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Class III peroxidases are activated in proanthocyanidin-deficient *Arabidopsis thaliana* seeds

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• *Background and Aims* It has previously been shown that proanthocyanidins (PAs) in the seed coat of *Arabidopsis thaliana* have the ability to scavenge superoxide radicals (O_2^-). However, the physiological processess in PA-deficit seeds are not clear. It is hypothesized that there exist alternative ways in PA-deficient seeds to cope with oxidative stress.
• *Methods* The content of hydrogen peroxide (H_2O_2) and its relevance to the activities of superoxide dismutase (SOD), catalase (CAT) and peroxidases was investigated in both wild-type and PA-deficit mutant seeds. A biochemical staining approach was used to detect tissue localizations of peroxidase activities in PA-deficit mutant seeds.
• *Key Results* PA-deficient mutants possess significantly lower levels of H_2O_2 than the wild-type, despite their higher accumulation of superoxide radicals. Screening of the key antioxidant enzymes revealed that peroxidase activity was significantly over-activated in mutant seeds. This high peroxidase activity was mainly confined to the seed coat zone. Interestingly, neither ascorbate peroxidase nor glutathione peroxidase, just the guaiacol peroxidases (class III peroxidases), was specifically activated in the seed coat. However, no significant difference in peroxidase activity was observed in embryos of either mutants or the wild-type, although gene expressions of several candidate peroxidases were down-regulated in the embryos of PA-deficient seeds.
• *Conclusions* The results suggest that enhanced class III peroxidase activity in the seed coat of PA-deficient mutants is an adaptive strategy for seed development and survival.

Key words: Proanthocyanidins, peroxidase, seed coat, seed development, seed germination, anti- oxidation, *Arabidopsis thaliana*.

INTRODUCTION

The dual roles of reactive oxygen species (ROS) have been documented extensively in previous studies. At high concentrations they trigger genetically programmed cell suicide. However, they also have an important function as secondary messengers in signal transduction cascades, which regulate many physiological and developmental processes in plants (Apel and Hirt, 2004; Foyer and Noctor, 2005; Mittler *et al.*, 2011). Several forms of ROS are present in plants, including the superoxide radical (O_2^-), singlet oxygen ($^1O_2$), hydroxyl radical ($OH^·$) and hydrogen peroxide (H_2O_2) (Vranova *et al.*, 2002). H_2O_2 has weak toxicity compared with other ROS species but it can trigger highly reactive hydroxyl radicals via the Haber–Weiss reaction. The relatively longer life span of H_2O_2 makes it harmful to organelles (Levine *et al.*, 1994; Willekens *et al.*, 1997; Apel and Hirt, 2004; Foyer and Noctor, 2005; Moller *et al.*, 2007).

Plants have a complex antioxidant system for maintaining the homeostasis of ROS. In general, this system can be divided into enzymatic and non-enzymatic ROS-scavenging mechanisms. Three types of antioxidative enzymes, superoxide dismutase (SOD), catalase (CAT) and peroxidase, play a major role in keeping superoxide radicals and H_2O_2 at steady-state levels (Bowler *et al.*, 1992; Willekens *et al.*, 1997; Apel and Hirt, 2004). There are two classes of peroxidases in plants: class I and class III (Cosio and Dunand, 2009). Ascorbate peroxidase (APx) and glutathione peroxidase (GPx) belong to class I, which can detoxify H_2O_2 into H_2O (Mittler, 2002). Class III peroxidases are encoded by a large multi-gene family. Altogether, 73 members have been identified in *Arabidopsis thaliana* (hereafter arabidopsis) (Hiraga *et al.*, 2001; Almagro *et al.*, 2009). Although they play a role in antioxidants by removing ROS, they also produce ROS. The bifunctional enzymes of class III peroxidases also participate in a broad range of physiological processes, such as defence against pathogens, the formation of lignin and suberin, cross-linking of cell-wall components, auxin catabolism, and seed germination and senescence (Passardi *et al.*, 2004*b*; Almagro *et al.*, 2009).

The main non-enzymatic antioxidants include ascorbate (vitamin C), α-tocopherol (vitamin E), glutathione (GSH),

carotenoids and flavonoids, and these substances also play an important role in compromising oxidative damage as major cellular redox buffers (Noctor and Foyer, 1998; Sattler *et al.*, 2004, 2006; Han *et al.*, 2009; Foyer and Noctor, 2011). Most flavonoids outperform other antioxidants in *in vitro* antioxidant assays although relatively little has been reported compared with other non-enzymatic antioxidants such as ascorbate and α-tocopherol (Hernandez *et al.*, 2009). Flavonoids are important secondary metabolites that originate from general phenopropanoid metabolism (Vogt, 2010). More than 9000 individual molecules have been identified, and it is believed that higher plants are the only natural source of flavonoids (Hernandez *et al.*, 2009). Flavonoids are categorized into the following groups based on the oxidation levels of the central C-ring, stereochemistry, type and degree of polymerization, position and nature of the substitutions, and linkages between basic units: anthocyanidin, flavanone, proanthocyanidins (PAs) and flavone or flavonol (Winkel-Shirley, 2001; Lepiniec *et al.*, 2006; Routaboul *et al.*, 2006).

Several species of flavonoids have been investigated for their chemical identity and antioxidant capacity by *in vitro* experiments (Proteggente *et al.*, 2003; Spencer *et al.*, 2003). Flavonoids function as antioxidants mainly by scavenging peroxyl radicals but have minimal impact on OH radicals (Deng *et al.*, 1997). Guaiacol peroxidase catalyses the H_2O_2-dependent oxidation of flavonols, which is evidence of an H_2O_2-scavenging mechanism mediated by a flavonoid–peroxidase reaction (Yamasaki *et al.*, 1997). Although the antioxidant capacities of flavonoids are genuine and even greater than other extensively investigated antioxidants, many aspects of their putative functions as antioxidants remain unclear (Hernandez *et al.*, 2009). The main obstacle to understanding their functions is the different subcellular localization of flavonoids from the site of ROS production. However, a recently discovered transport mechanism for anthocyanin from its synthesis site to the sites of storage and disposal, mediated by an endoplasmic reticulum-derived vesicle-like structure, provides the possibility of an interaction between flavonoids and ROS (Poustka *et al.*, 2007; Gomez *et al.*, 2011).

