Rice Aldehyde Dehydrogenase7 Is Needed for Seed Maturation and Viability[1][W][OA]

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Aldehyde dehydrogenases (ALDHs) catalyze the irreversible oxidation of a wide range of reactive aldehydes to their corresponding carboxylic acids. Although the proteins have been studied from various organisms and at different growth stages, their roles in seed development have not been well elucidated. We obtained T-DNA insertional mutants in *OsALDH7*, which is remarkably inducible by oxidative and abiotic stresses. Interestingly, endosperms from the *osaldh7* null mutants accumulated brown pigments during desiccation and storage. Extracts from the mutant seeds showed a maximum absorbance peak at 360 nm, the wavelength that melanoidin absorbs. Under UV light, those extracts also exhibited much stronger fluorescence than the wild type, suggesting that the pigments are melanoidin. These pigments started to accumulate in the late seed developmental stage, the time when *OsALDH7* expression began to increase significantly. Purified OsALDH7 protein showed enzyme activities to malondialdehyde, acetaldehyde, and glyceraldehyde. These results suggest that OsALDH7 is involved in removing various aldehydes formed by oxidative stress during seed desiccation. The mutant seeds were more sensitive to our accelerated aging treatment and accumulated more malondialdehyde than the wild type. These data imply that OsALDH7 plays an important role in maintaining seed viability by detoxifying the aldehydes generated by lipid peroxidation.

The major regulatory factors that control seed aging are oxidative stress, lipid peroxidation, and respiration (Sun and Leopold, 1995; Bailly et al., 1996; Akimoto et al., 2004). Lipid peroxidation and respiration result in the formation of reactive aldehydes such as malondialdehyde (MDA) and acetaldehyde, which tend to react with proteins and amino acids (Mueller, 1998; Almeras et al., 2003; Weber et al., 2004). Those reactions cause aging and seed damage (Zhang et al., 1995, 1997). Until recently, a physiological approach has been taken in research on seed aging, and molecular and genetic studies have been seldom reported (Clerkx et al., 2004a).

Rice (*Oryza sativa*) is an important food crop, especially in Asia. Although stress tolerance has been extensively evaluated in an effort to develop advanced cultivars, the aging of seeds is an important economic problem that has been rarely examined (Devaiah et al., 2007). Their deterioration by lipid peroxidation leads to undesirable taste, color, and odor (Robertson et al., 1973; Nakayama et al., 1981; List et al., 1992). This is caused by various reactive aldehydes containing MDA, which are volatile aromatic compounds. Those aldehydes also bind with proteins and amino acids nonenzymatically, resulting in the accumulation of brown pigments during seed storage (Sun and Leopold, 1995).

Aldehydes are intermediates in several fundamental metabolism pathways for carbohydrates, vitamins, steroids, amino acids, and lipids (Yoshida et al., 1998). They are also produced in response to environmental stresses that disturb metabolism, including salinity, dehydration, desiccation, cold, and heat shock (Bartels, 2001). Although indispensable for an organism, excessive amounts threaten seed survival because of their chemically reactive nature and the toxic effect of the molecules (Lindahl, 1992). Therefore, aldehyde levels in cells must be regulated tightly.

Aldehyde dehydrogenases (ALDHs) are represented by a protein super-family that can be categorized into 21 families in eukaryotes (Perozich et al., 1999; Sophos et al., 2001; Sophos and Vasiliou, 2003; Fong et al., 2006). Some ALDHs are substrate specific while others have a highly variable substrate specificity (Sophos and Vasiliou, 2003). ALDHs catalyze the irreversible oxidation of a wide range of reactive aldehydes to their corresponding carboxylic acids (Perozich et al., 1999; Kirch et al., 2005). Therefore, they play a pivotal role in detoxifying the aldehydes generated by environmen-

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tal stresses. Plant enzymes are represented in 11 ALDH families (Kirch et al., 2004). Despite their importance, the physiological functions of ALDHs in plants have rarely been studied (Liu et al., 2001; Liu and Schnable, 2002; Sunkar et al., 2003; Tsuji et al., 2003).

Family 7 ALDHs (antiquitins) are comparatively distinguishable from other ALDH families, because they show low sequence identity (approximately 30%) to ALDHs from other families (Vaciliou et al., 1999). However, the amino acid sequences among Family 7 members are highly homologous (about 60%–70%; Lee et al., 1994; Fong et al., 2006). In contrast to animal antiquitins that do not exhibit any inducible response to stresses (Lee et al., 1994; Fong et al., 2006), antiquitin in garden pea (*Pisum sativum*) is inducible by dehydration (Guerrero et al., 1990).

Overexpression of Arabidopsis (*Arabidopsis thaliana*) and soybean (*Glycine max*) *ALDH7* confers tolerance to osmotic and oxidative stresses in transgenic plants (Kotchoni et al., 2006; Rodrigues et al., 2006). Moreover, their MDA and hydrogen peroxide contents are decreased. This suggests that ALDH7s function not

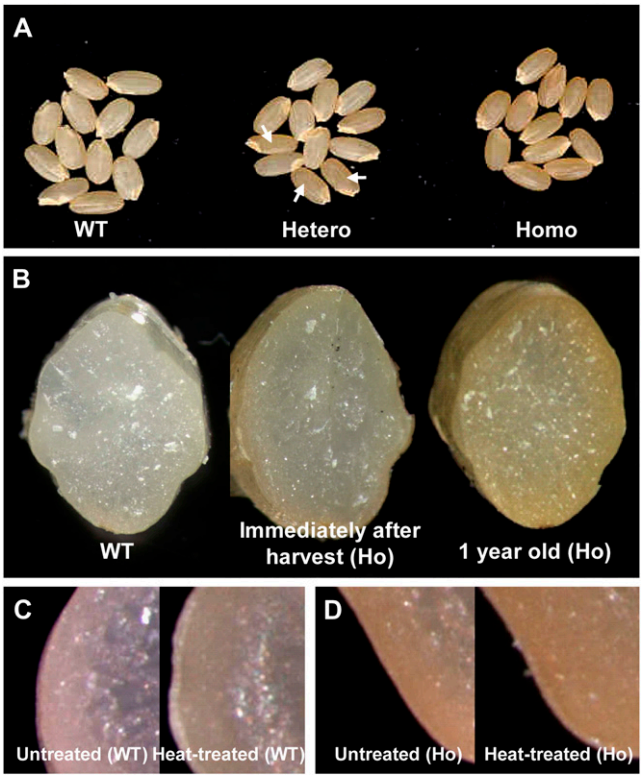

Figure 1. Phenotypes of T-DNA-tagged mutant line that accumulates brown pigment in seeds. A, Wild type (WT; left), heterozygous (middle), and homozygous (right) plants. Arrows indicate mutant seeds in heterozygous line. B, Cross sections of WT (left), mutant seeds immediately after harvest (middle), and seeds at 1 year after storage (right). C, Cross sections of WT seeds before (left) and after (right) heat treatment. D, Cross sections of mutant seeds before (left) and after (right) heat treatment. Ho, Homogenic mutant.

