isolated from our T-DNA mutant pool (An et al., 2003; Jeon et al., 2000; Jeong et al., 2002), was named \textit{defective tapetum cell death 1} (\textit{dtc1-1}). Anthers from line 1A-12132 were pale green (Fig. 1A, B) and smaller than those of the wild-type (WT). In that line, T-DNA was inserted in the third exon of Os07g35610 (Fig. 1C). When heterozygous progeny were selfed, normal and sterile plants were produced at a ratio of approximately 3:1 (54 fertile: 25 sterile; \(\chi^2 = 1.861; P < 0.05\)), thereby demonstrating that this sterile phenotype was caused by a single recessive mutation. Whereas all the sterile progeny were homozygous for the T-DNA insertion, approximately one-third of the fertile progeny were WT that lacked T-DNA while the rest carried T-DNA in only one chromosome. This indicated that the phenotype was due to the T-DNA insertion. We identified another line, 1C-01160, where T-DNA was inserted at 349 bp upstream from the start codon of Os07g35610 (Fig. 1C). This \textit{dtc1-2} mutant also displayed a male-sterile phenotype (Fig. 1A, B). The sterile plants from \textit{dtc1-2} heterozygous progeny accounted for approximately 28% of the total (48
fertile: 19 sterile; $\chi^2 = 1.0403; P < 0.05$ for 3:1), suggesting that this sterile phenotype was also
caused by a single recessive mutation. T-DNA was found in both chromosomes for all of the sterile
plants, supporting our conclusion that the T-DNA insertion led to male sterility.

Defective Tapetum Cell Death 1 (DTC1) encodes a protein containing a development and cell
death (DCD) domain and KELCH repeats (Supplemental Fig. S1). Among the 11 members in the rice
genome that have this DCD domain, three proteins containing KELCH repeats are expressed in early
stages of anther development (NCBI Gene Expression Omnibus: GSE13988; Fujita et al., 2010). One
Arabidopsis member, AT5g01660, is also expressed in the flowers, sperm cells, and embryos (Schmid
et al. 2005). This suggests that they may play a conserved role during the formation of male
reproductive organs. Our RT-PCR analyses of developing anthers detected no DTC1 transcripts in
dtc1-1, implying that it is a null mutant. By comparison, a small amount of the transcript remained in
dtc1-2, suggesting that it is a knock-down allele (Fig. 1D).

We found that the promoterless GUS, located within the T-DNA, was oriented in the same
direction as DTC1 in T-DNA-tagged line 1A-12132, thereby generating a translational fusion between
the two gene products (Fig. 1C). Therefore, we were able to monitor its expression pattern by
measuring GUS activity. Here, analyses of the GUS enzyme showed that DTC1 was not expressed in
any of the vegetative cells but was specifically expressed in developing anthers between ST7 and
ST10 (Fig. 2A). Expression in the anthers was diminished at ST11, but activity was prominent in the
carpels at ST12 (Fig. 2A). Cross sections of anthers at ST8 and ST9 indicated that the signal was
limited in the tapetal cell layer (Fig. 2B, C). This anther-preferential pattern of expression during
spikelet development helped to explain the male-sterile phenotype associated with the dtc1 mutants.

Our qRT-PCR analysis of DTC1 transcripts demonstrated that the gene was not expressed in
vegetative organs but was strongly expressed in young spikelets, especially in developing anthers and
mature carpels (Fig. 2D). This pattern is identical to what we observed in our GUS analysis of the T-
DNA-tagged line, indicating that GUS expression patterns could be used to represent transcript levels
for DTC1.
The dtc1 Anthers Are Defective at the Microspore Stage

To determine when these defects occur, we compared thin sections of anthers from dtc1-1 and WT samples. During meiosis, the meiocytes formed dyads and tetrads at ST8 in WT anthers. At this time, the tapetum was considerably changed due to development and differentiation. In particular, the cytoplasm became condensed toward the middle layer at ST8a (Fig. 3A). Afterward, the tapetum was vacuolated, as evidenced by the partial staining of its cells at ST8b (Fig. 3B). During that stage, the middle layer began to shrink, making it difficult to continue our examination. From ST9, condensing of the tapetum was accelerated and the cytoplasm was strongly stained (Fig. 3C). At that stage, young microspores were detached from the tetrads. At ST10, the tapetal cells started to degenerate and microspores were enlarged and vacuolated (Fig. 3D). By ST11, the tapetal cells had disappeared almost entirely and the vacuolated pollen became bi-nucleus pollen grains (Fig. 3E). At ST12, the tapetal cell layer was completely absent and mature pollen grains were filled with starch granules (Fig. 3F).