PAs are major end-products of the flavonoid biosynthesis pathway and mainly accumulate in the seed coat of arabidopsis (Debeaujon *et al.*, 2003; Xie *et al.*, 2003; Lepiniec *et al.*, 2006). Among the many *TRANSPARENT TESTA* (TT) genes involved in PA biosynthesis, TT2, TT8 and TTG1 form a ternary complex to directly regulate *BANYULS* (*BAN*) expression (Baudry *et al.*, 2004). The precise functions of the other three regulatory factors, including TT1, TT16 and TTG2, remain unclear and thus there is a need for further investigation (Lepiniec *et al.*, 2006). *TT18* encodes a leucocyanidin dioxygenase that is located downstream of the flavonoid pathway and is essential for the biosynthesis of PAs (Abrahams *et al.*, 2003). PA-deficient mutants exhibit yellow or pale brown seed coat colour due to reduced flavonoid pigmentation and accumulation (Debeaujon *et al.*, 2000; Lepiniec *et al.*, 2006). Most PAs found in seeds are soluble procyanidin polymers, consisting of two stereoisomers: epicatechin (EC, 2-3-*cis*) and catechin (C, 2-3-*trans*) and their degree of polymerization can be up to 9 (Harborne and Williams, 2000; Routaboul *et al.*, 2006). However, only EC with a mean degree of polymerization between 5 and 8 is detected in the seeds of arabidopsis (Abrahams *et al.*, 2003; Routaboul *et al.*, 2006).

Our most recent study also provided evidence of the antioxidation ability of PAs by *in vivo* experiments in arabidopsis seed. We found that the superoxide radical (O_2^-) accumulated more in PA-deficient mutant seeds than in wild-type seeds, which demonstrates that PAs function as antioxidants via the removal of O_2^- (Jia *et al.*, 2012*b*). The PAs in the seed coat are likely to play an important role in buffering ROS homeostasis during maturation and germination (Marles *et al.*, 2003; Routaboul *et al.*, 2006). However, it would be difficult for PAs to exert their antioxidant functions following accumulation in the vacuole if they are unable to break the physical barrier created by the tonoplast (Gould *et al.*, 2002; Hernandez *et al.*, 2009). In the later stages of seed development, the seed coat experiences programmed cell death and releases PAs from the vacuole, making them available to exert their antioxidant properties (Debeaujon *et al.*, 2003; Dixon *et al.*, 2005; Haughn and Chaudhury, 2005). However, there are no reports of decreased seed quality of freshly harvested PA-deficient mutants. How the antioxidant system in these PA-deficient mutants operates is not clear. We investigated the hypothesis that these mutants have acquired an alternative antioxidant mechanism compared with the wild-type, which enables them to develop without the protection of PAs. Our results demonstrate that increased activity of class III peroxidases in the seed coat of PA-deficient mutant seeds may compensate for the loss of PAs.

MATERIALS AND METHODS

Plant material

The following mutants of arabidopsis (*Arabidopsis thaliana*) have been described previously: *tt1-6* (SALK_107737C; Appelhagen *et al.*, 2011), *tt2-5* (SALK_005260; Nesi *et al.*, 2001), *tt8-4* (SALK_030966), *tt8-5* (SALK_048673) (Nesi *et al.*, 2000) and *tt18-3* (SALK_028793; Shikazono *et al.*, 2003; Jia *et al.*, 2012*a*). Plants were kept in a growth chamber at 22 ± 2 °C with a 16-h photoperiod at a photon flux density of approx. 200 μmol m^{-2} s^{-1} and a relative humidity of 80 %. Seeds of the wild-type (Col0) and the mutants were grown in PlantMate compost (Huadu Co., Kong Kong) for all-purpose potting soil and harvested at the same time. The seed samples after harvest were stored in a dehumidifier cabinet for 2 months before using them in the experiments.

Seed coat isolation

Seed coats were isolated to investigate antioxidant enzyme activities. Gentle manipulation with fine forceps under a dissecting microscope allowed the release of embryos from the micropylar end of the seed coat. Isolated seed coats were washed with 5 % sucrose solution to remove the endosperm layer. Two hundred dissected seeds were collected in separate Eppendorf tubes for sample extraction (Dean *et al.*, 2011).

Chemical treatments

Three oxidants or oxidative inducers were selected for H_2O_2 determination, antioxidant enzyme detection and gene expression

analysis at the transcriptional level. All three chemicals [methyl viologen (MV), $CuSO_4$ (Cu^{2+}) and *tert*-butylhydroperoxide (*t*-BHP)] were obtained from Sigma-Aldrich (St Louis, MO, USA). A 200-μL solution including 0·5 μM MV, 0·4 mM Cu^{2+} or 0·5 mM *t*-BHP was added to approx. 50 mg seed sample for oxidative stress treatment, and the same volume of water was added to the control (Van Camp *et al.*, 1996; Bae *et al.*, 2010).

H_2O_2 measurements

An amplex red H_2O_2/peroxidase assay kit (Molecular Probes, Eugene, OR, USA) was used for the detection of endogenous concentrations of H_2O_2. Approximately 50 mg seed samples were ground in 20 mM sodium phosphate buffer (pH 7·4). The mixture was centrifuged at 10 000 *g* for 10 min at 4 °C, and the supernatant was used for subsequent assays. The detailed procedure followed the protocol of the assay kit and 50 μL supernatant was used for each reaction. The reaction proceeded for 30 min in darkness and the absorbance at 560 nm was read using FLUOStar Optima. The concentrations were calculated according to the standard curve (Shin and Schachtman, 2004; Lee *et al.*, 2010).

