The mitochondrial aldehyde dehydrogenase OsALDH2b negatively regulates tapetum degeneration in rice

Xianrong Xie1,2,3,*, Zixu Zhang1,2,3,4,*, Zhe Zhao1,2,3,4, Yongyao Xie1,2,3,4, Heying Li1,2,3, Xingliang Ma1,2,3, Yao-Guang Liu1,2,3 and Letian Chen1,2,3,4,†,

1 State Key Laboratory for Conservation and Utilization of Subtropical Agro-Bioresources, Guangzhou 510642, China
2 Guangdong Laboratory for Lingnan Modern Agriculture, Guangzhou 510642, China
3 College of Life Sciences, South China Agricultural University, Guangzhou 510642, China
4 Key Laboratory of Plant Functional Genomics and Biotechnology of Guangdong Provincial Higher Education Institutions, South China Agricultural University, Guangzhou 510642, China

* These authors contributed equally to this work.
† Correspondence: lotichen@scau.edu.cn

Received 17 September 2019; Editorial decision 8 January 2020; Accepted 26 January 2020

Editor: Dabing Zhang, Shanghai Jiao Tong University, China

Abstract

Timely degradation of anther tapetal cells is a prerequisite for normal pollen development in flowering plants. Although several genes involved in tapetum development have been identified, the molecular basis of tapetum degeneration regulation remains poorly understood. In this study, we identified and characterized the nucleus-encoded, conserved mitochondrial aldehyde dehydrogenase OsALDH2b as a key regulator of tapetum degeneration in rice (Oryza sativa). OsALDH2b was highly expressed in anthers from meiosis to the early microspore stage. Mutation of OsALDH2b resulted in excess malonaldehyde accumulation and earlier programmed cell death in the tapetum, leading to premature tapetum degeneration and abnormal microspore development. These results demonstrate that OsALDH2b negatively regulates tapetal programmed cell death and is required for male reproductive development, providing insights into the regulation of tapetum development in plants.

Keywords: Aldehyde dehydrogenase, male sterility, OsALDH2b, programmed cell death, rice, tapetal degeneration.

Introduction

Pollen development is essential to plant reproduction. In rice (Oryza sativa L.), which is a staple for over half the global population, abnormal anther development significantly influences rice production (Chen and Liu, 2014). Previous studies have revealed that various regulators such as transcription factors, receptor-like kinases, ATP binding cassette G (ABCG) transporters, glycoproteins, redox homoeostasis-related factors, hormones, and enzymes are involved in anther development and pollen formation in Arabidopsis and rice (Wilson and Zhang, 2009; Zhang and Yang, 2014; Zhao et al., 2016; Cai and Zhang, 2018; Yu and Zhang, 2019). The tapetum, the innermost layer in anthers, directly contacts male gametophytes and plays a vital role in microspore development. Tapetum degeneration via programmed cell death (PCD) provides enzymes, sporopollenin precursors, and nutrients for pollen maturation and wall synthesis. Premature or delayed tapetal degradation causes defective pollen development (Ma, 2005). Timely tapetal degradation is strictly controlled by a set of genes including OsCP1, TDR, PTC1, MTR1, EAT1/DTD, TIP2, OsTDF1, OsTGA10, Cex11, and EDT1 (Li et al., 2011; Guo and Liu, 2012; Tan et al., 2012; Ji et al., 2013; Luo et al., 2013; Ko et al., 2014; Zhang and Yang, 2014; Cai et al., 2015; Chen et al., 2018;
Bai et al., 2019). Tapetum Degeneration Retardation (TDR), a homolog of Aborted Microspores (AMS) of Arabidopsis (Sorensen et al., 2003), interacts with TDR Interacting Protein2 (TIP2) and Eternal Tapetum1 (EAT1) to positively regulate tapetal PCD via two aspartic proteases (AP37 and AP25) and is required for induction of OsC1 (Li et al., 2006; Niu et al., 2013; Fu et al., 2014). The tdr mutant exhibits delayed tapetal PCD and degeneration. However, negative regulators of tapetum degeneration are seldom reported. In addition, recent studies have established that cellular redox status and dynamic reactive oxygen species (ROS) change are essential for tapetal cell specification and punctual initiation of tapetal PCD (Yu and Zhang, 2019; Bai et al., 2019), while glutaredoxin and rice Tapetum Determinant1-Like1A (OsTDL1A–Multiple Sporocytes1 (MSP1) pathways have a conserved role in determining anther cell fate (Zhang and Yang, 2014; Yu and Zhang, 2019). Rice MADS-box transcription factor3 (OsMADS3) is a key transcriptional regulator acting with the promoter of OsMT-I-4b to scavenge ROS during later anther development (Hu et al., 2011). Another ROS-scavenging protein, rice Metallothionein Type 2b (OsMT2b), interacts with Defective Tapetum Cell Death 1 (DTC1) to maintain the normal level of ROS in tapetal cells (Yi et al., 2016).

