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## Original Article

# Peroxisomal APX knockdown triggers antioxidant mechanisms favourable for coping with high photorespiratory $H_2O_2$ induced by CAT deficiency in rice

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## ABSTRACT

**The physiological role of peroxisomal ascorbate peroxidases (pAPX) is unknown; therefore, we utilized pAPX4 knockdown rice and catalase (CAT) inhibition to assess its role in CAT compensation under high photorespiration. pAPX4 knockdown induced co-suppression in the expression of pAPX3. The rice mutants exhibited metabolic changes such as lower CAT and glycolate oxidase (GO) activities and reduced glyoxylate content; however, APX activity was not altered. CAT inhibition triggered different changes in the expression of CAT, APX and glutathione peroxidase (GPX) isoforms between non-transformed (NT) and silenced plants. These responses were associated with alterations in APX, GPX and GO activities, suggesting redox homeostasis differences. The glutathione oxidation-reduction states were modulated differently in mutants, and the ascorbate redox state was greatly affected in both genotypes. The pAPX suffered less oxidative stress and photosystem II (PSII) damage and displayed higher photosynthesis than the NT plants. The improved acclimation exhibited by the pAPX plants was indicated by lower $H_2O_2$ accumulation, which was associated with lower GO activity and glyoxylate content. The suppression of both pAPXs and/or its downstream metabolic and molecular effects may trigger favourable antioxidant and compensatory mechanisms to cope with CAT deficiency. This physiological acclimation may involve signalling by peroxisomal $H_2O_2$, which minimized the photorespiration.**

*Key-words*: *Oryza sativa*; ascorbate peroxidase; $H_2O_2$ homeostasis; oxidative stress; photorespiration; signalling.

## INTRODUCTION

Despite the great advances in the understanding of oxidative metabolism in plants in the last decades (to review see Foyer & Noctor 2011), several contradictory data involving the classical antioxidant pathways have frequently been reported (Rizhsky *et al*. 2002; Miller *et al*. 2007; Koussevitzky *et al*. 2008; Carvalho *et al*. 2014). In addition, the physiological role displayed by some ascorbate peroxidase (APX) isoforms, especially peroxisomal isoforms, is poorly known. APX is coded by a gene family that is widely represented in plants, and the existence of two peroxisomal APX (pAPX) isozymes, which are targeted for bind to the external surface of peroxisomal membranes, has been confirmed in *Arabidopsis* (Panchuk *et al*. 2002; Narendra *et al*. 2006). A third isoform possibly targeted to the peroxisomal matrix has also been reported (Panchuk *et al*. 2002). These isoforms were initially characterized many years ago (Yamaguchi *et al*. 1995; Bunkelmann & Trelease 1996; Mullen *et al*. 1999). In rice, two peroxisomal APX isoforms, *OsAPX3* and *OsAPX4*, have been characterized, and both enzymes are also targeted for membranes (Teixeira *et al*. 2004, 2006).

The most important remaining questions concern the importance of pAPX in $H_2O_2$ scavenging during photorespiration and its possible role in signalling and peroxisome-cytosol cross-talking. The results reported to date remain controversial, but suggest that pAPX is important in the absence or in complement to catalase (CAT) (Wang *et al*. 1999). Works in which pAPX was overexpressed in *Arabidopsis* have suggested that transgenic plants are more resistant to certain abiotic stresses (Kavitha *et al*. 2008), oxidative stress generated by CAT inhibition (Wang *et al*. 1999) and methyl viologen (Kavitha *et al*. 2008). Tobacco transgenic plants that overexpress pAPX from *Salicornia brachiata* have displayed higher seed germination under osmotic and saline conditions (Singh *et al*. 2014). Nevertheless, a study conducted with pAPX3 knockout (KO) *Arabidopsis* grown under normal growth conditions and exposed to salt, chilling and heat stresses clearly showed that this gene or the encoded protein is dispensable for plant growth and development (Narendra *et al*. 2006).