Enzyme assays

Approximately 50 mg seed sample was crushed into a fine powder in a mortar and pestle under liquid nitrogen. One microlitre of 50 mM potassium phosphate buffer (pH 7·0) containing 1 mM EDTA and 1 % polyvinylpyrrolidone (PVP) was added and the sample was mixed. The homogenate was centrifuged at 15 000 *g* for 20 min at 4 °C and the supernatant was used for enzyme assays.

Total superoxide dismutase (SOD) (EC 1·15·1·1) activity was analysed using nitro blue tetrazolium (NBT) following the method of Giannopolitis and Ries (1977) with some modifications. The 1-mL reaction mixture contained 50 mM potassium phosphate buffer (pH 7·8), 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0·1 mM EDTA and 20 μL enzyme extract. The reaction mixtures were illuminated for 15 min at a light intensity of 5000 lx. One unit of SOD activity was defined as the amount of enzyme required to cause 50 % inhibition of the reduction of NBT as monitored at 560 nm (Giannopolitis and Ries, 1977).

Catalase (CAT) (EC 1·11·1·6) activity was determined by observing the consumption of H_2O_2 (extinction coefficient 39·4 mM^{-1} cm^{-1}) at 240 nm for 3 min (Aebi, 1984). The reaction mixture contained 50 mM potassium phosphate buffer (pH 7·0), 10 mM H_2O_2 and 200 μL enzyme extract in a 3-mL volume (Jiang and Zhang, 2002).

Peroxidase was detected with an Amplex red H_2O_2/peroxidase assay kit (Molecular Probes). The extraction method was the same as that used for the H_2O_2 measurement but H_2O_2 was added to the seed sample solutions instead of horseradish peroxidase (HRP). A standard curve was produced using the diluted HRP solutions (0–2 mU mL^{-1}) provided in the assay kit (Shin and Schachtman, 2004; Lee *et al.*, 2010).

Guaiacol peroxidase activity was assayed in a reaction mixture consisting of 100 mM potassium phosphate buffer (pH 6·5), 15 mM guaiacol, 0·05 % (v/v) H_2O_2 and 60 μL enzyme extract diluted between 1 : 40 and 1 : 80 (v/v) with assay buffer. The reaction was initiated by adding H_2O_2, and the oxidation of guaiacol was determined based on the increase in A_{470} ($\varepsilon = 26{\cdot}6$ mM^{-1} cm^{-1}). One guaiacol peroxidase unit is defined as the amount of enzyme that produces 1 μmol min^{-1} oxidized guaiacol under the above assay conditions (Garcia-Limones *et al.*, 2002). For the imaging of guaiacol peroxidase activity, 200 detached seed coats were ground in liquid nitrogen and reacted in the solution described above for 3 min before photos were taken.

APx activity was determined by observing the decrease in A_{290} (extinction coefficient 2·8 mM^{-1} cm^{-1}) for 30 s in 1-mL reaction mixture containing 50 mM potassium phosphate buffer (pH 7·0), 0·5 mM ASC, 0·1 mM H_2O_2 and 20 μL enzyme extract. The reaction was initiated with an enzyme extract. A correction was made for the low, non-enzymatic oxidation of ascorbate by H_2O_2 (Nakano and Asada, 1981).

In-gel GPx activity was detected using 8 % native PAGE gels. Approximately 150 μg protein per sample was loaded onto a gel and run at 4 °C. The gel was removed from the glass plates, placed into a glass staining dish and washed for 3× 10 min in distilled water containing GSH using approx. 50 mL per wash. The stain was prepared in two 50-mL conical tubes by preparing 1 % ferric chloride in one tube and in a second tube preparing 1 % potassium ferricyanide. The gel was incubated in 100 mL ddH_2O containing 0·008 % cumene hydroperoxide for 10 min. The gel was rinsed twice with ddH_2O and incubated with the stains of a mixture of the two reagents prior to staining. They were poured directly on top of the gel. When achromatic bands began to form (5–15 min), the stain was poured off and the gel was rinsed with ddH_2O. The formation of chromatic bands demonstrated the presence of GPx activity (Weydert and Cullen, 2010).

Histochemical detection of peroxidase activity

The imbibed seeds were spread between two glass slides and squeezed slightly to ensure that the cell inclusions were wholly or partially out of the testa. Then 3,3′, 5,5′-tetramethylbenzidine (TMB) Stabilized Substrate for Horseradish Peroxidase (Promega, Madison, WI, USA) and 1 mM H_2O_2 were added to squeezed seed samples. Peroxidase localization was achieved by short-term (10 min) staining for seed coats and long-term (1 h) staining for embryos (Linkies *et al.*, 2010). For relative absorption value measurements of the reaction products spread from testa, 200 wild-type and/or PA-deficient mutant seeds were squeezed and the samples were collected into Eppendorf tubes. Following staining with 200 μL TMB solution, the supernatant was collected after being centrifuged at 10 000 *g* for 1 min. The reaction was stopped after 10 min with 50 μL H_3PO_4 and the absorbance at 450 nm was measured (Vanhercke *et al.*, 2005).