Reactive metabolites such as ROS and malonaldehyde (MDA) can induce DNA damage (Hu et al., 2011; Zhang and Yang, 2014). Oxidative stress and cellular processes (e.g. lipid peroxidation) produce aldehydes that bear reactive carbonyl groups, which can directly interact with DNA to cause lesions resulting in PCD (Voulgaridou et al., 2011; Biswas and Mano, 2015). Aldehyde dehydrogenases (ALDHs) are a group of NAD(P)+-dependent enzymes that catalyze conversion of aldehydes to the corresponding acids. In mammals, ALDHs are commonly detoxifying enzymes that eliminate toxic bio- genic and xenobiotic aldehydes (Yoshida et al., 1998). Aldehyde dehydrogenase function in anther development are required for induction of OsC1 (Li et al., 2006; Niu et al., 2013; Fu et al., 2014). The tdr mutant is-delayed tapetal PCD and degeneration. However, negative regulators of tapetum degeneration are seldom reported. In addition, recent studies have established that cellular redox status and dynamic reactive oxygen species (ROS) change are essential for tapetal cell specification and punctual initiation of tapetal PCD (Yu and Zhang, 2019; Bai et al., 2019), while glutaredoxin and rice Tapetum Determinant1-Like1A (OsTDL1A–Multiple Sporocytes1 (MSP1) pathways have a conserved role in determining anther cell fate (Zhang and Yang, 2014; Yu and Zhang, 2019). Rice MADS-box transcription factor3 (OsMADS3) is a key transcriptional regulator acting with the promoter of OsMT-I-4b to scavenge ROS during later anther development (Hu et al., 2011). Another ROS-scavenging protein, rice Metallothionein Type 2b (OsMT2b), interacts with Defective Tapetum Cell Death 1 (DTC1) to maintain the normal level of ROS in tapetal cells (Yi et al., 2016).

Reactive metabolites such as ROS and malonaldehyde (MDA) can induce DNA damage (Hu et al., 2011; Zhang and Yang, 2014). Oxidative stress and cellular processes (e.g. lipid peroxidation) produce aldehydes that bear reactive carbonyl groups, which can directly interact with DNA to cause lesions resulting in PCD (Voulgaridou et al., 2011; Biswas and Mano, 2015). Aldehyde dehydrogenases (ALDHs) are a group of NAD(P)+-dependent enzymes that catalyze conversion of aldehydes to the corresponding acids. In mammals, ALDHs are commonly detoxifying enzymes that eliminate toxic bio- genic and xenobiotic aldehydes (Yoshida et al., 1998; Vasiliev et al., 2000). In plants, most identified ALDHs confer tolerance to abiotic stresses, such as heat, salinity, ultraviolet radiation, and anaerobic conditions (Nakazono et al., 2000; Sunikar et al., 2003; Kotchoni et al., 2006; Rodrigues et al., 2006). Notably, rice OsALDH7 detoxifies aldehydes like MDA and thereby maintains seed viability (Shen et al., 2012; Shin et al., 2009), while OsALDH2a potentially functions in submergence tolerance (Nakazono et al., 2000). A maize (Zea mays) gene, Rj2a, for restoration of Texas-type cytoplasmic male sterility (CMS-T) encodes a mitochondria-localized aldehyde dehydrogenase (Cui et al., 1996) and also is required for anther development (Liu et al., 2001). However, the details of mitochondrial aldehyde dehydrogenase function in anther development are unclear.

Rice has 22 aldehyde dehydrogenase members grouped into 11 families, including OsALDH7, OsALDH2a, and OsALDH2b (Gao and Han, 2009). In this study, we identified a rice male-sterility mutation caused by a 7-bp deletion in OsALDH2b. Furthermore, we revealed that OsALDH2b encodes a mitochondrially-targeted aldehyde dehydrogenase enzyme and is highly expressed in anthers during microsporogenesis. Our results demonstrate that OsALDH2b removes excess aldehydes generated during anther development to negatively regulate tapetum degeneration.

Materials and methods

Plant materials

All rice plants were grown under natural conditions in South China Agricultural University at Guangzhou’s paddy field. The male-sterile mutant (later named osaldh2b) was obtained from a 60Co–γ-ray-treated rice Nipponbare (O. sativa, ssp. japonica) mutant library. The F2 mapping population was generated from a cross between the mutant and an indica variety, Huanghuazhan (HHZ, O. sativa, ssp. indica). In the F2 population, male-sterile plants were selected primarily for genetic mapping. For screening recombinant individuals, F2 and F3 segregants were planted in 96-well plates and used for high-throughput DNA preparation as described previously (Wang et al., 2013).

Mutant phenotype characterization

Plants were photographed with a Nikon digital camera. Flowers were photographed with a stereomicroscope (SZX10/DP72, Olympus, Japan). Pollen grains were stained with 1% I–I1 solution and photographed with a fluorescence microscope (Axio Observer Z1, Zeiss, Germany). Preparation of rice anther sections for light microscopy and electron microscopy was performed as previously described (Li et al., 2006).