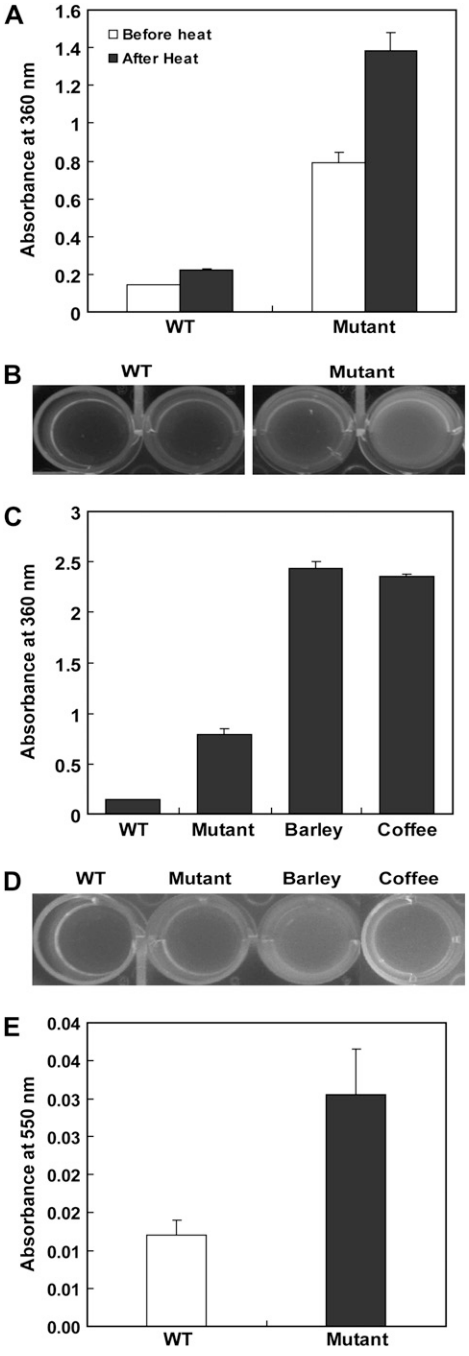

Figure 2. Absorbance patterns for mature seeds of rice, barley, and coffee. A, Comparison of absorbance patterns for pigment extracts from wild type (WT) or mutant before or after heat stress. B, Fluorescence intensity under UV light for samples in A. C, Comparison of absorbance patterns for extracts from rice, barley, and coffee. D, Fluorescence intensity under UV light for samples in C. E, Measurement of Amadori products of WT and mutant seeds. Seed proteins (200 μg) isolated from stored seeds were used for the test. The protein solution was mixed with nitro blue tetrazolium reagent. After incubation at 42°C for 10 to 20 min, absorbance was measured at 550 nm.

only as aldehyde-detoxifying enzymes but also as efficient scavengers of reactive oxygen species and as lipid peroxidation-inhibiting enzymes.

In this study, we used null mutants in the rice *ALDH7* gene to investigate the functional roles of ALDH7 during seed development and storage.

RESULTS

Isolation of a Mutant That Accumulates Brown Pigments in Mature Seeds

Screening T-DNA insertional mutant populations for abnormality in their mature seeds resulted in the identification of a mutant that accumulates brown pigments (Fig. 1A). These pigments were found in the pericarp as well as the inner endosperm (Fig. 1B). This pattern is unusual, because pigments are usually accumulated mainly in the pericarp in color-seed cultivars. The level of pigment increased as the storage time was extended (Fig. 1B). This implies that such accumulation is induced by a factor generated during seed maturation and the storage period.

During the late stage of seed development and in storage, the water content in rice seeds dropped to <20%, which caused stress to the cells that still survived. Post-harvest heating to dry those seeds was another source of stress for the aleurone and embryo cells. To examine whether pigments were generated by these stresses, we treated wild-type and mutant seeds for 2 months at 60°C. This exposure induced pigment accumulation in the wild-type seeds (Fig. 1C) while enhancing such accumulation in the mutants (Fig. 1D).

The Pigment Is Likely Melanoidin

To analyze the components of this accumulated pigment, we scanned the absorption spectra of the aqueous extracts from wild-type and mutant seeds. Extracts from both genotypes peaked at 360 nm, although the mutant extract showed a peak that was up to 4 times higher (Fig. 2A). Heat treatment of seeds for 2 months at 60°C increased the absorbance in both the mutant and wild type (Fig. 2A). Under UV light, the extracts displayed fluorescence, with intensity being much greater from the mutants (Fig. 2B). These results suggest that the pigment is a product of a Maillard reaction, which nonenzymatically produces melanoidin from carbonyl and amino compounds during storage (Adams et al., 2005; Papetti et al., 2006; Adams and Brown, 2007; Niquet and Tessier, 2007). High temperatures and long response times are major factors for melanoidin production. Its absorbing wavelength of approximately 360 nm (Adams et al., 2005) is characteristic of fluorescent materials. Therefore, the pigments accumulated in our mutant seeds are likely melanoidin compounds.

For further verification, we compared our rice extracts with those from the seeds of barley (*Hordeum vulgare*) and coffee (*Coffea arabica*), two well-known materials rich in melanoidin. Again, peaks occurred at 360 nm, with their heights being correlated with pigment intensity (Fig. 2C). Those extracts also contained fluorescent materials (Fig. 2D).

Amadori products are intermediates of the Maillard reaction (Sun and Leopold, 1995; Murthy and Sun, 2000). Therefore, measurement of those products has been commonly used for analyzing Maillard reaction products (Chandra et al., 2008). Here, we purified proteins from the seed extracts on 10-DG columns, and the Amadori products were measured by the nitro blue tetrazolium method (Murthy and Sun, 2000). This analysis showed that mutant seeds accumulated up to 3 times more products compared with the wild type (Fig. 2E). These results indicate that the pigments extracted from mutant seeds are likely melanoidin produced by the Maillard reaction.