Total RNA extraction and transcript level analysis

A plant RNA Isolation Mini Kit (Agilent, Palo Alto, CA, USA) was used for total RNA extraction from seed samples according to the manufacturer's instructions. After being reverse transcribed using the SuperScript RT-PCR system (Invitrogen) the cDNA was used to perform the QRT-PCR analysis. IQ SYBR Green Supermix (Bio-Rad) and iCycle (Bio-Rad) were used. *UBQ5* was used as an internal standard

TABLE 1. *Sequence of primers used for QRT-PCR*

Primer name	Primer sequence
APX1-QRT-S	5′ GGTCGTCTTCCTGATGCTAC 3′
APX1-QRT-R	5′ TGGCATCGTCCCAGAGTGT 3′
Prx16-QRT-S	5′ TTGCTTCGTCCGTGGATG 3′
Prx16-QRT-R	5′ CTATCAAGGGCCTGCTTTG 3′
PrxIIE-QRT-S	5′ AATGGCGAATTCACAGGG 3′
PrxIIE-QRT-R	5′ CACCTCCTTCTTCAAGATTCAG 3′
UBQ5-QRT-S	5′ GCATGCAAGCTTGGCGTAA 3′
UBQ5-QRT-R	5′ TGAGCGGATAACAATTTCACACA 3′
ACT8-QRT-S	5′ CTCAGGTATTGCAGACCGTATGAG 3′
ACT8-QRT-R	5′ CTGGACCTGCTTCATCATACTCTG 3′

to normalize the data, and a second reference gene, *ACT8*, was used to re-confirm the results (Graeber *et al.*, 2011). The primers used for gene expression analysis by QRT-PCR are shown in Table 1.

Accession numbers

The sequence data described in this article can be found in the Arabidopsis Genome Initiative or TIGR databases under the following accession numbers: *UBQ5* (AT3G62250); *ACT8* (AT1G49240); *APX1* (AT1G07890); *Prx16* (AT2G18980); *PrxIIE* (AT3G52960).

RESULTS

Accumulation of endogenous H_2O_2 in PA-deficient mutant seeds

H_2O_2 was detected in both the non-dormant wild-type and several PA-deficient mutant seeds. Lower concentrations of H_2O_2 were observed in *tt* mutant seeds, including *tt1-6*, *tt2-5*, *tt8-4* and *tt18-3*, than in the wild-type (Fig. 1A). Two PA-deficient mutants, *tt2-5* and *tt18-3*, were selected to investigate H_2O_2 accumulation under normal and oxidative stress conditions in imbibed seeds. Three oxidants or oxidative inducers, MV, $CuSO_4$ (Cu^{2+}) and *t*-BHP, were chosen and two time points, short (3 h) and long (24 h), were set for detection. The mutants displayed more H_2O_2 accumulation over the short-term water imbibition period (Fig. 1B), which induced a rapid germination of *tt* mutants as reported earlier (Jia *et al.*, 2012*a*). Under oxidative stress, the H_2O_2 content increased in wild-type seeds for both short- and long-term treatments. However, this did not occur in PA-deficient mutants. Not only was there no marked increase in the H_2O_2 content, but a decrease was detected under some oxidative treatments such as the Cu^{2+} and *t*-BHP in the short-time treatment (Fig. 1B).

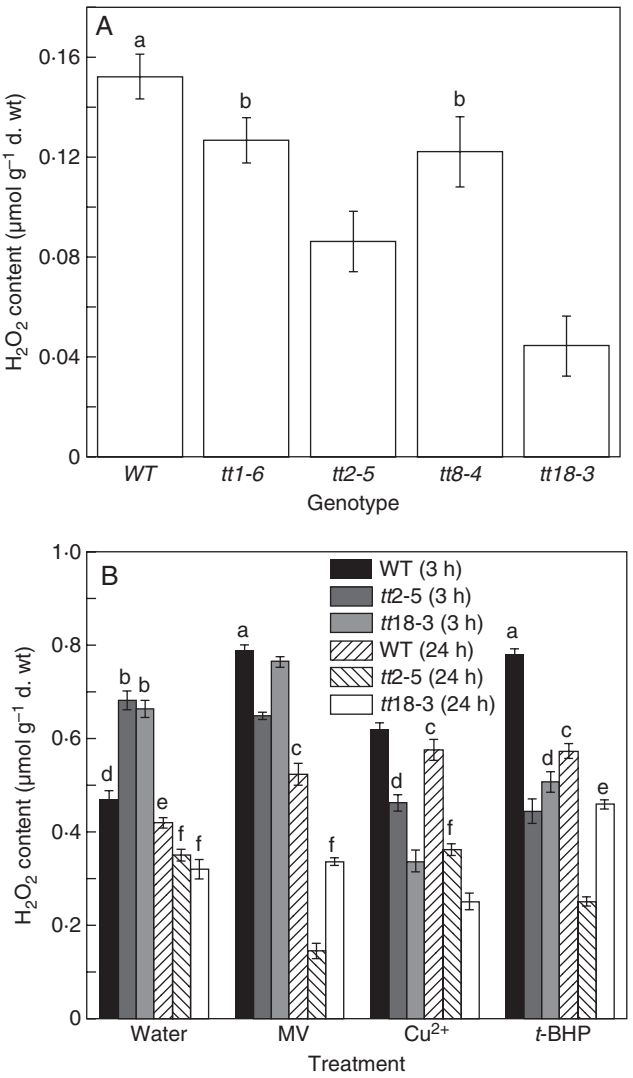

FIG. 1. Comparison of hydrogen peroxide (H_2O_2) content between wild-type arabidopsis seeds and PA-deficient mutants under normal and oxidative stress conditions. (A) H_2O_2 content in non-dormant, dry, wild-type seeds and PA-deficient mutants including *tt1-6*, *tt2-5*, *tt8-4* and *tt18-3*. (B) Three different inducers of oxidative stress [methyl viologen (MV), $CuSO_4$ (Cu^{2+}), *t*-butyl hydroperoxide (*t*-BHP)] and two PA-deficient mutants (*tt2-5* and *tt18-3*) were selected for further detection of H_2O_2 concentrations over short-term (3 h) and long-term (24 h) imbibition periods. Water imbibition was used as a control. Values are means and s.e. ($n = 3$). Means denoted by different letters are significantly different at $P < 0{\cdot}05$ according to Duncan's multiple range test.