Peroxisomal APX isoenzymes may act in concert with other peroxidases in peroxisomes, especially CAT. High photorespiratory $H_2O_2$ production in C3 plants subjected to abiotic stress is common, and under extreme situations, the photorespiration might represent up to 50% of Rubisco activity (Peterhansel & Maurino 2011). CAT has a high $K_M$ for $H_2O_2$, whereas pAPX has high affinity for this substrate (Yamaguchi *et al*. 1995). The simultaneous activities of both enzymes are important because they allow the elimination of

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high $H_2O_2$ amounts by CAT and the maintenance of low $H_2O_2$ levels by APX activity. Indeed, APX enzymes are considered to play a special role in the fine-tuning control of $H_2O_2$ levels, allowing signalling for redox homeostasis and other physiological processes (Shigeoka *et al*. 2002). In contrast, CAT activity has been associated with $H_2O_2$ scavenging and antioxidative protection in peroxisomes and glyoxysomes. In the presence of light and abiotic stresses, CAT degradation and/or delay in its re-synthesis may be induced, resulting in a deficiency in its activity (Hertwig *et al*. 1992).

Plants lacking CAT by KO, especially the *Arabidopsis cat2* mutant and others presenting CAT activity deficiency induced by pharmacological inhibitors, are good models to elucidate the role of pAPX and its involvement with CAT and other antioxidants in peroxisomes. These plants display several phenotypic anomalies, such as earlier senescence, followed by necrotic spots on leaves, which are associated with photorespiratory $H_2O_2$ accumulation (Chamnongpol & Willekens 1996; Willekens *et al*. 1997). The main oxidative feature of these CAT-deficient plants is that the glutathione-oxidized form (GSSG) and total glutathione are strongly increased, and as a consequence, the redox state of glutathione-reduced (GSH) is drastically decreased (Mhamdi *et al*. 2010). However, the biochemical mechanisms underlying the oxidation of the GSH pool under photorespiratory $H_2O_2$ are not completely elucidated (Noctor *et al*. 2013). Interestingly, in *Arabidopsis*, tobacco and barley that are deficient in CAT, the ascorbate (ASC) pool remains practically unaltered under high photorespiratory $H_2O_2$ (Kendall *et al*. 1983; Willekens *et al*. 1997), suggesting a minor antioxidant/signalling role.

Peroxisomal APX activity should play a central role in the $H_2O_2$ control under CAT deficiency and in the presence of normal activity of this enzyme. Unfortunately, few biochemical studies have characterized the effectiveness of pAPX in peroxisomes, and the primary peroxidases that overlap with CAT activity are still unknown (Mhamdi *et al*. 2010). However, plants have several other peroxidases that could reduce $H_2O_2$ to $H_2O$, including the glutathione peroxidase (GPX), phGPX, peroxidase-type glutathione-S-transferase (GST), thioredoxin-dependent peroxiredoxins, GST-dependent peroxiredoxins, glutaredoxin-dependent peroxiredoxins and type III peroxidases (Foyer & Noctor 2009). Some of these peroxidases are localized in or out of peroxisomes, and some require further confirmation (del Río *et al*. 2002; Schrader & Fahimi 2006). The relative importance of these enzymes for compensating and/or supplementing CAT and APX activities in peroxisomes is unclear (Kavitha *et al*. 2008).

The antioxidant metabolism in plants is complex, and experimental evidence suggests the existence of redundant mechanisms, and unexpected results commonly occur. Indeed, tobacco and *Arabidopsis* double-KO mutants were more resistant to oxidative stress than the single APX and CAT KO mutants (Rizhsky *et al*. 2002; Vanderauwera *et al*. 2011). In addition, double-KO *Arabidopsis* mutants for APX1 and chloroplast APX were also more resistant to oxidative stress (Miller *et al*. 2007). Recently, our group, while studying rice with silenced cytosolic and chloroplastic APXs, also reported unexpected results. The deficiency of both cytosolic and chloroplastic APXs greatly affected the redox homeostasis (Rosa *et al*. 2010), up-regulated a compensatory mechanism involving other peroxidases (Bonifacio *et al*. 2011), triggered changes in the expression of genes and proteins not directly related to antioxidant metabolism (Ribeiro *et al*. 2012), and displayed a compensatory mechanism for maintaining photosynthesis (Carvalho *et al*. 2014) and a mechanism for photoprotection complexes (Caverzan *et al*. 2014).