Map-based cloning of OsALDH2b

A set of 145 male-sterile plants segregated from 652 F2 individuals was used for primary mapping. Recombinants were then screened from F2 and F3 families for fine mapping with newly developed insertion/deletion (InDel) molecular markers (see Supplementary Table S1 at JXB online). Rice genomic DNA samples were prepared from fresh leaf tissues using 1% sodium dodecyl sulfate.

Vector construction for transgenic plants

For the functional complementation test, a 12.2 kb wild-type genomic fragment of OsALDH2b was amplified by three steps. The first fragment was amplified using OsALDH2b-F1 and OsALDH2b-R1 primers (Supplementary Table S2) and cloned into the MtM and SaI sites of the binary vector pCAMBIA1300.2. The second fragment was amplified with OsALDH2b-T5F2 and OsALDH2b-T5R2 primers and cloned, into the positive clones produced in the first step, at the SaI site using an isothermal in vitro recombination (IR) system (jiang et al., 2013). The third fragment, containing the 5′-upstream region, was amplified with pALDH2b-T5F and pALDH2b-T5R primers, and inserted into the vector constructed in step 2 at the BamHI site by the IR method. The CRISPR/Cas9 genome-targeting construct for OsALDH2b (target site: TGGGACAACAGAATTGTGCGCCG; protoscaler adjacent motif italicized) was designed with the web-based CRISPR/GE toolkit (http://skl.scau.edu.cn/) (Xie et al., 2017) and prepared using the CRISPR/Cas9 vector system (Ma et al., 2015). All constructs were introduced into rice with Agrobacterium-mediated transformation. Positive transformants were screened with HPT primers by PCR. The target site sequences of gene knockout mutants were sequenced and decoded with CRISPR/GE/DSDecodeM (Liu et al., 2015; Xie et al., 2017).

RNA extraction and qRT-PCR

Total RNA was extracted from rice tissues using TRIZOL reagent (Thermo Fisher Scientific, USA), and isolated RNA was treated with DNase 1. The treated RNA was then used for first-strand cDNA synthesis with oligo (dT) using the first-strand cDNA synthesis kit (Promega, USA). Two microliters of the reverse transcription product was used as the
TUNEL assay

Another developmental stage was confirmed by observing anther cross-sections with light microscopy. Preparation of anther sections and fertile (RF1b) and sterile (RF1b–mOrange) constructs were bombarded into onion epidermal cells by a helium-driven accelerator (PDS/1000; Bio-Rad USA). Cells that exhibited eGFP and mOrange fluorescence were imaged with a laser scanning confocal microscope (LSM7 DUO, Zeiss, Germany).

Measurement of malonaldehyde content in the anthers

Determination of MDA levels was by the thiobarbituric acid (TBA) method (Loreto and Velikova, 2001). Anther samples at different developmental stages (each 100 mg) were homogenized in 2 ml of 0.1% trichloroacetic acid solution, and the extract was centrifuged at 12000 g for 15 min; 0.5 ml of the supernatant was diluted to 1 ml with 0.5% TBA in 20% trichloroacetic acid. The mixture was heated at 95°C for 30 min and then cooled on ice. Supernatant absorbance was measured at 530 nm with a Synergy Mx Multi-Mode Reader (BioTek, USA), subtracting non-specific absorbance at 600 nm.

TUNEL assay

Another developmental stage was confirmed by observing anther cross-sections with light microscopy. Preparation of anther sections and fertile (RF1b) and sterile (RF1b–mOrange) constructs were bombarded into onion epidermal cells by a helium-driven accelerator (PDS/1000; Bio-Rad USA); these were prepared as previously described (Li et al., 2006; Luo et al., 2013). Fluorescein's green fluorescence (TUNEL signal) and propidium iodide's red fluorescence were imaged with 488 nm (excitation) and 520 nm (detection), and 488 nm (excitation) and 610 nm (detection), respectively, under a LSM 7 DUO laser scanning confocal microscope (Zeiss, Germany).

Subcellular localization

The coding region of OsALDH2b was amplified from wild-type cDNA with the primers OsALDH2b-cF and OsALDH2b-cR (Supplementary Table S2). After digestion with HindIII and BamHI, the fragments were fused in-frame with the enhanced green fluorescent protein (eGFP) coding sequence (Heim et al., 1995; Cormack et al., 1996), subcloned into a pUC-18-based vector and driven by the CaMV35S promoter to produce the transient expression vector ALDH–eGFP. A mutant orange fluorescent protein (mOrange) fused with a mitochondrial transit signal peptide derived from RF1b (Wang et al., 2006) was prepared as a positive control (RF1b–mOrange). These constructs were bombarded into onion epidermal cells by a helium-driven accelerator (PDS/1000; Bio-Rad, USA). Cells that exhibited eGFP and mOrange fluorescence were imaged with a laser scanning confocal microscope (LSM7 DUO, Zeiss, Germany).