Comparison of antioxidant enzymes between wild-type seeds and PA-deficient mutants

SOD, CAT and peroxidase activities were compared between the wild-type and mutant seeds under normal and oxidative conditions. Relatively lower CAT and SOD activities were detected in the mutants than in the wild-type seeds under not only normal conditions but also oxidative stress conditions induced by treatment with three oxidants (Fig. 2A, B). Increased SOD activity and decreased CAT activity under oxidative stress conditions might be the result of concentration effects of oxidative stress.

In contrast to the decreased activities of SOD and CAT in PA-deficient mutants, much higher activities of peroxidase were detected in the mutants than in the wild-type seeds (Fig. 2C). About a 4-fold (3·74- and 3·51-fold) increase in peroxidase activity was observed in the *tt2-5* and *tt18-3* seeds under normal conditions, which might have resulted from a strong response at an earlier stage before germination such as during seed development or the desiccation stage. Under oxidative stress conditions, peroxidase activity also increased substantially, for both short- and long-term treatments. We

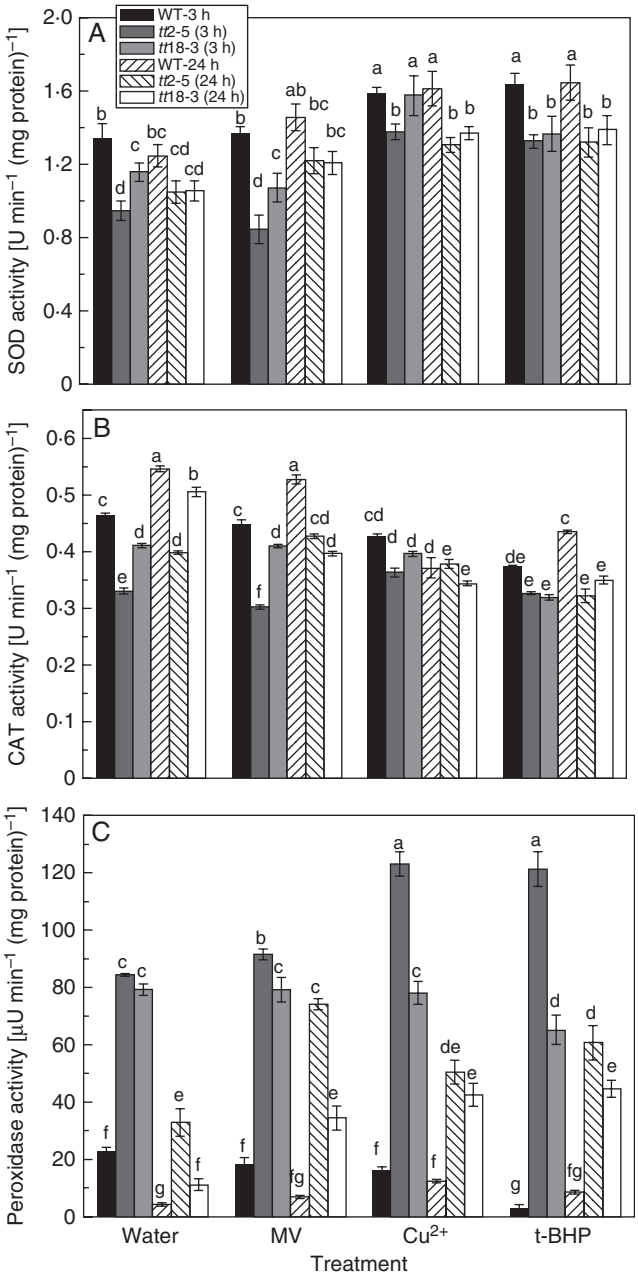

FIG. 2. Changes in antioxidant enzyme activities under normal and oxidative stress conditions in wild-type and PA-deficient mutant arabidopsis seeds over short- (3 h) and long-term (24 h) imbibition periods. (A) Superoxide dismutase (SOD) activity, (B) catalase (CAT) activity, (C) peroxidase activity. Values are means and s.e. ($n = 3$). Means denoted by the same letter are not significantly different at $P < 0{\cdot}05$ according to Duncan's multiple range test.

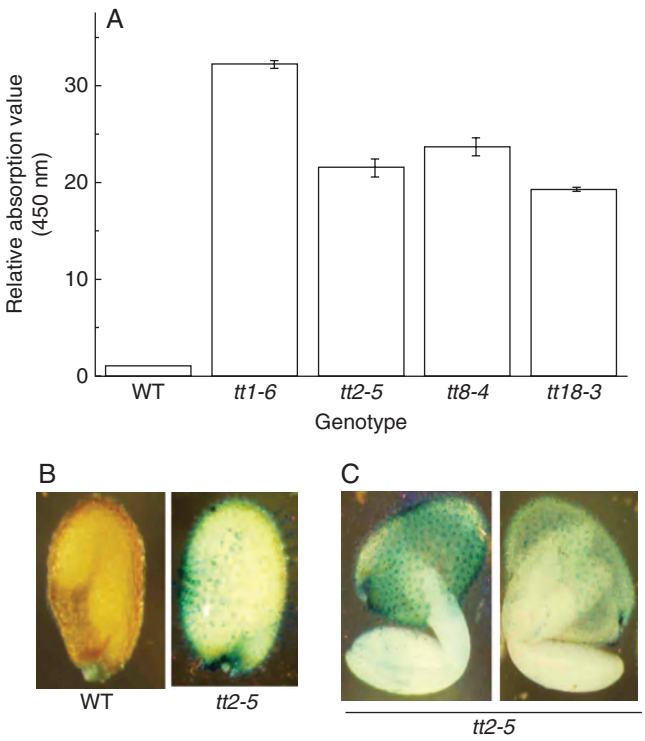

FIG. 3. Activity and tissue localization of peroxidase in arabidopsis seeds. Comparison of peroxidase activity between wild-type and PA-deficient mutant seeds by 3,5,3′,5′-tetramethylbenzidine-HCl (TMB) staining and (A) relative quantitative analysis by absorptive value (values are means and s.e.; $n = 3$). (B) Tissue localization of peroxidase in seeds using the wild-type and PA-deficient mutants (*tt2-5*) as experimental material. (C) Staining effects of peroxidase over short-term periods (10 min) in arabidopsis seeds.

speculate that peroxidase may play a major role in the defence against oxidative stress, particularly by keeping H_2O_2 at low levels in PA-deficient seeds.