The Mutant Phenotype Results from a Knockout of *OsALDH7* by T-DNA Insertion

Because the mutant phenotype cosegregated with T-DNA, we determined DNA sequences for the

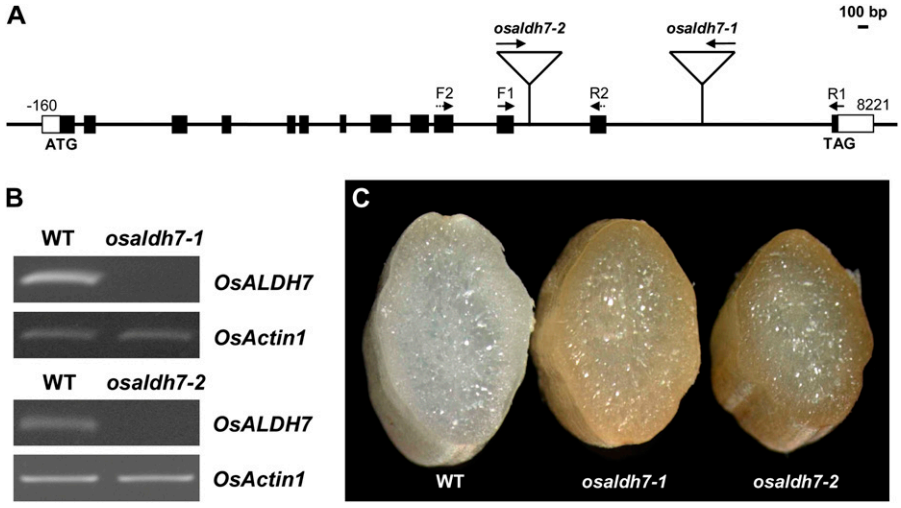

Figure 3. Characterization of T-DNA insertional mutants. A, T-DNA insertion positions within *OsALDH7*. T-DNA is inserted into 12th and 13th introns in *osaldh7-1* and *osaldh7-2* alleles, respectively. Black boxes indicate exons and white boxes indicate 5′- and 3′-untranslated regions. Solid lines between exons are introns. B, RT-PCR analyses of *OsALDH7* expression in *osaldh7* mutants, with primers F1, F2, R1, and R2. *OsActin1* was used to monitor equal loading. C, Cross sections of mature seeds from WT (left), *osaldh7-1* (middle), and *osaldh7-2* (right).

T-DNA flanking region by inverse PCR (An et al., 2003; Jeong et al., 2006). Analysis of the flanking sequence revealed that T-DNA was inserted in the 13th intron of *OsALDH7* (Fig. 3A). We designated that mutant as *osaldh7-1*. We also isolated another allele, *osaldh7-2*, where T-DNA was located in the 12th intron of *OsALDH7* (Fig. 3A). Reverse transcription (RT)-PCR analyses of transcripts accumulated in the seeds showed that *OsALDH7* was not expressed in both alleles (Fig. 3B), in which higher levels of brown pigments were accumulated (Fig. 3C).

To examine the subcellular region in which OsALDH7 functions, we conducted a localization experiment using protoplasts purified from Oc suspension cells. OsALDH7-GFP protein was localized in the cytoplasm, which was colocalized with mRFP, a well-known cytosol marker (Supplemental Fig. S1). This result is consistent with a previous report of Arabidopsis ALDH7B4 localization (Kotchoni et al., 2006).

OsALDH7 Expression Is Dramatically Increased under Oxidative Stress

Kotchoni et al. (2006) and Rodrigues et al. (2006) have reported that ALDH7 in Arabidopsis and soybean functions to detoxify aldehydes generated under oxidative stresses. To examine whether rice ALDH7 also plays an important role in abiotic stresses, we investigated *OsALDH7* expression patterns after seedlings were treated with cold, heat, drought, paraquat,