Plant cells display strong coordination between chloroplasts, peroxisomes, mitochondria and cytosol, especially during photorespiration (Van Aken & Whelan 2012; Munné-Bosch *et al*. 2013). The complexity of these interactions connecting several cross-talk pathways may be able to explain some of the complex and unexpected results concerning antioxidant enzymes localized in different cellular compartments (Rizhsky *et al*. 2002; Miller *et al*. 2007; Vanderauwera *et al*. 2011). Changes in the $H_2O_2$ levels and oxidation-reduction states of ASC and GSH, especially GSH content, may act as powerful signals capable of generating redox changes in compartments other than where they were produced (Mhamdi *et al*. 2010). For example, the excess $H_2O_2$ produced in cytosol caused by APX1 deficiency may trigger alterations in chloroplast metabolism (Davletova *et al*. 2005). In addition, the cross-talk may involve a transfer of reducing power (NADH) between peroxisomes, chloroplasts, mitochondria and cytosol, which would affect the redox state of each one of them (Yoshida *et al*. 2008).

Because the antioxidant physiological role of pAPX in plants is essentially unknown, especially in terms of its action in photorespiratory $H_2O_2$ scavenging and its relationship with CAT deficiency, we conducted this study using an integrated molecular-physiological approach to assess these unknown aspects. We utilized transgenic rice plants deficient in peroxisomal *OsAPX4*, which additionally presented nearly complete suppression of *OsAPX3* expression. In this study, we tested the following hypothesis: if pAPX is an important peroxidase for $H_2O_2$ homeostasis in peroxisomes, then the rice mutants that are simultaneously deficient in both pAPX and CAT enzymes should suffer higher oxidative stress induced by high photorespiratory $H_2O_2$ compared with non-transformed (NT) plants.

The results obtained in this study do not support the proposed hypothesis. Instead, pAPX-silenced rice exhibited an antioxidant response that provided an improved acclimation to oxidative stress that was induced by high photorespiration. The best performance in mutant plants was confirmed by several physiological and biochemical stress indicators (cell integrity, photosynthesis, lipid peroxidation and $H_2O_2$ accumulation). These responses were associated with down-regulation of glycolate oxidase (GO) activity, higher $CO_2$ assimilation and decreased glyoxylate content. The possible physiological mechanisms involved with the acclimation of pAPX to CAT deficiency are discussed later.

## MATERIALS AND METHODS

### RNAi vector construct, plant transformation and the growth of mutants and NT seedlings

A 218 bp sequence was amplified by PCR based on the sequence of the *OsAPX4* gene (LOC_Os08g43560). The following primers were used: APx4RNAiF: 5′ AA AAAGCAGGCTCCTGACAAGGCATTGTTGGAAG 3′ and APx4RNAiR: 5′ AGAAAGCTGGGTCCAGCTGC AGCAACAGCTACC 3′. The PCR product was cloned into the pANDA vector (Miki *et al*. 2005), which enables hairpin structure formation and post-transcriptional silencing of the *OsAPX4* gene. The pANDA vector contains the maize ubiquitin promoter and the *Hpt* gene for selection by hygromycin. The transformation of rice calli was achieved via *Agrobacterium tumefaciens* as described previously (Upadhyaya *et al*. 2000). Regenerated seedlings were grown at 28 °C in 10× diluted Murashige and Skoog (MS) medium with a photoperiod of 12 h and 150 $\mu$mol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux density (PPFD) in a growth chamber for 7 d. Three lines of pAPX4 transgenic plants were selected based on the transcript amounts: Lg, Lh and Lj, which expressed 10, 15 and 14%, respectively, of the transcript amount in the NT plants.

### Plant growth in the greenhouse

The Lg, Lh and Lj lines and the NT plants were transferred to 3 L plastic pots filled with half-strength Hoagland–Arnon's nutritive solution (Hoagland & Arnon 1950). The pH was adjusted to 6.0 ± 0.5 every 2 d, and the nutrient solution was changed weekly. The seedlings were previously grown for 45 d in a greenhouse under natural conditions: day/night mean temperature of 29/24 °C and mean relative humidity of 68% and a photoperiod of 12 h. The light intensity (from sunlight) inside the greenhouse varied as it would for a typical day from 0600 to 1800 h, reaching an average of maximum PPFD of 820 $\mu$mol $m^{-2}$ $s^{-1}$ at noon.