Tissue localization of peroxidase activity in seeds

Staining was used to localize and quantify the tissue distribution of the increased peroxidase activity in PA-deficient mutants, using TMB. A deeper colour was clearly exhibited in PA-deficient mutant seeds over short-term treatments but the staining was not observed in wild-type seeds (Fig. 3A). The result was further verified via quantitative analysis using a colorimetric method (Fig. 3B). The difference in peroxidase activity over short-term staining periods mainly occurred in the seed coat. After absorption of water, the peroxidase in the seed coat rapidly reacted with the staining substrate. We did not observe any staining on the surface of wild-type seeds but coloration was apparent in PA-deficient mutant seed coats, which is consistent with our hypothesis (Fig. 3B). There was also no staining in the cotyledon and radicle of mutants when embryos released from seeds were immersed into the same reaction solution as the seed coat (Fig. 3C).

Distribution of peroxidase in the seed coat and embryo

We investigated the distribution of antioxidant activities, including peroxidase, in the seed coat and embryo (Fig. 4). Based on the activity per grain, we found almost equal distributions of SOD activity between the seed coat and embryo (49·62 and 50·38 %, respectively) in wild-type seeds. There was about a 10 % decrease in the seed coats of the mutants compared with the wild-type seeds (Fig. 4A). In contrast to the distribution of SOD activity, the activities of CAT and peroxidase in the seed coat of mutants were higher than those in the wild-type seeds. Although the activity of CAT was higher

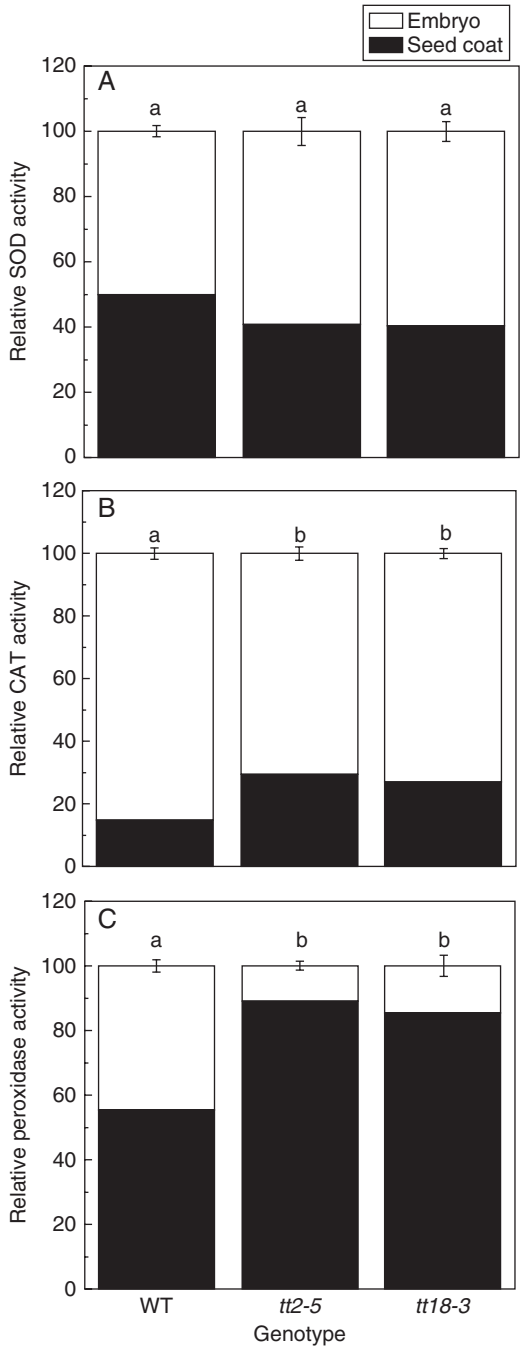

FIG. 4. Comparison of antioxidant enzyme activities in the testa and embryo between wild-type and PA-deficient mutant seeds (*tt2-5* and *tt18-3*). (A) Relative SOD activity, (B) relative CAT activity, (C) relative peroxidase activity. Values are means and s.e. ($n = 3$). Means denoted by different letters are significantly different at $P < 0{\cdot}05$ according to Duncan's multiple range test.

in mutant seeds than in wild-type seeds, it was still below 30 % of the total, accounting for 29·38 and 27·13 % in *tt2-5* and *tt18-3*, respectively (Fig. 4B). Wild-type seeds showed more peroxidase activity than SOD or CAT activity. However, peroxidase activity was even higher in PA-deficient mutants, being 88·94 and 85·19 % for the *tt2-5* and *tt18-3* mutants, respectively, compared with 55·12 % in wild-type seeds (Fig. 4C). The high peroxidase activity in seeds and its widespread distribution in the coat of PA-deficient seeds indicate that it must have some essential function therein and may partially account for the antioxidant role of PAs.

Guaiacol peroxidase is specifically activated in the seed coat of tt *mutants*

To investigate which type of peroxidase contributes most when PAs are deficient, we determined the activities of APx, GPx and guaiacol peroxidase (class III peroxidase). Using a leaf as a control, we found that there was very low APx activity and no GPx activity in the seed. There was also no significant difference in the APx activities of the wild-type and PA-deficient (*tt2-5* and *tt18-3*) seeds (Fig. 5B, C). We also did not detect guaiacol peroxidase activity in non-dormant wild-type seeds. However, guaiacol peroxidase activity was dramatically activated in the *tt2-5* and *tt18-3* seeds (Fig. 5A). Further investigation indicated that the guaiacol peroxidase activity was confined to the mutant seed coats (data not shown).