The dtc1 mutant anthers did not differ from those of the WT through ST9, with development and vacuolation, as well as the formation of four-layer anther walls and progression from meiocytes to microspores, being normal (Fig. 3G-I). However, phenotypic differences were obvious at ST10. Compared with the WT, the tapetum layers in the mutant did not degenerate, but maintained their original shape and became darker (Fig. 3J). At the mature stages, the tapetal cells and middle layers were not degraded, and remnants of the degenerated pollen were visible inside the locules (Fig. 3K, L). This indicated that DTC1 functions in the degeneration of the tapetum and middle layer.

We performed analyses with a transmission electron microscope to observe any subcellular defects. At ST10, WT locules were filled with vacuolated pollen grains and the middle layer was greatly diminished (Fig. 4A). By contrast, most of the pollen grains in the mutant had ruptured. The middle layer was not degenerated and the mutant tapetum was thicker than that of the WT (Fig. 4B).
Although pollen development was severely hampered in the dtc1 anthers, vacuolated pollens with a globular shape were rarely observed (Supplemental Fig. S2A). In the WT tapetum, the cytoplasm was condensed and organelles were not easily identifiable (Fig. 4C). Vestige-like stacks in the WT were thought to be mitochondria and ER. In contrast, the dtc1-1 tapetum contained distinctive organelles, such as mitochondria, ER, and plastids (Fig. 4D). The tapetum surrounding the remaining pollen grains from the mutant also contained those organelles (Supplemental Fig. S2B). Ubisch bodies were evenly spread along the inner surface of the WT tapetum (Fig. 4E). The spheroid structure is a mediator that transports tapetum-produced lipidic sporopollenin precursors to the microspores (Wang et al., 2003). However, we rarely noted any normal Ubisch bodies within the dtc1 tapetum (Fig. 4F). Smaller Ubisch bodies were occasionally found in the region where vacuolated pollen grains remained (Supplemental Fig. S2C). While this lack of Ubisch bodies affected the formation of exine in the mutant, pollen grains from the WT developed well-organized pollen walls that comprised two layers of tectum and nexine, as well as a rod-shaped bacula (Fig. 4G). In contrast, dtc1 pollen grains exhibited abnormal exine development (Fig. 4H and Supplemental Fig. S2D, E); in severe cases, debris from the pollen walls was indistinguishable (Fig. 4H). Normal-appearing vacuolated pollen also exhibited an irregular arrangement of exine. Finally, the tectum in the outer layer was thinner than the nexine, and bacula development was uneven (Supplemental Fig. S2D, E).

**Mutation of DTC1 Causes a Defect in Tapetum Degeneration**

Microscopic observations indicated that the mutant tapetal cells were apparently not degenerated. Because they contained numerous mitochondria and other organelles that appeared normal, we proposed that the process of cell death is delayed in the mutant. Tapetum degeneration is caused by PCD, for which the best feature is cleavage of nuclear DNA. We, therefore, compared DNA fragmentation with a terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay. In the WT, yellow-colored TUNEL signals were first seen at meiosis stage ST8a (Fig. 2A).
5B), and were increased at microspore stages ST8b and S9 (Fig. 5C, D). Those signals then weakened at ST10 (Fig. 5E). However, in dtc1-1 anthers, no positive TUNEL signals were detected at any stage (Fig. 5F-J). Furthermore, positive signals were absent in the weak allele dtc1-2 during ST8 and ST9 (Fig. 5K-N), and were weak at ST10 (Fig. 5O). This indicated that the mutations of DTC1 caused a defect in tapetum degeneration, a step that is important for releasing nutrients and several compounds to growing pollen grains. In fact, from ST8 to ST10 was the period during which PCD signals were intensive in the WT tapetum, coinciding with the time when primexine and exine were being formed.

222 Expression of Genes Encoding Secretory Proteins is Affected in dtc1 Anthers

224 To study the relationship between DTC1 and previously identified genes that function in anther development, we monitored their expression by qRT-PCR analyses. We observed no obvious change in expression levels of MSP1, DTM1, UDT1, TDR, and EAT1/DTD, suggesting that DTC1 may act downstream of these genes (Fig. 6A-E). To verify this genetic hierarchy, we evaluated DTC1 transcripts in msp1, udt1, dtm1, and tdr mutants and found that their levels were lower than in the WT (Fig. 7). This further supported our belief that DTC1 functions later than the regulatory genes.