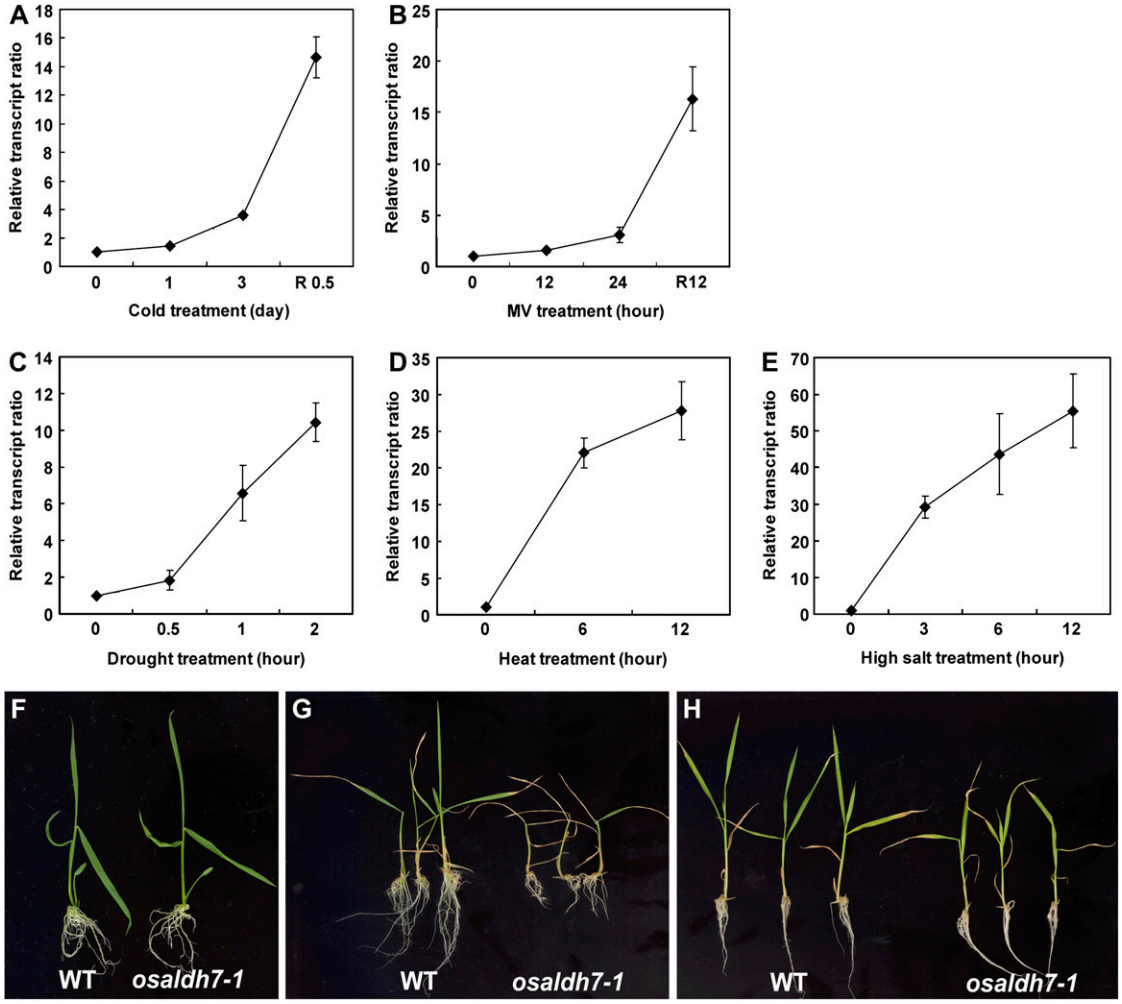

Figure 4. Expression patterns of *OsALDH7* during cold (A), MV (B), drought (C), heat (D), or high salt (E) treatments. For cold treatment, seedlings were placed at 4°C under lights for 3 d, then transferred to room temperature for recovery. For MV treatment, seedling roots were submerged in 20 μM MV solution for 1 d, then transferred to distilled water for recovery. For heat stress, seedlings were treated at 50°C. Drought stress was introduced by placing plants on air at 30°C. For high salt stress, seedlings were treated with 250 mM NaCl. Relative transcript ratio implies *OsALDH7* transcript levels normalized by *OsActin1* mRNA level. One-week-old wild-type (WT) and *osaldh7-1* seedlings grown under normal growth condition (F). G and H, Phenotypes of WT (left) and *osaldh7-1* (right) seedlings after cold (G) and high salt (H) stresses.

or high salt. Transcript levels were significantly induced by such stresses (Fig. 4). Interestingly, two expression patterns were revealed. Whereas *OsALDH7* transcripts were not increased during cold and paraquat (MV) treatment but increased remarkably during the recovery period following the treatment (Fig. 4, A and B), those transcripts were elevated significantly during the application of drought, heat, or high salt (Fig. 4, C–E). The cold and MV treatments were probably too harsh to sustain most physiological reactions. However, when treated seedlings were returned to normal conditions, their responses such as lipid peroxidation by membrane degradation started to accelerate. We speculate that the *OsALDH7* transcription level was increased to remove aldehydes that formed within the lipid peroxidation pathway. By comparison, drought, heat, and high salt stresses did not seriously inhibit cellular operation. Thus, those treated cells could respond to stress without much delay.

Wild type and *osaldh7-1* mutants were germinated and grown on Murashige and Skoog (MS) media. Under the normal growth conditions, the mutant plants were not different from wild type (Fig. 4F). When *osaldh7-1* seedlings were treated with cold or high salt, they were more sensitive than the wild type (Fig. 4, G and H). Therefore, we conclude that OsALDH7 is needed for the detoxification of aldehydes that form under various stress conditions.

OsALDH7 Expression Patterns during Seed Development Correspond with Pigment Accumulation Patterns in Seeds

Pigment accumulation started in the late stage of seed development (35 d after pollination), mainly in pericarp tissues (Fig. 5A), with a higher amount of pigment being accumulated at the fully mature stage (Fig. 5B). Stored seeds showed even further accumulation, especially in the mutant (Fig. 5C).

We looked for correlations between pigment accumulation and *ALDH7* transcript levels during seed development. Real-time PCR analyses indicated that those levels were low early on but then significantly increased at the late stage (Fig. 5D). A sudden rise in *OsALDH7* expression occurred at the time when pigment accumulation started. This timing coincides with a rapid decline in water content in seeds (Hoshikawa, 1989), suggesting that OsALDH7 is associated with desiccation. Therefore, the phenotypes observed in our *osaldh7* mutants were likely due to a failure to remove the aldehydes generated by dehydration stress.

Enzyme Activity of OsALDH7

We examined whether *OsALDH7* indeed encodes for an enzyme that catalyzes aldehydes. The *OsALDH7* full-length cDNA clone was inserted into the pET-topo vector containing six His residues. This molecule was

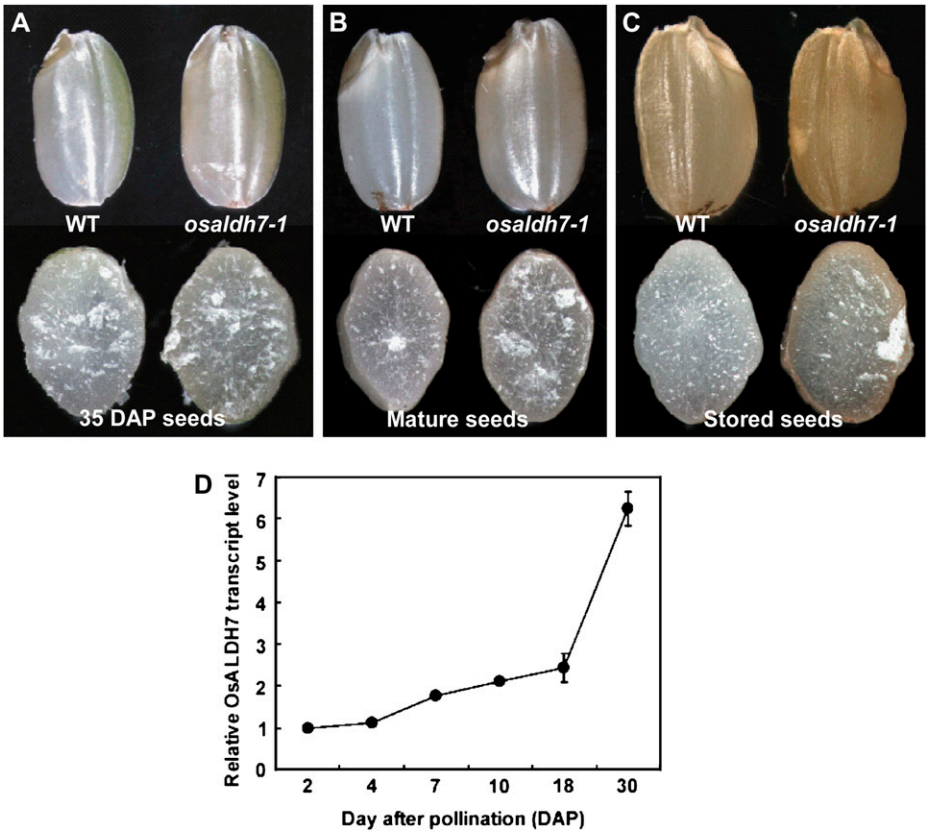

Figure 5. Pigment accumulation during seed development. A to C, Intact (top) or cross-sectioned (bottom) seeds of 35-d-old (A), mature (B), and stored (C) samples from wild type (WT; left) and *osaldh7-1* (right). D, Expression pattern of *OsALDH7* during seed development analyzed by real-time PCR.

introduced into *Escherichia coli* strain BL21 (DE3), and OsALDH7-His tag fusion molecules were induced by isopropylthio-β-galactoside treatment. Using a His-tag column, we purified OsALDH7-His protein and performed immunoblots to demonstrate that the purified protein was OsALDH7 (data not shown).