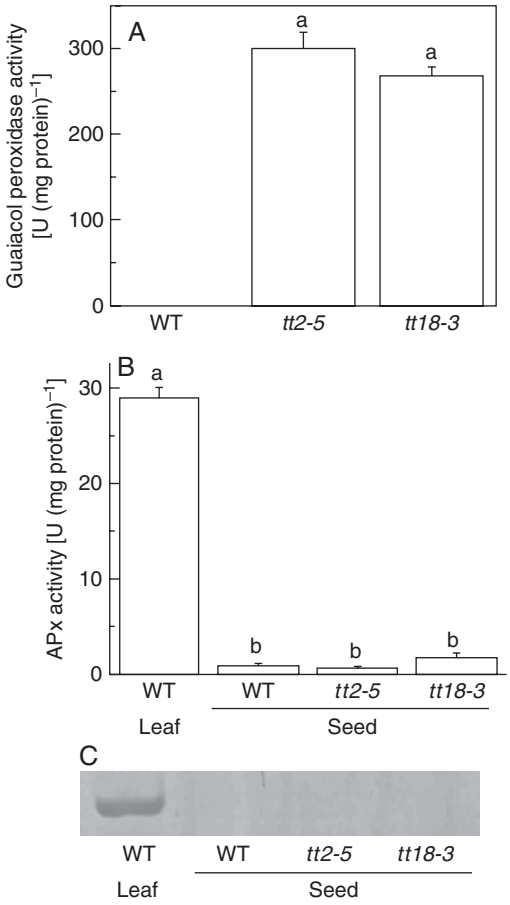

FIG. 5. Peroxidase activities in the seed coat of arabidopsis. (A) Comparison of guaiacol peroxidase activity between wild-type seeds and PA-deficient mutants. (B) APx activities in wild-type and PA-deficient mutant seeds. The leaf of the wild-type was used as a control. (C) In-gel GPx activities of wild-type and PA-deficient mutant seeds. The leaf of the wild-type was used as a control. Values are means and s.e. ($n = 3$ for SA). Means denoted by the same letter are not significantly different at $P < 0{\cdot}05$ according to Duncan's multiple range test.

Peroxidase in the embryo at transcriptional and post-transcriptional levels

Despite the high peroxidase activity in the mutant testa, there was no significant difference in its activity in the embryo between the wild-type and the mutants (Figs 2 and 4). This was consistent with the results of histochemical analysis by immersing the embryo in staining solution for a longer time. Peroxidase activity was present at the radicle tip for both the wild-type and the mutants (Fig. 6A). However, the transcriptional levels of peroxidase-encoding genes, including *APX1*, *Prx16* and *PrxIIE*, were different between the wild-type and PA-deficient mutant seeds. All three genes displayed lower expressions in the mutants compared with non-dormant wild-type seeds (Fig. 6B). Under oxidative treatment, the expression patterns became complex with *APX1* being inhibited and *PrxIIE* being promoted at the transcriptional level.

DISCUSSION

Seeds experience substantial water content changes from maturation to germination, during which large amounts of ROS can accumulate and generate oxidative stress. In arabidopsis, PAs are mainly produced in the endothelium and have functions as antioxidants to cope with such oxidative stress (Debeaujon *et al.*, 2000, 2003; Jia *et al.*, 2012*a*). However, PA-deficient mutant seeds can still germinate and seedlings can establish under normal conditions (Bailly, 2004; El-Maarouf-Bouteau and Bailly, 2008). In a previous study, we found that more O_2^- was produced in PA-deficient mutants under oxidative stress and that these ROS can be removed by PAs in wild-type seeds in the germination stage (Jia *et al.*, 2012*b*). However, H_2O_2 is reduced in the mutants under normal and oxidative stress conditions (Fig. 1). This finding is inconsistent with the results of a previous study regarding the function of flavonoids as ROS scavengers (Yamasaki *et al.*, 1997). We hypothesize that there may be another antioxidant system that is activated or initiated in the mutants in response to the lack of PAs in seeds.

The enzymatic ROS-scavenging mechanism plays a pivotal role in multiple developmental stages and defends against biotic and abiotic stresses. Such a mechanism mainly includes SOD, CAT and peroxidase (Bowler *et al.*, 1992; Willekens *et al.*, 1997; Apel and Hirt, 2004). In the present study, all three antioxidant enzymes were investigated under different time periods and with different ROS-generated xenobiotics (Fig. 2). Among the enzymes, only peroxidase was substantially more activated in mutants compared with wild-type seeds (Fig. 2C).

Based on these results, we hypothesize that peroxidase is a candidate antioxidant that compensates for the absence of PAs in *tt* mutants. If so, peroxidase expression should be mainly distributed in the seed coat because PAs are biosynthesized and accumulate in the endothelium (Debeaujon *et al.*, 2003; Xie *et al.*, 2003; Lepiniec *et al.*, 2006). To detail the tissue localization of peroxidase inside seeds, a histochemical method was used. We found that peroxidase activity is focused on the seed coat (Fig. 3). This is because the cells in all layers of the seed coat experience programmed cell death during the later