234 We then investigated genes that encode PCD effector or secretory proteins. Our qRT-PCR assay showed a dramatic reduction in transcript levels of a cysteine protease-encoding OsCP1 and two aspartic protease-encoding genes, AP25 and AP37, as well as OsC6, involved in lipid transport from the tapetum to the pollen exine through the orbicules and CYP704B2, encoding fatty acid hydroxylase (Fig. 6F-J). In our WT, those genes were expressed at higher levels at ST9 than at ST8 but expression was significantly reduced in the dtc1 anthers during those stages of development (Fig. 6F-J). These findings indicated that DTC1 functions prior to the secretory pathway between tapetum and locule. Our analytical results also suggested that DTC1 functions later than TDR at ST8b and before the secretory genes that function at ST9 (Fig. 8). Because DTC1 is a cytosolic protein, it may not directly control the transcription of the later-expressed genes. Instead, a reduction in ROS accumulations in
the defective dtc1 tapetum might indirectly affect their expression. These second-hand changes in transcript levels have also been observed in other male-sterile mutations in DTM1 and MTR1, which are not transcription factor genes (Tan et al., 2012; Yi et al., 2012).

**DTC1 Binds to Metallothionein**

To understand the biochemical mechanism by which DTC1 regulates tapetum development, we screened our cDNA library of rice inflorescences (<5 cm panicles) to identify interacting partners via yeast two-hybrid analyses. Because DTC1 protein does not have transcriptional activation activity, the full-length DTC1 was used as a bait. Among the 33 positive colonies identified here, we selected OsMT2b (LOC_Os05g02070) for further evaluation because it occurred at the highest frequency (5 colonies out of 33).

Because OsMT2b is located in the cytoplasm (Wong et al., 2004), we tested whether DTC1 is also localized there by performing a transient subcellular localization experiment. The full-length cDNA of DTC1 was fused to sGFP and the fusion was placed under the UBIQUITIN (Ubi) promoter from Zea mays. We also constructed an OsMT2b–mRFP fusion construct under that promoter. After both molecules were transferred to protoplasts prepared from rice Oc cells, their expression was monitored with a fluorescence microscope. This experiment showed that the GFP signal from our DTC1–sGFP fusion coincided with the RFP signal driven by OsMT2b–mRFP in the cytosol (Supplemental Fig. S3A). We also generated an OsMT-I-4b–mRFP fusion molecule under the Ubi promoter. OsMT-I-4b is expressed in anthers (Hu et al., 2011). In common with OsMT2b, OsMT-I-4b was also co-localized with DTC1 in the cytoplasm (Supplemental Fig. S3B).

To confirm their interaction, we conducted a bimolecular fluorescence complementation (Bi-FC) assay. A modified yellow fluorescence protein gene, YFP, encoding the N terminal region (VenusN1–173, YFPN; Waadt et al., 2008), was fused to the full-length DTC1 cDNA, and the YFPN–DTC1 fusion was placed under the control of the Ubi promoter. Similarly, YFP encoding the C-terminal region
(VenusC156–239, YFP<sup>C</sup>; Waadt et al., 2008) was fused to OsMT2b, and the YFP<sup>C</sup>–OsMT2b fusion molecule was also placed under the control of that promoter. We also generated a YFP<sup>C</sup>–OsMT-I-4b fusion molecule under the Ubi promoter. Co-expression of YFP<sup>N</sup>–DTC1 and YFP<sup>C</sup>–OsMT2b in the protoplasts revealed their interaction (Fig. 9A). Furthermore, we observed that YFP<sup>N</sup>–DTC1 binds to YFP<sup>C</sup>–OsMT-I-4b.

For additional verification, we performed co-immunoprecipitation (co-IP) assays. The Myc-tagged DTC1 plasmid was introduced into Oc cell protoplasts together with either OsMT2b–HA or OsMT-I-4b–HA. Western blots using antibodies against c-Myc or HA showed that the constructs were expressed in the transient-expression system (Fig. 9B, upper). These Co-IP experiments indicated that DTC1 binds to the two MT proteins (Fig. 9B, lower). Therefore, we could again conclude that DTC1 interacts with either OsMT2b or OsMT-I-4b.

If OsMT2b and OsMT-I-4b are involved in tapetum degeneration, then one would expect that they are expressed during early anther development when the dtc1 mutants exhibit their phenotypes. Our qRT-PCR assays showed that OsMT2b was expressed in all examined tissues, i.e., shoots, roots, mature leaves, and reproductive organs (Fig. 2E). In the developing anthers, transcripts were maintained at higher levels during the early stages before being reduced to a low level. This expression pattern was in accord with that previously observed for GUS activity in a promoter-trap line (Yuan et al., 2008). Other researchers have also reported that OsMT-I-4b is expressed in roots and anthers at later stages, i.e., ST10 and ST11 (Hu et al., 2011). Therefore, all of these expression patterns would explain why we did not detect OsMT-I-4b in our yeast screening assay with the inflorescence cDNA library.