The purified protein had enzyme activity against MDA, a by-product formed in the nonenzymatic lipid peroxidation pathway (Fig. 6A). MDAs are electrophilic, strongly binding to peptides and amino acids. Therefore, the MDA content in cells is evidence of their environmental conditions and can be used as a general index of oxidative stress. Here, OsALDH7 catalyzed other aldehydes as well, including acetaldehyde (Fig. 6B) and glyceraldehyde (Fig. 6C). Acetaldehyde is generated from the alcohol fermentation pathway and glyceraldehyde is related with carbohydrate metabolism. These results suggest that OsALDH7 has broad substrates and may participate in multiple functions such as metabolic pathways and abiotic stresses.

MDA Contents Increase in *osaldh7* Seeds

If one of the roles of OsALDH7 is to remove MDA, then MDA contents should be higher in the *osaldh7* seeds than in the wild type. Therefore, we measured those contents in developing and mature seeds. During the former stage, seeds are exposed to severe oxidative stress due to desiccation. Such an environment promotes the acceleration of lipid peroxidation and an increase in MDA. Performing a thiobarbituric acid-reactive-substances assay, we found that, as expected, MDA contents were higher in the *osaldh7* seeds (Fig. 7, A and B). Although contents in wild-type seeds were somewhat elevated, those in the mutants were more significantly increased (up to 2-fold), especially after seed harvest. Patterns were similar with A_{360} (Fig. 7, C and D). These data demonstrate that OsALDH7 plays a pivotal role in the removal of MDA that forms during seed desiccation.

osaldh7 Seeds Show Accelerated Seed Aging

Because lipid peroxidation is involved in seed deterioration (Bailly et al., 1996, 1998), we hypothesized that the viability of *osaldh7* mutant seeds would be diminished. Thereupon, we applied an accelerated aging (AA) treatment, a routine experiment (McDonald, 1999). Here, the germination rate was drastically decreased in the *osaldh7* seeds to about one-half that of the wild type (Fig. 8, A and B). Mutant seeds also accumulated higher levels of MDA during the AA treatment (Fig. 8, C and D). These results indicate that OsALDH7 is required for the maintenance of seed viability, detoxifying the MDA that is generated during seed storage.

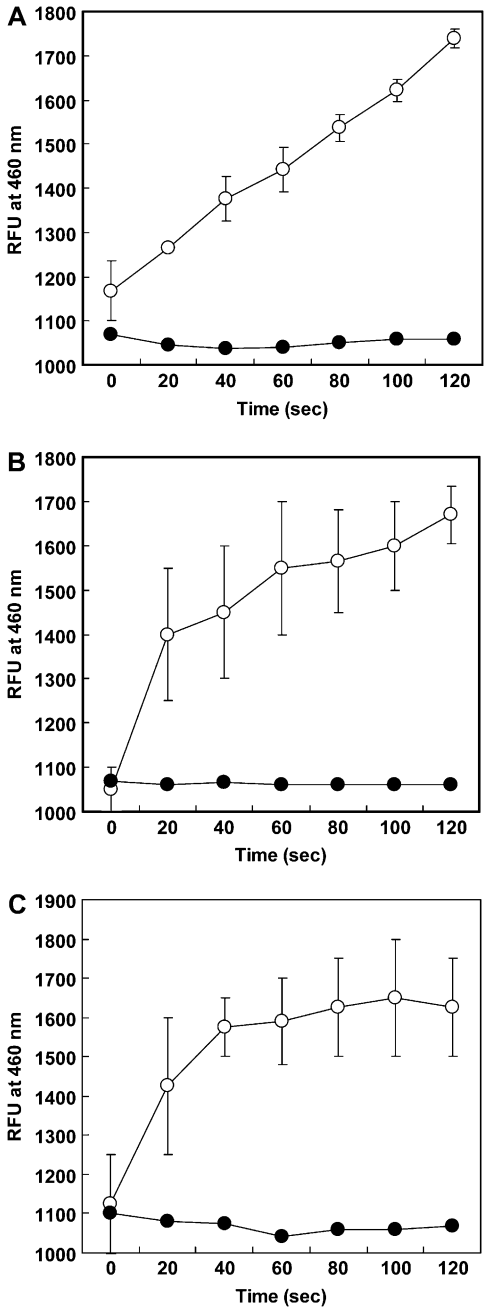

Figure 6. ALDH enzyme assay. OsALDH7 protein was produced in *E. coli*, with pET expression vector (pET-OsALDH7). ALDH activity was measured using MDA (A), acetaldehyde (B), and glyceraldehyde (C) as substrates. Extract of *E. coli* containing pET-U52 vector expressing rice U box gene U52 served as negative control. White circles, pET-ALDH; black circles, pET-U52.