### 3-Amino-1,2,4-triazole (AT) application to entire plants

Forty-five-day-old transformed and NT plants were grown as previously described. For the entire-plant experiment, a group of plants was transferred to a growth chamber at 27 ± 2 °C/24 ± 2 °C (day/night), 70 ± 10% humidity, 400 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD and a 12 h photoperiod. The plants were acclimated under these conditions for 24 h. After this time, the specific CAT inhibitor (10 mM AT dissolved in 10 mM N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid) (HEPES) buffer at pH 6.5 with 1.5 mM $CaCl_2$ and 0.1% Triton X-100) was sprayed in excess on the shoot until a complete wetting was achieved. The plants were treated for 12 h, and after AT application, they did not exhibit any visual symptoms of leaf toxicity. The control plants were sprayed with the same solution without AT. The leaf samples were immediately harvested, separated for physiological, biochemical and PCR analysis, placed in liquid $N_2$ and transferred to a −85 °C freezer.

### Leaf segment experiments: CAT inhibition, GO inhibition and high light application

For the assay with leaf segments, the plants were previously acclimated in a growth chamber at the same conditions and for the same time period described for the intact plants experiments. For the AT treatment, leaf segments (10 cm) from 45-day-old plants were immersed in a buffer of 10 mM HEPES at pH 6.5 that contained $CaCl_2$ 1.5 mM and 0.01% Triton X-100 (v/v) in the presence of 5 mM AT. For the GO inhibition treatment, 10 mM α-hydroxy-2-pyridinemethanesulfonic (HPMS) was dissolved in the same medium utilized for AT. The leaf segments remained immersed in the 10 mM HPMS for 24 h or 12 h (time-course). In the low- and high-light experiments, the leaf segments were immersed in the same solution utilized for the CAT and GO inhibition using the previously described procedure. The plates were divided into four groups: (1) low light – LL (200 $\mu$mol $m^{-2}$ $s^{-1}$); (2) LL + 5 mM AT; and (3) high light – HL (1000 $\mu$mol $m^{-2}$ $s^{-1}$) + 5 mM AT. In treatments 1 and 2, the AT and LL were supplied for 24 h, whereas in treatment 3, the AT was supplied for 12 h followed by 12 h of HL application. Treatment 1 was used as the control. The leaf segments were collected using the same procedure as for the entire plant.

### Determining gas exchange and chlorophyll *a* fluorescence

The net $CO_2$ assimilation rate ($P_N$), transpiration and stomatal conductance ($g_s$) were measured using a portable infrared gas analyser (IRGA) system equipped with a light-emitting diode (LED) source and leaf chamber (IRGA LI-6400XT, LI-COR®, Lincoln, NE, USA). The IRGA chamber had the following internal parameters: 1000 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD, 1.0 ± 0.2 kPa vapour pressure deficit (VPD), 38 Pa $CO_2$ and 28 °C. *In vivo* chlorophyll *a* fluorescence was measured using a LI-6400-40 leaf chamber fluorometer (LI-COR) coupled with the IRGA equipment. The actinic light utilized for the measurement of gas exchange and chlorophyll *a* fluorescence was 1000 PPFD, which corresponded to the saturation light for $CO_2$ assimilation in rice (Carvalho *et al*. 2014). The fluorescence parameters were measured using the saturation pulse method (Schreiber *et al*. 1994) in leaves exposed to light and 30 min dark-adapted conditions. The intensity and duration of the saturation light pulse were 8000 $\mu$mol $m^{-2}$ $s^{-1}$ and 0.7 s, respectively. The amount of blue light was set to 10% of the PPFD to maximize the stomatal aperture (Flexas *et al*. 2007). The following parameters were assessed: the maximum quantum yield of photosystem II (PSII) [$Fv/Fm = (Fm - Fo)/Fm$], which was measured under 30 min dark-adapted conditions, and the effective quantum yield of PSII [$\Delta F/Fm' = (Fm' - Fs)/Fm'$], which was measured in leaves exposed to actinic light (1000 $\mu$mol $m^{-2}$ $s^{-1}$ PPFD). The photochemical quenching coefficient was calculated as follows: $qP = (Fm' - Fs)/(Fm' - Fo)$. The *Fm* and *Fo* parameters correspond to the maximum and minimum fluorescence of the

dark-adapted leaves, respectively. *Fm′* and Fs are the maximum and steady-state fluorescence in the light-adapted leaves, respectively, and *Fo′* is the minimum fluorescence after the far-red illumination of the previously light-exposed leaves (Schreiber *et al*. 1994; Flexas *et al*. 2007).