**ROS Are Rarely Accumulated in dtc1 Anthers**

Because MT proteins have ROS-scavenger activity (Wong et al., 2004; Hu et al., 2011), the interaction between DTC1 and MTs would suggest that DTC1 is involved in ROS homeostasis.
Therefore, we analyzed levels of the superoxide anion in WT and dtc1-1 anthers with nitroblue tetrazolium (NBT), a widely used indicator that forms a dark-blue formazan precipitate when in contact with superoxide (Bielski et al., 1980). In WT anthers, staining was darkest during ST8 and ST9, but weak at ST10 and ST11 (Fig. 10A). In contrast, the degree of staining was much less in the mutant at ST8 and ST9, showing that ROS levels were lower there. We quantified superoxide levels using a water-soluble tetrazolium salt reagent (WST-1: Na,2-[4-iodophenyl]-3-[4-nitrophenyl]-5-[2,4-disulfophenyl]-2H-tetrazolium), which is reduced by superoxide to a stable water-soluble formazan with high molar absorptivity (Tan and Berridge, 2000). As observed from the NBT assays, levels of superoxide in the WT were highest at ST9 but reduced at ST10 and ST11 (Fig. 10B). Tapetal PCD was initiated early in anther development (ST8-ST9) when the levels of superoxide were increased. These observations were consistent with other reports that ROS function as a signal for PCD (Fath et al., 2001; Obara et al., 2001; Li et al., 2006; Muhlenbock et al., 2007). In our dtc1 anthers, superoxide levels remained low during all stages of development (Fig. 10B). These results suggested that optimal levels of ROS are required for PCD and that low amounts in mutant anthers delay tapetum degeneration.

Discussion

DTC1 Is a Newly Discovered Regulator of Tapetal PCD

After meiosis, the rice tapetum undergoes PCD-triggered cellular degradation that is assumed to provide nutrients and signaling molecules to support pollen development (Li et al., 2006; Li et al., 2011). Tapetal PCD is characterized by sequential cytological processes, including cytoplasmic shrinkage, DNA fragmentation, vacuole rupture, and swelling of the ER (Papini et al., 1999). Tapetal cell specification, development, and subsequent degeneration coincide very well with post-meiotic
events, and premature or delayed tapetal PCD frequently causes male sterility (Li et al., 2006; Li et al., 2011; Niu et al., 2013).

Previous forward and reverse genetics studies have uncovered several key regulators of tapetal PCD in rice. These include GAMYB (Aya et al., 2009; Liu et al., 2010); the bHLH transcription factors UDT1 (Jung et al., 2005), TIP2 (Fu et al., 2014), TDR (Li et al., 2006; Zhang et al., 2008), and EAT1/DTD (Ji et al., 2013; Niu et al., 2013); a PHD finger protein, PERSISTENT TAPETAL CELL1 (PTC1) (Li et al., 2011); a homolog of animal anti-apoptosis, APOPTOSIS INHIBITOR5 (API5) (Li et al., 2011); and OsMADS3 (Hu et al., 2011). Here, we identified a new tapetal PCD regulator, DTC1, that contains a DCD domain and KELCH repeats. DTC1 is expressed in the early stages of anther development. The dtc1 mutants have delayed tapetal PCD, as evidenced by a lack of obvious DNA fragmentation and no degeneration of tapetal cells and middle layer.