Aldehyde dehydrogenase enzymatic assays

Full-length OsALDH2b cDNA (excluding the mitochondrial-targeting sequence) was isolated with the primers OsALDH2b-cFD and OsALDH2b-cR (Supplementary Table S2), and cloned into the pET32a(+) vector fused with a His-tag. The resultant plasmid was transformed into E. coli strain BL21 (DE3). Once the OD600 reached approximately 0.6, transformed cells were incubated at 18°C for 16 h with 1 mM isopropylthio-galactoside. The supernatant containing extracted proteins was purified with a Ni2+-nitrilotriacetic acid spin column. For the enzymatic assay, aldehyde was the substrate. ALDH enzymatic activity for reduction of NAD+ to NADH was evaluated by the increase of absorbance at 340 nm (Shin et al., 2009).

Results

Identification and phenotype of the osaldh2b mutant

We obtained a male-sterile mutant by screening a rice mutant library ( japonica cultivar Nipponbare) created with 60Co-γ-ray radiation. The mutant exhibited normal vegetative and panicle development, but failed to generate viable pollen, and never set seed (Fig. 1A–D). Analysis revealed that the sterility was caused by a loss-of-function mutation located in the OsALDH2b gene (see below); we therefore named this mutant osaldh2b. We crossed mutant plants with pollen grains from the indica variety (HHZ) to generate F1 hybrids. We examined 652 F3 individual plants that resulted from the cross, and observed that 507 plants were male fertile and 145 male sterile (40.5%) (Supplementary Table S3), indicating that the male-sterility phenotype was controlled by a recessive locus.

Map-based cloning and functional validation of OsALDH2b

To isolate the mutated gene conferring male sterility, we used 6652 segregants from the F2 and F3 families and a set of polymorphic markers covering the entire genome, and mapped the mutant locus to a 114-kb region on chromosome 6 (Fig. 2A). DNA sequencing analysis in this region of the mutant revealed a 7-bp deletion in the third exon of OsALDH2b (LOC_Os06g15990, annotated by RGAP; or Os06g0270900, annotated by RAP-DB), causing a frame shift to introduce a premature stop codon at the 125th amino acid (Fig. 2B).

To confirm that the male-sterile phenotype resulted from the mutation in OsALDH2b, we prepared a binary construct (OsALDH2b–C) that carried a 12.2-kb genomic DNA fragment of wild-type OsALDH2b to thoroughly test functional complementation of this fragment comprising OsALDH2b's 4.2-kb upstream 5′-UTR/promoter sequence, the entire 5.4-kb coding region (including introns), and a 2.6-kb downstream region. We transformed this construct into calli induced from heterozygous OsALDH2b/osaldh2b plants. Of 26 OsALDH2b–C transgenic plants (T0), six plants were homozygous for the osaldh2b allele, and these exhibited normal male fertility (Fig. 2C). Next, we used CRISPR/Cas9-based genome editing to knock out OsALDH2b in wild-type plants. As expected, the OsALDH2b-knockout plants (OsALDH2b–KO) exhibited a male-sterile phenotype, similar to the osaldh2b mutant (Fig. 2C; Supplementary Fig. S1). Therefore, we concluded that OsALDH2b is required for male development in rice.

OsALDH2b and its orthologs are highly conserved in monocot and eudicot species

To gain insight into OsALDH2b's evolutionary history, we used BLASTP with OsALDH2b's full-length amino acid sequence to find orthologs and create a phylogenetic tree. We retrieved 13 orthologous sequences from seven monocot species and six eudicot species from the database. Comparing these proteins showed that OsALDH2b had the highest similarity to orthologs from monocot plants (Sorghum bicolor, 91%; Zea mays, 92%; Setaria italica, 91%; Brachypodium distachyon, 90%; Hordeum vulgare, 88%; Secale cereal, 88%; and Aegilops tauschii, 88%). Similarity to eudicot orthologs was lower (Arabidopsis, 88%; Solanum lycopersicum, 79%; Hevea brasiliensis, 79%; Glycine max, 78%; Populus trichocarpa, 77%; and Brassica napus, 76%) (Supplementary Fig. S2). A phylogenetic tree generated using MEGA7 (Kumar et al., 2016) divided the proteins into two groups, monocots and eudicots (Fig. 3). Of the orthologs we
identified, the maize protein (RF2A) is required for anther development and is a functionally characterized fertility restorer for CMS-T maize lines (Liu et al., 2001; Cui et al., 1996). These data suggest that the ALDH-like proteins are highly conserved among monocot and eudicot plants, and potentially share a conserved function in male reproductive development.

OsALDH2b encodes a mitochondrial aldehyde dehydrogenase and is highly expressed in anthers

Sequence analysis showed that OsALDH2b encodes a predicted 549 amino acid mitochondrial aldehyde dehydrogenase (Supplementary Fig. S3). To verify OsALDH2b’s subcellular localization, we co-transformed OsALDH2b–eGFP and an RF1b–mOrange control into onion epidermal cells. Images demonstrated that the OsALDH2b–eGFP signal co-localized with RF1b–mOrange in mitochondria, indicating that OsALDH2b is a mitochondrion-localized protein (Fig. 4A).