## Electrolyte leakage and lipid peroxidation

Membrane damage (MD) or cellular viability was measured by electrolyte leakage as described previously by Blum & Ebercon (1981). Twenty leaf discs (1.0 cm diameter) were placed in test tubes containing 20 mL of deionized water. The flasks were incubated in a shaking water bath (25 °C) for 12 h, and the electrical conductivity in the medium (L1) was measured. The discs were then boiled (95 °C) for 60 min and cooled to 25 °C, and the electrical conductivity (L2) was measured again. The relative MD was estimated by MD = L1/L2 × 100. The lipid peroxidation was measured based on the formation of thiobarbituric acid-reactive substances (TBARS) in accordance with Cakmak & Horst (1991). The concentration of TBARS was calculated using its absorption coefficient (155 $mM^{-1}$ $cm^{-1}$), and the results are expressed as $\eta$mol MDA-TBA g $FW^{-1}$.

## Determination of the ASC-GSH redox state

The contents of reduced (ASC), oxidized (DHA) and total ascorbate (ASC + DHA) were determined according to the protocol described by Queval & Noctor (2007). The assay of ASC was based on decrease of the ASC absorbance at 265 nm in the presence of ASC oxidase (AO). For determination of total ASC (ASC + DHA), the DHA was reduced by 25 mM DTT, and total ASC was measured as described for reduced ASC. The DHA content was calculated by the difference between total ascorbate and ASC, and all forms were expressed as $\mu$mol g fresh matter $(FM)^{-1}$ calculated from an ASC standard curve. GSH was measured by the GSH reductase (GR)-dependent reduction of 5,5′-dithio-bis (2-nitrobenzoic acid), DTNB, according to the Griffith (1980) method and detailed protocol described in Queval & Noctor (2007). The GSH and GSSG content were expressed as $\mu$mol g $FM^{-1}$ and calculated from standard curves of GSH and GSSG, respectively.

## Diaminobenzidine (DAB) staining

*In situ* detection of peroxide ($H_2O_2$) was performed by staining with 3,3′-DAB as previously described (Thordal-Christensen *et al*. 1997). Leaf segments were infiltrated under dark vacuum conditions with 10 mM potassium phosphate buffer, 10 mM $NaNO_3$ and 0.1% (w/v) 3,3′-DAB, pH 7.8. The segments were incubated for approximately 16 h in dark conditions and then destained with 15% (w/v) trichloroacetic acid (4:1 v/v) ethanol : chloroform for 48 h before being photographed.

## qRT-PCR

qRT-PCR experiments were conducted using cDNA synthesized from total RNA purified with Trizol (Invitrogen®, Carlsbad, CA, USA) as previously described (Rosa *et al*. 2010). The primer pairs to amplify the *Osfdh3* gene (LOC_Os02g57040) and *Osfa1* gene (LOC_Os03g08020) were used as internal controls to normalize the amount of mRNA present in each sample. All qRT-PCR reactions were performed with a StepOne plus real-time PCR system (Applied Biosystems®, Foster City, CA, USA) using SYBR-green intercalating dye fluorescence detection.

## Enzymatic activity assays

All enzymatic activities were assayed from the total soluble protein extract. The protein extraction was performed utilizing fresh leaf matter in the presence of 100 mM phosphate buffer, pH 7.5, containing 2 mM EDTA and 1 mM ASC. The APX activity was assayed following ASC oxidation by measuring the decrease in absorbance at 290 nm (Amako *et al*. 1994). The APX activity was expressed as $\mu$mol ASC mg $protein^{-1}$ $min^{-1}$. The CAT activity was measured by following the oxidation of $H_2O_2$ at 240 nm over a 300 s interval at 25 °C in the presence of 50 mM potassium phosphate buffer, pH 7.0, containing 20 mM $H_2O_2$ (Havir & McHale 1987). The CAT activity was calculated according to the molar extinction coefficient of $H_2O_2$ ($36 \times 10^{-3}$ $mM^{-1}$ $cm^{-1}$) and expressed as $\mu$mol $H_2O_2$ mg $protein^{-1}$ $min^{-1}$. The superoxide dismutase (SOD) activity was determined by inhibiting blue formazan production via Nitro Blue Tetrazolium (NBT) photoreduction. The reaction was started by illumination (30 W fluorescent lamp) at 25 °C for 6 min, and the absorbance was measured at 540 nm (Giannopolitis & Ries 1977). One SOD activity unit (UA) was defined as the amount of enzyme required to inhibit 50% of NBT photoreduction, and the activity was expressed as UA mg $protein^{-1}$ $min^{-1}$ (Beauchamp & Fridovich 1971). The GO activity and glyoxylate concentration were assayed by the formation of the glyoxylate–phenylhydrazone complex and measured after reading at 324 nm (Baker & Tolbert 1966). GO activity was calculated using the molar extinction coefficient of the glyoxylate-phenylhydrazone complex (17 $mM^{-1}$ $cm^{-1}$) and expressed as $\eta$mol $glyoxylate^{-1}$ mg $protein^{-1}$ $min^{-1}$, whereas the glyoxylate concentration was determined from a standard curve. The GPX activity was assayed by measuring the GSH oxidation using cumene hydroxide as an electron acceptor. The method is based on the measurement of GSH oxidation by the Griffith (1980) method after correction of GSH oxidation in the absence of cumene hydroxide. The enzymatic reaction medium was composed of 100 mM phosphate buffer at pH 7.0, which contained cumene hydroxide 0.5 and 4.0 mM GSH in the final reading medium. The reaction was stopped after 3 min at 25 °C. The GPX activity was expressed in $\eta$mol GSH mg $protein^{-1}$ $min^{-1}$. The GST activity was assayed by direct GSH consumption as described by Valentovičová *et al*. (2009), and the activity was expressed in $\eta$mol GSH mg $protein^{-1}$ $min^{-1}$.