**DTC1 Inhibits ROS-Scavenging Activity by Interacting with OsMT2b**

In plants, ROS are produced when O$_2$ is employed as a terminal electron acceptor, generating molecules such as superoxide anion, hydroxyl radicals, and H$_2$O$_2$. Most of these ROS molecules are toxic by-products of aerobic metabolism in plants under abiotic stress (Miller and Mittler, 2006). Plants utilize various enzymes and non-enzymatic molecules to remove these toxins (Mittler et al., 2004). These include the Cys-rich MTs, which are non-enzymatic, low-molecular-mass antioxidants (Gechev et al., 2006; Hu et al., 2011). Recombinant MT2b and MT-1-4b proteins show superoxide- and hydroxyl radical-scavenging activities (Wong et al., 2004; Hu et al., 2011). A reduction in $MT2b$ or $MT-1-4b$ expression results in the accumulation of ROS molecules in rice roots (Steffens and Sauter, 2009) and anthers (Hu et al., 2011), respectively. Mutations in OsMADS3 cause decreased expression of $MT-1-4b$, leading to higher accumulations of ROS molecules. Consequently, the mutants display oxidative stress-associated defects such as brown-colored anthers with degenerated microspores that result from oxidative damage to the lipid, protein, and other metabolic pathways (Hu et al., 2011; Qu...
In addition to their toxic effects, ROS are important signaling molecules that affect various plant processes, e.g., root hair patterning and leaf development (Geciev et al., 2006; Miller et al., 2008). The superoxide anion radical and H$_2$O$_2$ modulate plant cell death, including tapetal PCD (Bouchez et al., 2007). Here, we showed that OsMT2b is highly expressed at the early stages of anther development and is likely a key player in reducing ROS levels. Consistent with its role in tapetal cells, laser microdissection transcriptome analyses have indicated that OsMT2b is a tapetum-preferential gene (Hobo et al., 2008). Similarly, MT1a in Arabidopsis and Mzm3-4 in Zea mays are specifically expressed in tapetal tissues (García-Hernández et al., 1998; Charbonnel-Campa et al., 2000), suggesting that these tapetum-specific MTs may have a conserved function among plant species. We previously measured increased levels of ROS in rice anthers at ST9 but greatly reduced amounts at ST10, which is consistent with the initiation of tapetal PCD (Hu et al., 2011). The dtc1 anthers contained less ROS, which probably delayed tapetal PCD. Furthermore, we found that DTC1 physically interacts with OsMT2b. We hypothesize that this interaction may inhibit the ROS-scavenging activity of OsMT2b. Mammalian MTs interact with zinc-dependent enzymes that are capable of deactivating MTs (Atrian and Capdevila, 2013). However, suppression of ROS-scavenging activity of MTs by an inhibitory protein has not been previously reported in plants.

In the absence of DTC1 in the dtc1 mutants, OsMT2b continuously eliminates ROS, such that PCD is not induced in the tapetum. This indicates that temporal production of ROS is needed for tapetal PCD. Our result is similar to that reported for Arabidopsis mutants in RESPIRATORY-BURST OXIDASE HOMOLOG (RBOH) genes, which show delayed tapetal PCD and a reduced amount of ROS signals when compared with WT anthers where the ROS level peaks during ST8 and ST9 before decreasing at later stages (Xie et al., 2014). Timely production of ROS is essential for proper initiation of tapetal PCD. Pre-generation of ROS causes premature tapetal PCD and sporophytic male sterility (Luo et al., 2013). Mitochondrial gene WA352, originating from wild rice, confers cytoplasmic male sterility. The protein interacts with the nuclear-encoded mitochondrial protein COX11 to inhibit
COX11 functioning in peroxide metabolism, leading to ROS burst. Release of cytochrome c (cyt c) from the mitochondria is a key character in animal apoptosis, being required for the formation of the apoptosome, a molecular system activating caspases (Balk et al., 1999; Zou et al., 1999). Although evidence for apoptosome formation is lacking in plants, the release of cyt c is associated with premature induction of PCD in the tapetum of cytoplasmic male sterility mutants from Helianthus annuus (Balk and Leaver, 2001).

Regulatory Role of DTC1 during Anther Development

To understand the regulatory positioning of DTC1 among rice genes involved with anther development, we monitored UDT1, TDR, and EAT1/DTD in dtc1 anthers and observed no changes in their expression. This suggested that DTC1 acts downstream of those tapetal PCD regulators. Accordingly, levels of DTC1 transcript were reduced in tdr anthers. In addition, the dtc1 tapetum displayed a phenotype of delayed PCD that was similar to tdr even though the tapetal cells were not noticeably enlarged in the former (Li et al., 2006). Therefore, we could again conclude that DTC1 functions later than TDR. TDR is a key transcription factor that controls tapetal PCD by inducing OsCP1 and OsC6 (Li et al., 2006). In addition, EAT1/DTD promotes tapetal PCD by increasing aspartic proteases expression (Niu et al., 2013). Expression of EAT1/DTD is reduced in tdr and eat1 tdr double mutants that have expanded tapetal cells similar to those of tdr. Although it is possible that EAT1/DTD acts downstream of TDR in regulating tapetal PCD (Niu et al., 2013), those two proteins, EAT1/DTD and TDR, interact with each other (Ji et al., 2013; Niu et al., 2013). When compared with the dtc1 phenotype, eat1 anthers had thicker and darker-stained tapetal cells at ST9. Therefore, because EAT1/DTD transcript levels were similar between dtc1 and the WT, it seems that DTC1 functions later than EAT1/DTD. The promoter region of DTC1 contains an E box (CATGTG at –456 bp from ATG), which is a putative target sequence of bHLH transcription factors (Bouchard et al., 1998; Chinnusamy et al., 2003). This sequence may be a binding site of the bHLH transcription
transcripts of OsAP25 and OsAP37, encoding aspartic protease, were diminished in dtc1 anthers. Likewise, OsC6 and CYP704B2, involved in lipid allocation and metabolism, were affected. Those genes are down-regulated in tapetal PCD mutants such as tdr, eat1/dtd, and ptc1 (Li et al., 2006; Li et al., 2011; Niu et al., 2013). CP1, encoding Cys protease, is not as strongly expressed in dtc1 as observed from eat1 (Niu et al., 2013). However dtd, an allele of eat1, shows higher CP1 expression when compared with the WT. This discrepancy may be due to the mutant position. Unlike eat1-1, the mutation in that dtd allele does not affect the bHLH domain, suggesting that the partial CP1 fragment in dtd may function negatively (Ji et al., 2013). Whereas their expression is maximized at later stages of anther development (Hobo et al., 2008), DTC1 is highly expressed during the early stages. Thus, DTC1 may function prior to these secretory proteins.