To examine whether OsALDH2b has enzymatic activity for aldehydes, we purified recombinant OsALDH2b–His to use in enzyme activity assays. Here, reduction of NAD+ to NADH was measured as the increase in absorbance values at A340. When aldehyde was used as the substrate, recombinant
OsALDH2b–His exhibited significant enzymatic activity (Fig. 4B), demonstrating that the protein has aldehyde dehydrogenase activity.

To investigate OsALDH2b function, we analysed its expression pattern during rice development with qRT-PCR. OsALDH2b was expressed in both vegetative and reproductive organs. During anther development, OsALDH2b was highly expressed from the meiosis I stage (S7) until the middle microspore stage (S9b), and peaked at the meiosis II/tetrad (S8) and early microspore (S9a) stages (Fig. 4C); rice anther stages (S1–S12) were assigned as previously described (Zhang et al., 2011). OsALDH2b’s expression profile was consistent with transcriptome data (RiceXPro, http://ricexpro.dna.affrc.go.jp/) (Supplementary Fig. S4). These data suggest that OsALDH2b may function in anther and pollen development from meiosis to the microspore stages.

The osaldh2b mutant anthers accumulate excess malonaldehyde

Cellular redox state is a key factor for male gametogenesis (Zhang and Yang, 2014). MDA is the predominant product of oxidative stress and one of the most highly reactive of the endogenous aldehydes, which are triggered by hypoxic status during early anther development and potentially produce toxic byproducts (Voulgaridou et al., 2011). To determine whether OsALDH2b acts in anther development by reducing the aldehyde accumulation, we used a TBA assay to measure MDA content in anthers of both wild-type and osaldh2b plants.

In wild-type anthers, MDA content gradually increased from the microspore mother cell stage (S6) to meiosis/tetrad stages (S7/S8), and then decreased until the late microspore stage (S10) (Fig. 5). In osaldh2b anthers, however, the MDA level was much higher from the S8 to S10 stages. This result indicated that OsALDH2b acts as a detoxifying enzyme that eliminates aldehydes generated during anther and microspore development.
The osaldh2b mutant exhibits premature tapetal programmed cell death

MDA is a highly reactive aldehyde that reacts strongly with DNA and proteins (Voulgaridou et al., 2011). To examine whether MDA accumulation affects DNA fragmentation in osaldh2b anthers, we performed a TUNEL assay on anthers across developmental stages. Wild-type anthers showed strong TUNEL-positive signals in tapetal cells at the tetrad stage (S8b) (Fig. 6A–E, top). However, in osaldh2b tapetal cells, we detected TUNEL-positive signals in earlier stages, particularly in the metaphase I stage (S8a) (Fig. 6A–E, bottom). These results demonstrate that PCD-induced tapetal DNA fragmentation occurred at an earlier time point in the osaldh2b mutant, suggesting that excess MDA accumulation in the mutant’s developing anthers may accelerate PCD in tapetum cells.

The osaldh2b mutant exhibits abnormal tapetal degeneration and microspore development

To further investigate the role of OsALDH2b during male reproductive development, we analysed semi-thin sections of wild-type and mutant anthers. We observed no obvious differences between cells in wild-type and osaldh2b anthers, microsporocytes and somatic layers (including the epidermis, endothecium, middle layer, and tapetum) exhibited characteristic structures (Fig. 7A). Microsporocytes in both wild-type and osaldh2b anthers had progressed through normal meiosis (S7), during which the tapetum had become vacuolated (Fig. 7B); subsequently, tetrads of haploid microspores had formed (S8) (Fig. 7C). At the early microspore stage (S9a) in both genotypes, free microspores had been released from tetrads, the middle layer appeared thin, and the tapetum looked condensed, less vacuolated, and deeply stained (Fig. 7D).

We detected morphological differences between osaldh2b and wild-type anthers starting from the middle microspore stage (S9b): the wild-type tapetum was evident and microspores were round and vacuolated (Fig. 7E, top). The osaldh2b tapetum, however, appeared thinner, and microspores appeared irregularly shaped (Fig. 7E, bottom). At the late microspore stage (S10), wild-type tapetum had become hill-shaped, appeared highly condensed and deeply stained, and formed microspores containing a single, large central vacuole (Fig. 7E, top). By contrast, osaldh2b tapetum was less condensed and weakly stained; microspores appeared collapsed and exhibited uneven cytoplasm associated with abnormal vacuolization (Fig. 7E, bottom). At the bicellular pollen stage (S11), wild-type anthers exhibited typical falcate-shaped pollen grains and completely

Fig. 5. MDA content dynamics during anther development. MDA content of osaldh2b anthers was significantly higher than that in WT anthers at microspore stages (S9 and S10). These data are derived from three replicates; **P<0.01 by t-test.