## Statistical analysis

The experiments were conducted in a 2 × 2 or 2 × 2 × 2 factorial design with two genotypes and two AT concentrations

or two genotypes, two AT concentrations and two light regimes. An individual pot containing two plants or an individual Petri plate containing 15 leaf segments represents each replicate. Each experiment had at least four replicates. All the measurements were performed with at least four replicates from independent pots and plates. The data were analysed by analysis of variance (ANOVA), and the means were compared using Tukey's test ($P < 0.05$).

## RESULTS

### Physiological, biochemical and molecular characterization of the APX4 knockdown mutants

In this study, three rice lines (Lg, Lh and Lj) silenced in the peroxisomal APX4 were initially characterized to identify possible phenotypic changes after APX4 silencing. None of the studied lines displayed an altered phenotype from the germination to vegetative development phase (45 d after germination), which is shown in Supporting Information Fig. S1. However, pAPX4 silencing nearly completely suppressed *OsAPX4* gene expression in all the selected rice lines (Table 1), indicating that hairpin expression was very effective. As an indicator of plant growth, the fresh shoot matter accumulated at 45 d was similar between the pAPX knockdown lines and NT plants; this result is in contrast to the net photosynthesis ($P_N$), which was slightly higher in the APX4 lines compared with the NT plants. The stomatal conductance displayed a trend similar to $P_N$ in all the studied plants. The analysed parameters associated with PSII efficiency exhibited a different trend compared with the gas exchange parameters. The actual quantum yield of PSII ($\Delta F/Fm'$) and electron transport rate (ETR) at the PSII level did not change in any of the lines compared with the NT plants (Table 1). The APX, CAT, SOD and GO activities were evaluated to investigate whether the APX4-silenced plants exhibited changes in antioxidant enzymes and a key protein involved with photorespiration. The APX activities in the silenced lines were similar, CAT was lower, SOD was similar, and GO was significantly decreased compared with the NT plants (Table 2). After the biochemical and physiological characterization of these mutant lines, Lg was selected to represent the APX4 mutants because it displayed higher silencing and similar physiological and biochemical parameters compared with the other lines.

### CAT inhibition differentially affects the expression of the *OsAPX*, *OsGPX* and *OsCAT* isoforms in NT and APX4 mutants

The APX-silenced plants triggered an almost complete suppression of *OsAPX3* gene expression (a reduction of approximately 90% in the transcript amount compared with NT), which was most likely a result of the high homology displayed by these two genes (Fig. 1). Thus, the APX4 mutants used here were deficient in both peroxisomal APXs. In contrast, the quantity of transcripts of other *OsAPX* and *OsGPX* isoforms was slightly changed in the APX-silenced mutants in the control condition (Fig. 2a,b). To assess the combined effects of pAPX and CAT deficiency, an experiment was performed that utilized the classical 10 mM 3-AT CAT inhibitor, which was sprayed on the rice shoots of 45-day-old plants for a short period of time (12 h). The AT treatment did not change the transcript amount of either APX4 or APX3 in the mutants, which remained reduced by 90% compared with the NT control. This treatment also did not alter the expression of these genes in the NT plants. In the presence of AT, the cytosolic *OsAPX2* was up-regulated in both genotypes, but was more strongly expressed in the NT plants, which displayed a sixfold increase compared with the control. In contrast, higher transcript amounts of the