MATERIALS AND METHODS

Genotyping and Identification of dtc1 Mutants

Seeds from heterozygous rice lines 1A-12132 (dtc1-1) and 1C-01160 (dtc1-2) were germinated and seedlings were grown for 10 d at 28°C on a half-strength Murashige and Skoog medium containing 3% sucrose. Their DNA was isolated by the hexadecyltrimethylammoniumbromide method (Chen and Ronald, 1999). Genotypes were determined by PCR, using gene-specific primers and T-DNA primers (Supplemental Table S1) (Yi and An, 2013).

Histochemical Analyses

Spikelets collected at different developmental stages were fixed with 3% (w/v) paraformaldehyde and dehydrated in an ethanol series. The samples were embedded in Technovit 8100 resin (Heraeus Dental...
International, http://heraeus-dental.com), then sectioned to a 5- to 10-μm thickness, stained with 0.1% toluidine blue, and observed with an Olympus BX61 microscope (Olympus, http://www.olympus.com). Histochemical GUS-staining using the gene-trap line 1A-12132 (DTC1/dtc1-1) was performed as described by Jefferson et al. (1987). Afterward, the tissues were embedded and sectioned as mentioned above. For staining of the superoxide anion, intact anthers were incubated in 10 mM potassium-citrate buffer (pH 6.0) containing 0.5 mM NBT (Frahry and Schopfer, 2001).

### Ultrastructural Analyses by Transmission Electron Microscopy

Anthers were fixed in sodium phosphate buffer (pH 7.2) that contained 3% glutaraldehyde (Sigma-Aldrich, http://www.sigmaaldrich.com). They were then post-fixed using 2% osmium tetroxide (Pelco International, http://www.pelcoint.com). After dehydration, the specimens were placed in a Spurr’s low-viscosity embedding mixture (EMS, http://www.emsdiasum.com/). Ultrathin sections (40- to 60-nm thick) were stained with 2.5% uranyl acetate and 2.5% lead citrate aqueous solutions, and observed under a JEM-1230 transmission electron microscope (JEOL, Jeol Ltd, Tokyo, Japan) at 80 kV.

### TUNEL Assays

Preparation of the anther sections and TUNEL assays with a Dead End Fluorometric TUNEL Kit (Promega, https://www.promega.com) were performed as described by Li et al. (2006). The green fluorescence of fluorescein (TUNEL signal) and red fluorescence of propidium iodide (PI) were analyzed at 488 nm (excitation) and 520 nm (detection), and 488 nm (excitation) and 610 nm (detection), respectively, under the Olympus BX61 microscope.

### Subcellular Localization and Bi-FC
The coding regions of DTC1, OsMT2b, and OsMT-I-4b were amplified using primers shown in Supplemental Table S1. The fragments were inserted into pGA3452 and pGA3574 vectors for sGFP and mRFP tags, respectively (Kim et al., 2009). For Bi-FC, the coding regions of DTC1 and MTs OsMT2b and OsMT-I-4b were fused to N- and C-terminal YFP (Venus) fragments (Waadt et al., 2008) using primers listed in Supplemental Table S1. YFP fluorescence can be detected if the expressed bait and prey proteins interact in vivo (Walter et al., 2004). Protoplast preparation from rice Oc cells and transformation procedures were conducted as described by Moon et al. (2008). After 12 h of incubation at 25°C under darkness, the protoplast cells were examined under the Olympus BX61 microscope, which was equipped with filter sets for GFP, RFP, and YFP.