Fig. 6. Tapetal nuclear DNA fragmentation in WT and osaldh2b anthers. The anthers in WT (top) and osaldh2b (bottom) from the microspore mother cell stage through the middle microspore stage were compared for nuclear DNA fragmentation (indicating PCD) using the TUNEL assay (A–E). Nuclei were stained with propidium iodide (red fluorescence); yellow signals indicate TUNEL-positive nucleus staining. MMC, microspore mother cell; T, tapetum; Msp, microspore. Scale bars: 50 µm.
OsALDH2b regulates tapetum degeneration in rice

degenerated tapetal cells (Fig. 7G, top). At this stage, osaldh2b
anther wall layers, including the epidermis and endothecium,
appeared disordered, enlarged, and broken; mutant plants had
produced severely aberrant microspores (Fig. 7G, bottom). At
the mature pollen stage (S12), in contrast to wild-type,
osaldh2b
pollen grains were irregularly shaped and had accumulated no
or less storage materials, and the anthers had shriveled (Fig. 7H).

We used transmission electron microscopy to study the de-
velopmental abnormalities at the microspore stages in more
detail. At the early microspore stage (S9a), wild-type tapetal
cytoplasm was highly condensed, nuclei were intact, and cells
exhibited a prominent nucleolus (Fig. 8A, top). Strikingly,
we observed no nucleolus in osaldh2b tapetum nuclei at this
stage (Fig. 8A, bottom). At the middle microspore stage (S9b),
the wild-type tapetum had collapsed and nuclei were lobed.
Enlarged U-shaped orbicules were evident on the inner tapetal
surface. The exine in wild-type microspores was well estab-
lished with distinct nexine, tectum, and bacula layers (Fig. 8B,
top). By contrast, at this stage in osaldh2b tapetal cells, nuclei
appeared completely degenerated, orbicules were smaller, and
electron-dense sporopollenin reduced. Moreover, the exine
of osaldh2b microspores was much thinner compared with
those in WT. The osaldh2b microspores contained few organ-
elles in cytoplasm observed in electron-transparent channels
(Fig. 8B, bottom). At the late microspore stage (S10) in wild-
type, we observed further degenerated tapetum and vacuolated

---

Fig. 7. Transverse section analysis reveals anther development in WT and osaldh2b. (A–D) No obvious differences were observed between WT (top) and osaldh2b (bottom) anthers from S6 to S9a. (E) Compared with WT anthers, osaldh2b anthers displayed thinner tapetum and irregular microspores in the locule at S9b. (F) The osaldh2b tapetum was less condensed and weakly stained, and microspores were collapsed with uneven cytoplasm at S10. (G) The osaldh2b anther wall layers, including epidermis and endothecium, appeared disordered, enlarged, and broken, and exhibited severely abnormal microspores at S11. (H) The osaldh2b anthers at S12 exhibited collapsed pollen grains with no or less cellular content accumulation. E, epidermis; En, endothecium; ML, middle layer; Mp, mature pollen; Ms, microsporocyte; Msp, microspores; T, tapetum; Tds, tetrads. Scale bars: 20 μm.
micropores with abundant cytoplasm (Fig. 8C, top). At this stage, osaldh2b tapetum exhibited cavities, with low-electron-density orbicules on its surface, indicating that its tapetum had completely and prematurely degraded. In addition, microspore exine was much thinner (Fig. 8C, bottom). We further investigated the expression of eight genes related to male reproductive development. The qRT-PCR results (Supplementary Fig. S5) showed that the mutation of OsALDH2b disrupted the expression of genes involved in tapetum degeneration (TDR, UDT1, OsGAMYB, RTS) and pollen wall formation (WDA1, CYP704B2, CYP703A3, OsC6).

Together, these results suggested that defective mutation in OsALDH2b leads to excessive aldehyde accumulation, which causes early tapetal PCD, premature cellular degeneration, and aborted microspore development, resulting in male sterility (Fig. 9).