DISCUSSION

When ordinary seeds complete their maturation, they must become desiccated if long-term storage is to be successful (Murthy et al., 2003; Gilles et al., 2007). Their tolerance to dry conditions is based on their formation of desiccation-related proteins and materials (osmoprotectants), such as late embryogenesis abundant (LEA) proteins and sugars (Sun et al., 1994; Black et al., 1999; Wise and Tunnacliffe, 2004). *LEA* genes begin to be expressed in the mid- to late stage of

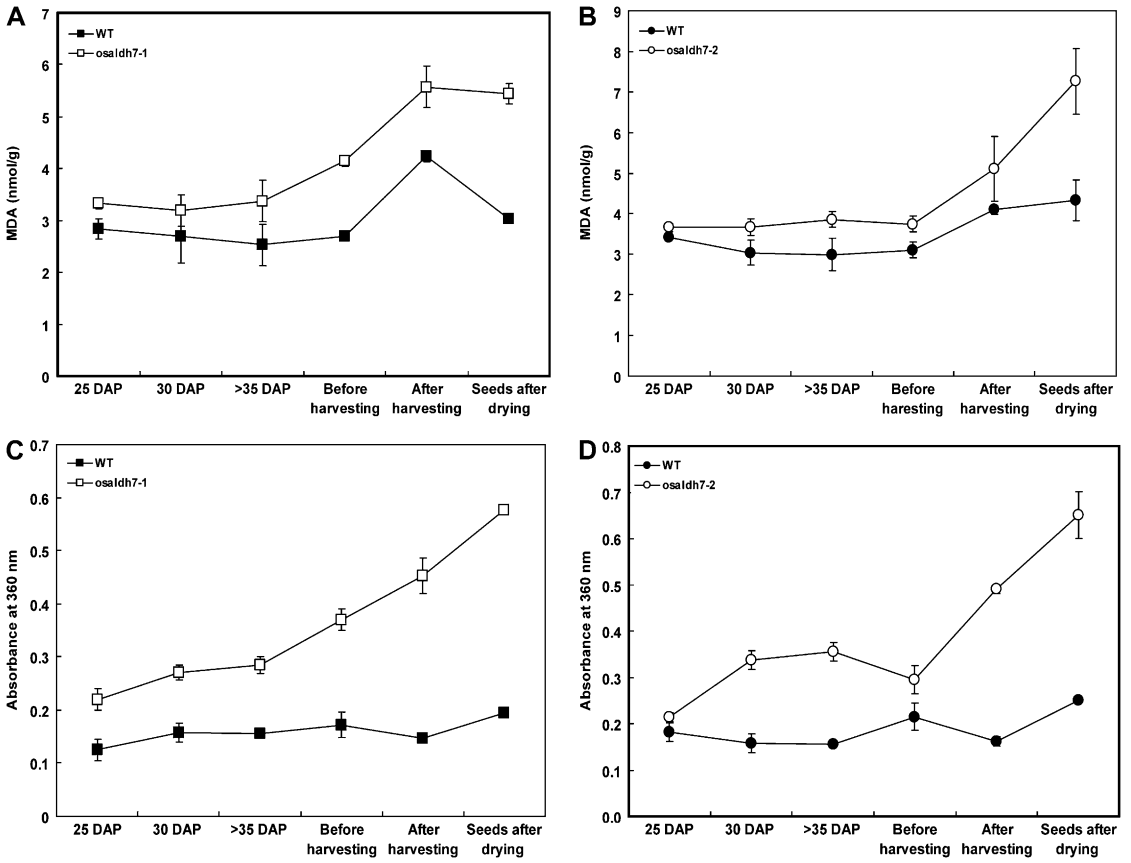

Figure 7. Correlation between MDA and Maillard reaction products during seed development and storage. A and B, MDA levels of wild-type (WT) and *osaldh7-1* and *osaldh7-2* seeds during late stages of seed development and storage. C and D, Absorbance patterns at 360 nm of the extracts from the mutant seeds (white) and WT (black).

seed development (Bartels et al., 1988; Hartwigsen and Goggi, 2002). Their overexpression enhances tolerance to salt, drought, and osmotic stresses in transgenic plants (Brini et al., 2007; Zhang et al., 2007). Here, we observed that levels of *OsALDH7* transcripts started to increase at the late stage of maturation, similar to those of the *LEA* genes. Expression patterns for *OsALDH7* implied that a relationship exists between OsALDH7 and seed desiccation. Kirch et al. (2005) have reported that expression of the Arabidopsis *ALDH7B4* gene, in Family 7, is activated by abscisic acid, which regulates the dehydration stress response and the seed desiccation pathway. Other researchers have suggested that osmoprotection may be a major role of the plant antiquitins (Stroeher et al., 1995; Buchanan et al., 2005), compounds that oxidize aldehydes (Fong et al., 2006). Based on those previous studies and our results, we anticipated that OsALDH7 would function during seed desiccation and maturation. We observed that this gene was strongly expressed under abiotic stresses, e.g. drought, cold, and heat, and that *osaldh7* mutant seedlings were more sensitive than the wild type. Our data, therefore, suggest that OsALDH7 can be used to confer tolerance to diverse stresses.

ALDHs detoxify the aldehydes generated by various metabolisms (Yoshida et al., 1998; Nakazono et al., 2000; Liu et al., 2001). During seed desiccation, the embryo and aluerone cells must face severe oxidative stress, which increases the level of nonenzymatic lipid peroxidation (Baryla et al., 2000). As a result, MDA is generated, which has a high reactive activity to other molecules such as proteins, amino acids, and DNA (Restow and Obe, 1978; Fraenkel-Conrat and Singer, 1988; Mueller, 1998; Vollenweider et al., 2000; Stintzi et al., 2001; Almeras et al., 2003; Weber et al., 2004). Therefore, MDAs stimulate the expression of genes involved in cell protection/detoxification as well as those implicated in tolerance to stresses such as heat shock and dehydration (Weber et al., 2004). Here, we observed that levels of MDA were higher in *osaldh7* seeds during the late developmental stages and while being stored. This suggests that *OsALDH7* is needed to reduce the MDA formed during seed dehydration. Our OsALDH7 enzyme assay showed this high reactivity with MDA, thus supporting our assumption.

The brown reaction, i.e. the Maillard reaction, occurs during storage or because of spontaneous heating (Murthy et al., 2003). This reaction is the nonenzymatic