Co-Immunoprecipitation Assays

For generation of DTC1-Myc, OsMT2b-HA, and OsMT-I-4b-HA fusion proteins, the coding regions of their genes were amplified using the primer sets listed in Supplemental Table S1. We used pGA3697 and pGA3698 (Kim et al., 2009) for the Myc and HA tags, respectively. The constructs were transformed into protoplasts isolated from rice Oc cells. After incubation, the protoplasts were harvested and used for co-IP assays as previously reported, but with minor modifications (Choi et al., 2014). The immuno-precipitation buffer contained 50 mM Tris- HCl (pH 7.5), 5 mM EDTA, 50 mM sodium chloride, 1% [v/v] Triton X-100, 1 mM dithiothreitol, and an adequate amount of protease inhibitor cocktail (Roche, https://lifescience.roche.com).

Determination of Superoxide Anion Levels

For quantifying the superoxide anion, anthers freshly detached from 70 florets were incubated in 1 mL of potassium phosphate buffer (20 mM; pH 6.0) that contained 0.5 mM WST-1 (Dojindo, www.dojindo.com), and were placed on a shaker for 8 h at 25°C in the dark. As the control, we used
blanks without anthers. After incubation, the samples were measured at 440 nm with a
spectrophotometer (Frahry and Schopfer, 2001).

**RNA Extraction and Quantitative RT-PCR**

Total RNA was isolated and cDNAs were synthesized as described previously (Lee and An, 2015). Gene expression was monitored by quantitative RT-PCR (see Lee and An, 2015). All experiments were conducted at least three times, using three or more independent samples per experiment. Primers for analyzing transcript levels are listed in Supplemental Table S1.

**Supplemental Data**

**Supplemental Figure S1.** Alignments of proteins containing DCD domain and KELCH repeats. A, Phylogenetic analysis of DCD domain-containing proteins from rice and *Arabidopsis*, obtained via NJ method implemented in MEGA version 6.0. Scale bar corresponds to 0.05 amino acid substitutions per residue. B, Multiple alignment of DCD domains, performed by Bioedit program (version 7.2.5). Identical amino acids are shaded in black; similar amino acids, in gray. Conserved motives are indicated as follows: ****, FGLP; ++++, LFL; · · · , PAQV; ^^^^, PLxE. C, Multiple alignment of KELCH motif. DTC1 has 3 KELCH repeats with 4 beta strands (β1- β4). KELCH repeat sequence is largely defined by conserved residues indicated with asterisk (*). l = large; h = hydrophobic; s = small (Adams et al., 2000).

**Supplemental Figure S2.** Transmission electron microscopy of *dtc1-1* anthers at ST10. A, Anther wall layer of *dtc1-1* with vacuolated pollen grains. B, Magnified images of tapetal cytoplasm indicated by black solid boxes in A. C, Magnified images of inner surface of tapetum in A. D and E, Magnified images of white dotted box (D) and white solid box (E) in A. Cy, cytosol of vacuolated pollen; ER, endoplasmic reticulum; Mt, mitochondria; Ne, nexine; Pl, plastids; Te, tectum;
Ub, Ubisch bodies; VP, vacuolated pollen. Scale bars = 10 μm (A) and 1 μm (B-E).

**Supplemental Figure S3.** Subcellular localization experiment using rice Oc protoplasts. A, Transient expression of constructs with DTC1–sGFP fusion protein (left) or OsMT2b–mRFP fusion protein (middle). Merged image is on right side. B, Transient expression of constructs with DTC1–sGFP fusion protein (left) or OsMT-I-4b–mRFP fusion protein (middle). Merged image is on right side.

**Supplemental Table S1.** Primer sequences used in this study

Restriction enzyme sites are underlined.

**ACKNOWLEDGEMENTS**

We thank Kyungsook An for generating the transgenic lines and handling the seed stock, and Priscilla Licht for editing the English composition of this article.

**Figure Legends**

**Figure 1.** Characterization of T-DNA insertion mutants in *DTC1*. A, Comparisons among WT (left), *dcl-1* (middle), and *dcl-2* (right) spikelets after removing lemma. Scale bar = 1 mm. B, Comparisons among WT (left), *dcl-1* (middle), and *dcl-2* (right) anthers. Scale bar = 1 mm. C, Gene structure of *DTC1*. Gray boxes indicate exons; open boxes, UTRs; lines connecting boxes, introns. Triangles are T-DNA insertions of *dcl-1* (line 1A-12132) and *dcl-2* (line 1C-01160). Arrows F and R show primers used for RT-PCR analyses of *DTC1* transcript levels. Scale bar = 1 kb. D, RT-PCR analyses of *DTC1* transcript levels in *dcl-1* and *dcl-2* compared with segregating WT. *Rice Ubiquitin 1 (OsUbi1, Os03g0234200)* served as control.
Figure 2. Expression profiles of DTC1 and OsMT2b. A, Expression patterns of DTC1–GUS fusion product. Photograph was taken after removal of lemma and half of palea. Numbers indicate anther development stages ST7 through ST12. Scale bar = 1 mm. B and C, Cross section of anthers from T-DNA-tagged line expressing DTC1–GUS at ST8 and ST9. T, tapetum. Scale bars = 20 μm. D and E, qRT-PCR analyses of DTC1 (D) and OsMT2b (E) expression in various organs. Transcript levels were normalized to OsUbi1.