Discussion

The tapetum is arguably the most important layer of anther tissue during male meiosis and microsporogenesis, providing enzymes, signals, and nutrients for pollen development via PCD-based cellular degeneration (Zhang et al., 2011; Guo and Liu, 2012). Many components and factors participate in the process of tapetum development, such as transcription factors, receptor-like kinases, and transporters. The bHLH transcription factors TDR, EAT1/DTD, and TIP2 function as crucial positive regulators to promote tapetal PCD (Li et al., 2006; Ji et al., 2013; Niu et al., 2013; Fu et al., 2014). Mutation of their genes leads to vacuolated and prematurely degraded tapetum. In this study, we identified a rice male sterility mutant, osaldh2b; its wild-type gene encodes a conserved mitochondrial aldehyde dehydrogenase, OsALDH2b (Figs 1–4). Cytological analysis showed that osaldh2b exhibited more rapid, prominent degradation of tapetal cell nuclei and formation of abnormal tapetal secretory structures at microspore stages (Figs 7–8). Consistent with the nucleus degradation, tapetum DNA fragmentation (indicating PCD) occurred earlier in the osaldh2b mutant, at the prophase I stage (Fig. 6). Therefore we infer that OsALDH2b plays an important role in anther development and pollen formation by negatively regulating tapetal PCD. Additionally, expression analysis of some marker genes related to anther development indicates that the defective OsALDH2b caused disorder of the regulatory networks for anther development (Supplementary Fig. S5). The expression of TDR is increased from meiosis to microspore stages in osaldh2b. As the function of TDR is to promote the initiation of tapetal PCD (Li et al., 2006), the up-regulated change of TDR expression is consistent with the earlier occurrence of tapetal PCD in osaldh2b anthers. Furthermore, the expression of GAMYB,
which is involved in the down-regulation of TDR expression in anthers (Aya et al., 2009; Liu et al., 2010), is decreased in the mutant. According to the expression analysis, it seems that TDR may act downstream of OsALDH2b in regulating tapetal PCD, but this needs further investigation.

Dynamic redox status is an emerging factor affecting tapetum specification and timing degradation. Two Cys-rich metallothioneins, OsMT2b and OsMT-I-4b, have been identified as ROS scavengers. DTC1 interacts with OsMT2b and inhibits the ROS scavenging activity of OsMT2b to ensure timely production of ROS for proper initiation of tapetal PCD and cellular degradation, and pollen abortion. ACS, acetyl coenzyme A synthetase. TCA, tricarboxylic acid.

propose that the MDA accumulation might serve as a signal to initiate the premature tapetal PCD in this mutant.

Although OsALDH2b is constitutively expressed in vegetative organs, especially in leaf, the osaldh2b mutant does not show vegetative defects. We reason that there might be functional divergence of mtALDH orthologs as described in maize (Liu and Schnable, 2002). Two mtALDHs, RF2A and RF2B, have differential accumulation and distinct enzymatic activities with their substrates; RF2A, but not RF2B, accumulates to high levels in tapetal cells and is involved in male fertility (Cui et al., 1996; Liu et al., 2001). Based on previous phylogenetic analysis of plant ALDHs, OsALDH2b is more similar to maize RF2A and OsALDH2a is more similar to RF2B (Tsui et al., 2003). A possible role of OsALDH2a may be to eliminate acetaldehyde in vegetative tissues, so as to increase submergence tolerance (Nakazono et al., 2000). Altogether, we conclude that mtALDHs have undergone functional specialization during evolution to accommodate endogenous or exogenous stresses in different developmental organs.

Supplementary data

Supplementary data are available at JXB online.

Fig. S1. CRISPR/Cas9 OsALDH2b knockout.
Fig. S2. Sequence alignment of ALDH plant orthologs.
Fig. S3. OsALDH2b amino acid sequence.
Fig. S4. OsALDH2b expression profile based on RiceXPro.
Fig. S5. Expression analysis of eight genes related to anther development.
Table S1. Molecular markers used for fine mapping.
Table S2. Primers used for vector construction and expression analysis.
Table S3. Genetic analysis of osaldh2b.

Acknowledgements

This work is supported by the National Natural Science Foundation of China (31701051), the Natural Science Foundation of Guangdong Province (2017A030310273) and the Key Research Program of Guangzhou Science Technology and Innovation Commission (201707020016).

References

Aya K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, Nishimura M, Matsuoka M. 2009. Gibberellin modulates anther development in rice via the transcriptional regulation of GAMYB. The Plant Cell 21, 1453–1472.
Bai W, Wang P, Hong J, et al. 2019. Earlier Degraded Tapetum1 (EDT1) Encodes an ATP-citrate lyase required for tapetum programmed cell death. Plant Physiology 181, 1223–1238.
Biswas MS, Mano J. 2015. Lipid peroxide-derived short-chain carboxyls mediate hydrogen peroxide-induced and salt-induced programmed cell death in plants. Plant Physiology 168, 885–898.
Blokhina O, Fagerstedt KV. 2010. Oxidative metabolism, ROS and NO under oxygen deprivation. Plant Physiology and Biochemistry 48, 359–373.
Cai C, Zhu J, Lou Y, Guo Z, Xiong S, Wang K, Yang Z. 2015. The functional analysis of OsTDF1 reveals a conserved genetic pathway for tapetal development between rice and Arabidopsis. Science Bulletin 60, 1073–1082.
Cai W, Zhang D. 2018. The role of receptor-like kinases in regulating plant male reproduction. Plant Reproduction 31, 77–87.
et al. 2013. A detrimental mitochondrial-nuclear interaction causes cytoplasmic male sterility in rice. Nature Genetics 45, 573–577.

Ma H. 2005. Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. Annual Review of Plant Biology 56, 385–403.

X, Zhang Q, Zhu Q, et al. 2015. A Robust CRISPR/Cas9 system for convenient, high-efficiency multiplex genome editing in monocot and dicot plants. Molecular Plant 8, 1274–1284.