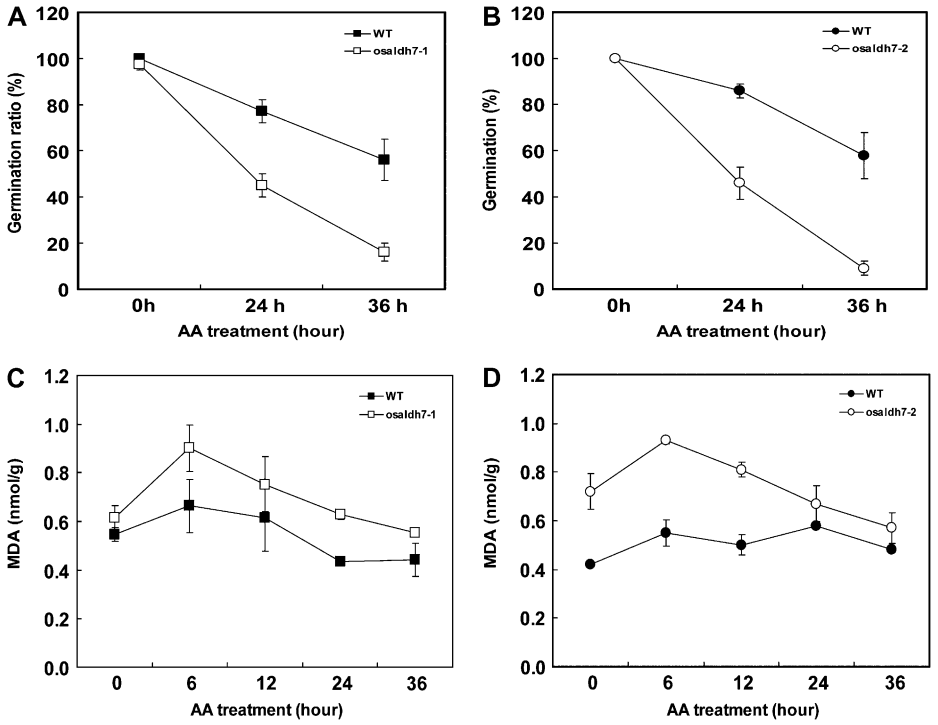

Figure 8. AA treatment of wild-type (WT) and *osaldh7* seeds. A and B, Germination rates of the mutants (white) and WT (black) after AA treatment. After 24- and 36-h treatments, seeds were germinated on MS media. C and D, MDA levels after AA treatment. Seeds were collected at 6-h intervals.

response between carbonyl compounds (e.g. reducing sugars and aldehydes) and amino compounds (peptides and amino acids). An increase in aldehyde levels promotes the reaction. During seed desiccation and storage, various aldehydes are formed by metabolisms and lipid peroxidation. The latter in particular is coupled to Maillard reactions during seed storage (Murthy and Sun, 2000; Zamora and Hidalgo, 2005). We observed here that the *osaldh7* mutant seeds accumulated more brown pigments, probably due to an increase in MDA contents.

It is difficult to investigate the effect of natural aging, because long-term storage is needed. Here, seed germination rates after short-term storage did not differ significantly between wild type and mutants. Our observation is consistent with that of Sattler et al. (2004), who showed that the germination rate of the *vte2* mutant seeds was not much different from that of wild-type seeds, although *VTE2* plays an essential role in seed longevity by participating in the tocopherol synthetic pathway. Therefore, we employed the AA treatment, which is broadly used for studying seed aging (Bentsink et al., 2000; Clerkx et al., 2004b).The *osaldh7* seeds showed decreased seed viability and generated more MDA after the AA treatment. This indicates that the mutant seeds are more sensitive to oxidative stress and also demonstrates that OsALDH7 is needed to sustain seed viability. Wettlaufer and Leopold (1991) have reported that AA treatment of soybean seeds dramatically increases the Maillard reaction. Furthermore, Sun and Leopold (1995) have suggested that Maillard reactions play a role in seed deterioration. Based on those previous reports and our current results, we postulate that the reduced viability of *osaldh7* seeds was caused by brown pigments, the final product of the Maillard reaction.

Elleder (1981) and Uchida (2006) have reported that lipid peroxidation is involved in the formation of lipofuscin, a lipidic brown pigment, with characteristic fluorescence. Pigment accumulations result from binding between MDA and protein. The brown pigments that accumulated in our *osaldh7* seeds are similar to lipofuscin in that they are fluorescent, MDA-derivative, and aging related. Therefore, we examined the solubility of the extract in diverse solvents, including water, methanol, ethanol, acetone, and hexan. However, the pigments were soluble only in water, indicating that they are not lipidic material and, thus, not likely to be lipofuscin.

We tried to investigate the chemical nature of this pigment by liquid chromatography-mass spectrometry and gas chromatography-mass spectrometry. However, we were unable to identify any candidate peaks. This may have been because melanoidins are highly polymerized compounds. The structure of those pigments has been poorly defined (Adams et al., 2005), such that no commercial standards are available for structural analysis.

In conclusion, we suggest the following working model. Lipid peroxidation is accelerated by oxidative stress in late-stage seeds, which causes an increase in MDA and other aldehydes. The toxic materials are removed by OsALDH7, inhibiting the Maillard reaction. However, in the *osaldh7* mutant, MDA and other aldehydes are not removed, and a Maillard reaction is promoted, forming brown pigments in mature seeds.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Seeds of *osaldh7* and segregant wild-type rice (*Oryza sativa* 'Dongjin') were sterilized with 50% hypochlorite for 30 min, washed three times with sterile distilled water, and placed on an MS medium (Murashige and Skoog, 1962). One-week-old seedlings were then transferred to soil and grown in the greenhouse. After 3 to 4 weeks, they were transplanted to a paddy field and grown to maturity. Their harvested seeds were air-dried for 1 week and stored at room temperature.

Screening of *OsALDH7* Mutants from T-DNA Tagging Lines

Defective seeds of the mutants were isolated from T-DNA tagging lines by screening for alterations in their shape or color. The T-DNA insertion position was determined by inverse PCR (An et al., 2003; Jeong et al., 2006). Another allele of *osaldh7* was obtained by searching the TES database (http://signal.salk.edu/cgi-bin/RiceGE).

Abiotic Stress Treatments

One-week-old seedlings were grown in an MS medium. To induce cold stress, the seedlings were transferred to 4°C and incubated for 3 d. During this treatment, they were harvested daily (beginning at d 0) for RNA isolation and real-time PCR analysis. After the 3-d test period, the seedlings were allowed to recover at room temperature for 12 h before sampling. For the heat treatment, seedlings were held at 50°C and sampled at 0, 6, and 12 h. For drought experiments, water was blotted from the seedling roots with paper towels before the seedlings were placed on a paper towel at 30°C. These treated seedlings were sampled at 0.0, 0.5, 1.0, and 2.0 h. The paraquat treatment involved submerging the seedling roots in a 20 μM MV solution, then sampling them at 12-h intervals. After 1 d, the seedlings were transferred to distilled water and sampled after 12 h. For high salt treatment, seedling roots were submerged in 250 mM NaCl for 12 h. Afterward, they were allowed to recover at room temperature.