Figure 3. Transverse-sectional comparisons between WT and dtc1-1 anthers. Cross sections of WT (A-F) and dtc1-1(G-L) anthers at ST8a (A, G), ST8b (B, H), ST9 (C, I), ST10 (D, J), ST11 (E, K), and ST12 (F, L). BP, binuclear pollen; En, endothecial cell; Ep, epidermal cell; MC, meiocyte; ML, middle layer; MP, mature pollen; Ms, microspore; T, tapetal cell; Tds, tetrads; VP, vacuolated pollen. Scale bars = 20 μm.

Figure 4. Transmission electron microscopy of WT and dtc1-1 anthers. A and B, Anther wall layer of WT (A) and dtc1-1 (B) at ST10. C and D, Magnified images of tapetal cytoplasm indicated by black solid boxes in A and B. E and F, Magnified images of inner surface of tapetum in A and B. G, Magnified images of vacuolated pollen wall from A. H, Magnified images inside locule from B. Ba, bacula; Cy, cytosol of vacuolated pollen; En, endothecial cell; Ep, epidermal cell; ER, endoplasmic reticulum; Ex, exine; ML, middle layer; Mt, mitochondria; Ne, nexine; Pl, plastids; T, tapetal cell; Te, tectum; Ub, Ubisch bodies; V, vacuole; VP, vacuolated pollen. Scale bars = 10 μm (A and B) and 1 μm (C-H).

Figure 5. Detection of DNA fragmentation by TUNEL assays. Signals from anthers of WT (A- E), dtc1-1 (F- J), and dtc1-2 (K- O) at ST7 and ST10. Nuclei were stained with propidium iodide (PI) indicated by red fluorescence. Yellow fluorescence shows TUNEL-positive regions. Bars = 20 μm.
Figure 6. Expression analyses of marker genes during stages of anther development. Quantitative RT-PCR of MSP1 (A), DTM1 (B), UDT1 (C), TDR (D), EAT1/DTD (E), OsCP1 (F), OsAP25 (G), OsAP37 (H), OsC6 (I), and CYP704B2 (J). Y-axis, gene expression relative to rice OsUbi1 transcript level.

Figure 7. Expression analysis of DTC1 in other anther mutants. Anthers from msp1, udt1, dtm1, and tdr mutants were harvested at ST9, and expression of DTC1 was measured by qRT-PCR. Y-axis, gene expression relative to OsUbi1 transcript level. Results represent averages of 3 independent experiments.

Figure 8. Role of DTC1 during anther development and tapetum degeneration. A, Schematic diagram of PCD in tapetum, and ROS levels regulated by MTs and DTC1. Genes involved in tapetum development are presented. Arrows indicate times when each marker gene functions. B, Differences in PCD induced by ROS in WT and dtc1 mutant anthers.

Figure 9. Interactions between DTC1 and MTs. A, Interaction of DTC1 with MT2b (upper panel) or OsMT-I-4b (lower panel) in rice Oc cells revealed by Bi-FC system. Each combination of constructs fused to YFPN and YFPC was transfected and transiently expressed. Images shown are bright field, YFP fluorescence by Bi-FC, and merged image. Scale bars = 10 μm. B, Co-immunoprecipitation analysis of interaction between DTC1 and MTs. Total proteins were extracted from rice Oc cell protoplasts that transiently expressed DTC1-Myc together with OsMT2b-HA or OsMT-I-4b-HA. Plus and minus signs indicate constructs introduced into protoplasts. Extracts were detected using anti-Myc antibody or anti-HA antibody (upper panel). Immunoprecipitation was performed with anti-HA antibody, then detected using anti-Myc antibody (lower panel).
**Figure 10.** Analyses of superoxide anion levels. A, NBT staining of superoxide anion from WT and dtc1-1 anthers from ST8a to ST11. Scale bars = 0.5 mm. B, Superoxide anion levels measured by WST-1.

**LITERATURE CITED**


preferentially expressed in the tapetum in *Zea mays*. Gene 254: 199-208


Methods 238: 59-68


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