Nakazono M, Tsuji H, Li Y, Saisho D, Arimura S, Tsutsuji M, Hiraï A. 2004. Expression of a gene encoding mitochondrial aldehyde dehydrogenase in rice increases under submerged conditions. Plant Physiology 134, 587–598.

Niu N, Liang W, Yang X, Jin W, Wilson ZA, Hu J, Zhang D. 2013. EAT1 promotes tapetal cell death by regulating aspartic proteases during male reproductive development in rice. Nature Communications 4, 1445.

Rodrigues SM, Andrade MO, Gomes AP, Damatta FM, Baracat-Pereira MC, Fontes EP. 2006. Arabidopsis and tobacco plants ectopically expressing the soybean antifungal-like ALDH7 gene display enhanced tolerance to drought, salinity, and oxidative stress. Journal of Experimental Botany 57, 1909–1918.

Shen Y, Zhang Y, Yang C, Lan Y, Liu L, Liu S, Chen Z, Ren G, Wan J. 2012. Mutation of OsALDH7 causes a yellow-colored endosperm associated with accumulation of oryzanmatic A in rice. Planta 235, 433–441.

Shin JH, Kim SR, An G. 2009. Rice aldehyde dehydrogenase7 is needed for seed maturation and viability. Plant Physiology 149, 905–915.

Sorensen AM, Kröber S, Unte US, Huijser P, Dekker K, Saedler H. 2003. The Arabidopsis ABORTED MICROSPORÉES (AMS) gene encodes a MYC class transcription factor. The Plant Journal 33, 413–423.

Sunkar R, Bartels D, Kirch HH. 2003. Overexpression of a stress-inducible aldehyde dehydrogenase gene from Arabidopsis thaliana in transgenic plants improves stress tolerance. The Plant Journal 35, 452–464.

Tan H, Liang W, Hu J, Zhang D. 2012. MTR1 encodes a secretory fasin-like glycoprotein required for male reproductive development in rice. Developmental Cell 22, 1127–1137.

Tsuji H, Tsutsuji N, Sasaki T, Hiraï A, Nakazono M. 2003. Organ-specific expressions and chromosomal locations of two mitochondrial aldehyde dehydrogenase genes from rice (Oryza sativa L.), ALDH2a and ALDH2b. Gene 305, 195–204.

Vasiiliou V, Pappa A, Petersen DR. 2000. Role of aldehyde dehydrogenases in endogenous and xenobiotic metabolism. Chemico-Biological Interactions 129, 1–19.

Vougalgiou GP, Anestopoulos I, Franco R, Panayiotidis MI, Pappa A. 2011. DNA damage induced by endogenous aldehydes: current state of knowledge. Mutation Research 711, 13–27.

Wang H, Chu Z, Ma X, Li R, Liu YG. 2013. A high through-put protocol of plant genomic DNA preparation for PCR. Acta Agronomica Sinica 39, 1200–1205.

Wang Z, Zou Y, Li X, et al. 2006. Cytoplasmic male sterility of rice with boro II cytoplasm is caused by a cytotoxic peptide and is restored by two related PPR motif genes via distinct modes of mRNA silencing. The Plant Cell 18, 676–687.

Wilson ZA, Zhang DB. 2009. From Arabidopsis to rice: pathways in pollen development. Journal of Experimental Botany 60, 1479–1492.

Xie X, Ma X, Zhu Q, Zeng D, Li G, Li YG. 2017. CRISPRGE: a convenient software toolkit for CRISPR-based genome editing. Molecular Plant 10, 1246–1249.

Yi J, Moon S, Lee YS, Zhu L, Liang W, Zhang D, Jung KH, An G. 2016. Defective Tapetum Cell Death 1 (DTC1) regulates ROS levels by binding to metallothionein during tapetum degeneration. Plant Physiology 170, 1611–1623.

Yu J, Zhang D. 2019. Molecular control of redox homeostasis in specifying the cell identity of tapetal and microsporocyte cells in rice. Rice 12, 42.

Yoshida A, Rzhetsky A, Hsu LC, Chang C. 1998. Human aldehyde dehydrogenase gene family. European Journal of Biochemistry 251, 549–557.

Zhang D, Luo X, Zhu L. 2011. Cytological analysis and genetic control of rice anther development. Journal of Genetics and Genomics – Yi Chuan Xue Bao 38, 270–290.

Zhang D, Yang L. 2014. Specification of tapetum and microsporocyte cells within the anther. Current Opinion in Plant Biology 17, 49–55.

Zhang G, Shi J, Liang W, Zhang D. 2016. ATP binding cassette G transporters and plant male reproduction. Plant Signaling & Behavior 11, e1136764.

Zhou ML, Zhang Q, Zhou M, et al. 2012. Aldehyde dehydrogenase protein superfamily in maize. Functional & Integrative Genomics 12, 683–691.