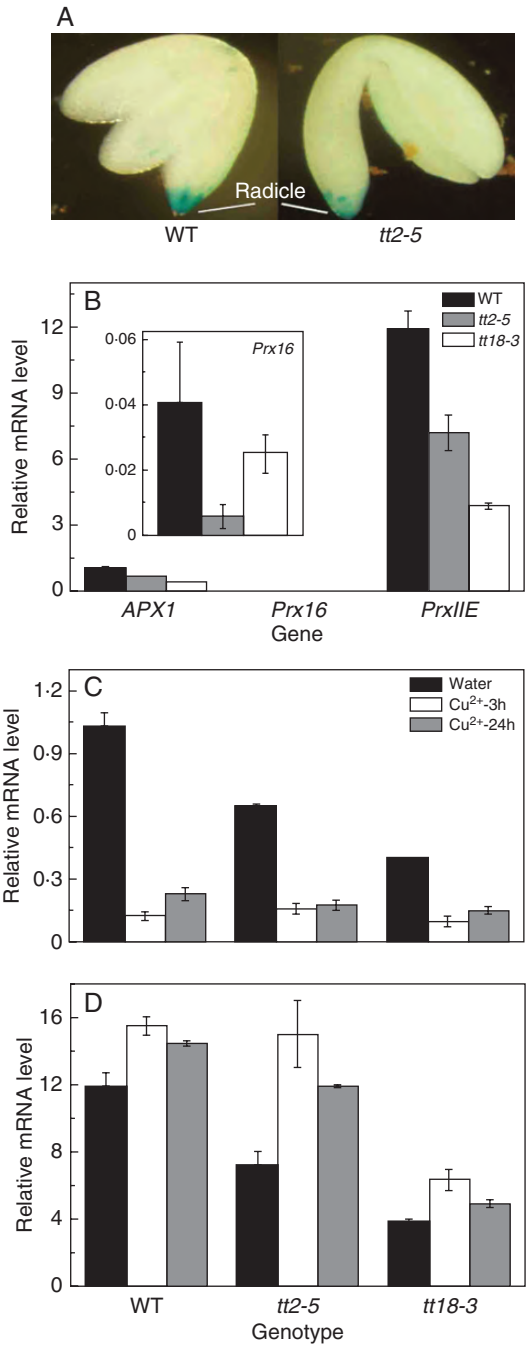

FIG. 6. Peroxidase localization and its encoded gene expression in the embryo of arabidopsis. (A) TMB staining over long-term periods (2 h). (B) Expression of peroxidase-encoded genes (*APX1*, *Prx16* and *PrxIIE*) in wild-type seeds and PA-deficient mutants. (C,D) Comparison of (C) *APX1* and (D) *PrxIIE* between wild-type seeds and PA-deficient mutants at transcriptional levels under oxidative stress conditions. In (B–D) values are means and s.e. ($n = 3$).

stages of seed development. Cell contents, including some metabolites and proteins, are readily released (Haughn and Chaudhury, 2005). In the short-term staining reactions, colour appeared only in PA-deficient mutants (Fig. 3A, C). When we immersed mutant seeds with radicles or embryos extruded from the testa in the same reaction solution, we

only observed colour on the seed coat in the short-term reaction, which further confirms the different peroxidase activities between wild-type and PA-deficient mutant seed coats (Fig. 3D).

The distribution of peroxidase in seed coats and embryos provides further evidence for this. A lower percentage of SOD activity and higher CAT and peroxidase activities occurred in the mutant seed coats compared with the wild-type (Fig. 4). However, CAT in the seed coat contributed less activity to the whole seed, not only in the wild-type but also in the mutants (Fig. 4B). Compared with other antioxidant enzymes, peroxidase seems to play a more important role in testa, because it accounted for almost 90 % of activity in the mutants (Fig. 4C). Due to the large distribution of peroxidase activities in the seed coat, there was no significant difference in the embryos between the wild-type and PA-deficient mutants, although the total peroxidase activity was higher in the mutants.

Class III peroxidases are bifunctional enzymes, participating in two possible catalytic cycles, peroxidative and hydroxylic, to detoxify or generate ROS and regulate H_2O_2 levels (Passardi *et al.*, 2004*a*). They can also cross-link cell-wall macromolecules to make cell walls rigid, particularly the class III peroxidases when secreted into a cell wall or surrounding medium (Chen and Schopfer, 1999; Passardi *et al.*, 2004*b*). Such functions of class III peroxidases make them good candidates to substitute for PAs in a PA-deficient seed coat.

Three major plant peroxidases, ascorbate peroxidase, glutathione peroxidase and class III peroxidase, were considered in our study. Guaiacol, as an artificial phenolic substrate, can be used to detect all class III peroxidases present independent of their different *in vivo* substrate specificities (Mika *et al.*, 2010). Only guaiacol peroxidase activity was increased in the testa of PA-deficient mutant seeds.

We also compared peroxidase activities in embryos between the wild-type and PA-deficient mutants due to their different germination behaviour (Fig. 6). A recent study has shown that peroxidases play an important role during seed germination and are specifically expressed in the micropylar endosperm and radicle, which are the key sites determining the seed germination of *Lepidium sativum*. The corresponding SALK lines of orthologues in arabidopsis display germination phenotypes (Linkies *et al.*, 2010). We investigated whether there is any difference in peroxidase activity between wild-type and PA-deficient mutant embryos in arabidopsis. We found that peroxidase is specifically localized at the radicle tip in arabidopsis seeds although we could not exclude its distribution in endosperm (Fig. 6A), which is consistent with the results from *L. sativum* (Linkies *et al.*, 2010). However, three candidate peroxidase-encoding genes are down-regulated at the transcriptional level in PA-deficient mutant seeds. This might be negative feedback regulation due to high peroxidase activity in the seed coat of PA-deficient mutants (Fig. 6b). Under oxidative stresses, *PrxIIE* is upregulated in the mutants but *APX1* expression is still lower compared with the wild-type, which demonstrates a complex regulatory network of peroxidase at the transcriptional level (Fig. 6C, D). This might be because many genes encoding peroxidase are universal with gene redundancy and result in an inconsistency between enzyme activity and gene expression (Apel and Hirt, 2004; Cosio and Dunand, 2009).

In conclusion, class III peroxidase activity is significantly enhanced in the seed coats of PA-deficient mutants as a consequence of the suppression of PA biosynthesis. Our results suggest that enhanced peroxidase activity may be an adaptive mechanism for substituting the antioxidant function of PAs that is required during seed development and later in seed germination. Further study is required to investigate the role and mechanism of peroxidase activity during seed development and how the regulatory mechanism operates to coordinate peroxidase activity and PA production.

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