Pigment Extraction and Analysis

Hulled-whole seeds (about 100 mg) of the wild type and *osaldh7* were ground in a milling machine, and the powder was dissolved in 1 mL of distilled water. After vigorous vortexing, the solution was mixed at 4°C for 24 h on a rotator. This solution was then centrifuged at 13,000 rpm for 15 min, and the supernatant was retrieved into a new Eppendorf tube. The solution was filtered using a 0.2-μm syringe filter (Sartorius). The extracts were scanned with a UV spectrophotometer (Shimadzu) over a spectral range of 200 to 700 nm. The extracts were also observed under UV light to determine whether the materials were fluorescent.

Measurement of Amadori Products

Stored seeds (10 mg) were ground with a milling machine before 1.2 mL of 50 mM phosphate buffer, pH 7.2, was added to the powder. Nucleic acids were removed by adding 200 μL of 10% streptomycin sulfate, then centrifuging at 15,000 rpm for 15 min. The supernatant was transferred to a new tube, and proteins were precipitated with ammonium sulfate (0.55 g mL^{-1}). The proteins precipitated by centrifugation were dissolved in 50 mM phosphate buffer, pH 7.2, and the protein solution was further purified on 10-DG columns (Bio-Rad). Those purified proteins were then used for measurement of Amadori products by the nitro blue tetrazolium method, as described previously (Murthy and Sun, 2000).

RT-PCR and Quantitative Real-Time PCR

Samples were homogenized in a milling machine (Retsch), and total RNA was extracted using TRIzol reagent (Invitrogen). For first-strand cDNA synthesis, 2 μg of total RNA was reverse-transcribed in a total volume of 25 μL that contained 10 ng of oligo$(dT)_{12-18}$ primer, 2.5 mM dNTPs, and 200 units of Moloney murine leukemia virus reverse transcriptase (Promega) in a reaction buffer. After RT at 37°C for 90 min, RT-PCR was conducted for 33 cycles in a 25-μL solution containing 20 pmol of gene-specific primers, 0.2 mM dNTPs, 1 unit of Taq DNA polymerase (Enzynomics), and 1× reaction buffer. Primers were designed at different exons, allowing the differentiation of cDNA products from genomic DNA contamination. Real-time PCR was performed using a Roche LightCycler II as previously described (Han et al., 2006). The procedure utilized a 20-μL solution containing 1 μL of cDNA solution, 20 pmol of gene-specific primers, 1× SYBR premix Ex Taq (TakaRa Shuzo). For RT-PCR analyses in *osaldh7-1* and *osaldh7-2*, F1 (5′-ccaaaatttattccttgtgc-3′)/R1 (5′-aatctgaaggaagcagttga-3′) and F2 (5′-gtactgcttccacgaatcac-3′)/R2 (5′-cggttgtgagagaagaactc-3′) primer sets were used, respectively (Fig. 3A). The mRNA level of *OsActin1* served to normalize the relative expression level of *OsALDH7*.

Vector Construction

To construct our localization vectors, we performed PCR with the following primers: 5′-gcACTAGTatggggagcttcgcgaggaa-3′ and 5′-gcACTAGTgccaaaatttattccttgt-3′ (underlined parts indicate *Spe*I-recognized sequences). Full-length cDNA of *OsALDH7* was inserted into the pBluescript SK+ vector (Stratagene), which was digested with *Eco*RV. The subclone was digested with *Spe*I and then introduced into the *Spe*I site in pGA3452, which contains the *sGFP* gene driven by the maize (*Zea mays*) *ubiquitin* promoter. Protoplasts prepared from the Oc cell line of rice were transformed with the localization vectors by methods described previously (Han et al., 2006; Woo et al., 2007).

Enzyme Assays

Full-length *OsALDH7* cDNA was isolated by the primers 5′-caccatggggagcttcgcgagg-3′ (forward primer) and 5′-ctagccaaaatttattcctt-3′ (reverse primer). The cDNA clone was inserted into pET100/D-TOPO, and the resulting plasmid was transformed into *Escherichia coli* strain BL21 (DE3). Transformed cells were incubated at 37°C overnight, then transferred to 250 mL of a fresh medium (1.6% tryptone, 1.0% yeast extract, and 0.5% NaCl) and incubated in a 37°C shaker. Once the OD_{600} reached 0.5 to 0.8, 1 mM isopropylthio-β-galactoside was added, and incubation was continued at 30°C for another 8 h. For OsALDH7 assays, cells were harvested and resuspended in 10 mL of B-PER protein extraction reagent (Thermo Fisher Scientific). This solution was incubated on ice for 20 min and sonicated three times, for 10 s each, using a Branson Sonifier model 450 at maximum output. The lysate was centrifuged at 12,000 rpm for 20 min. After the supernatant was transferred to a new tube, 6× His-tagged OsALDH7 proteins were purified on a Ni-NTA Spin column (Qiagen). For our enzyme assay, 10 μg of purified protein was added to a reaction mixture containing 1.5 mM NAD (Sigma) and 0.1 M sodium pyrophosphate buffer, pH 8.5. The total volume was adjusted to 300 μL with water before 20 μg of an aldehyde (MDA, acetaldehyde, or glyceraldehyde) was added to the mixture. Finally, the emission fluorescence of NADH was recorded for up to 2 min, at 20-s intervals, on a SPECTRAMAX Gemini (Elsevier Biosoft).

Lipid Peroxidation Assay

Levels of lipid peroxidation were assayed according to the thiobarbituric acid test, which determines the amount of MDA as an end product of the reaction (Cakmak and Horst, 1991; Loreto and Velikova, 2001). Seeds (0.2 g) were homogenized in 5 mL of 0.1% (w/v) TCA solution on ice. After the homogenate was centrifuged at 3,000 rpm for 10 min, the supernatant (0.5 mL) was transferred to a new tube, and 1 mL of 20% (w/v) TCA solution containing 0.5% thiobarbituric acid was added. The mixture was kept in a boiling water bath for 30 min, then quickly cooled in ice. Following centrifugation at 2,000 rpm for 15 min, absorbance of the supernatant was measured at 532 nm and 600 nm.

AA Test of Seeds

To promote AA, the hulls from 50 seeds each of the wild type and *osaldh7-1* and *osaldh7-2* mutants were removed. The seeds were transferred to a high-humidity chamber (100% relative humidity) and incubated at 50°C for 24 and 36 h. These AA-treated seeds were then surface-sterilized with 50% hypochlorite and placed on an MS medium. Germination rates were scored 7 d after sowing. This experiment was repeated three times.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF323586.

Supplemental Data

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

Supplemental Figure S1. Subcellular localization of OsALDH7::sGFP fusion protein.

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