**Table 1.** Transcript amounts of *OsAPX4* and physiological characterization of three pAPX4-silenced rice lines and NT plants after 45 d of growth in nutrient solution

| | Lines | | | |
|---|---|---|---|---|
| Parameters | NT | Lg | Lh | Lj |
| *OsApx4* transcript amounts | 1.000a | 0.090b | 0.146b | 0.141b |
| Shoot FW (g per plant) | 30.83a | 28.76a | 27.12a | 26.71a |
| $P_N$ ($\mu$mol $m^{-2}$ $s^{-1}$) | 19.40b | 25.56a | 22.51a | 25.39a |
| gs (mol $m^{-2}$ $s^{-1}$) | 0.17b | 0.31a | 0.25a | 0.33a |
| $\phi$PSII ($\Delta F/Fm'$) | 0.29a | 0.32a | 0.32a | 0.28a |
| ETR ($\mu$mol $m^{-2}$ $s^{-1}$) | 126.58a | 144.38a | 142.3a | 122.89a |

Letters represent significant differences between the mutant lines and NT plants within each parameter at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test.
ETR, electron transport rate; FW, fresh weight; NT, non-transformed; pAPX, peroxisomal ascorbate peroxidises; PSII, photosystem II.

**Table 2.** Activities of enzymes in the leaves of three pAPX4-silenced rice lines and NT plants after 45 d of growth in nutrient solution

| | Lines | | | |
|---|---|---|---|---|
| Antioxidant enzymes | NT | Lg | Lh | Lj |
| APX ($\mu$mol ASC mg $prot^{-1}$ $min^{-1}$) | 0.56a | 0.52a | 0.54a | 0.53a |
| CAT ($\mu$mol $H_2O_2$ mg $prot^{-1}$ $min^{-1}$) | 74.02a | 66.58ab | 59.28b | 55.52b |
| SOD (U mg $prot^{-1}$ $min^{-1}$) | 0.91a | 1.04a | 0.96a | 0.97a |
| GO ($\eta$mol glyoxylate mg $prot^{-1}$ $min^{-1}$) | 91.22a | 71.48b | 70.17b | 70.06b |

Letters represent significant differences between the mutant lines and NT plants within each parameter at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test.
APX, ascorbate peroxidise; ASC, ascorbate; CAT, catalase; SOD, superoxide dismutase; GO, glycolate oxidase; pAPX, peroxisomal ascorbate peroxidises; PSII, photosystem II.

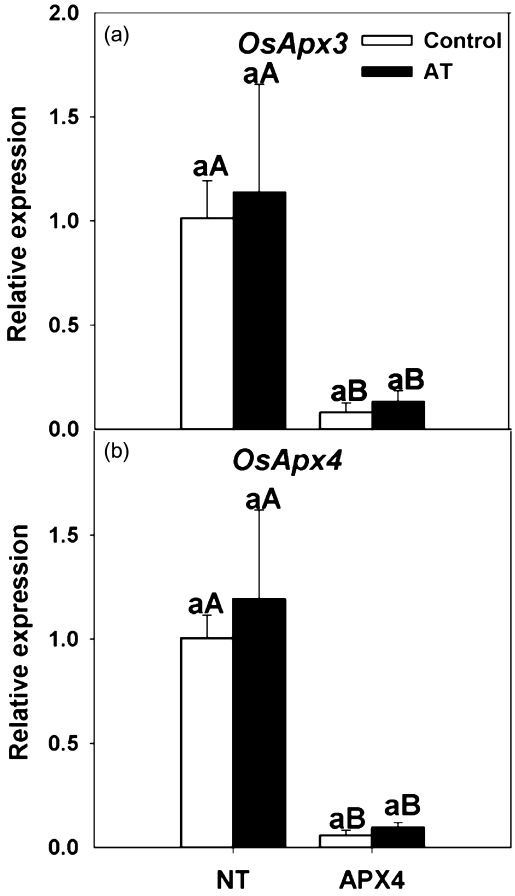


**Figure 1.** Transcript levels of the *OsAPX3* and *OsAPX4* genes in NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; NT, non-transformed.

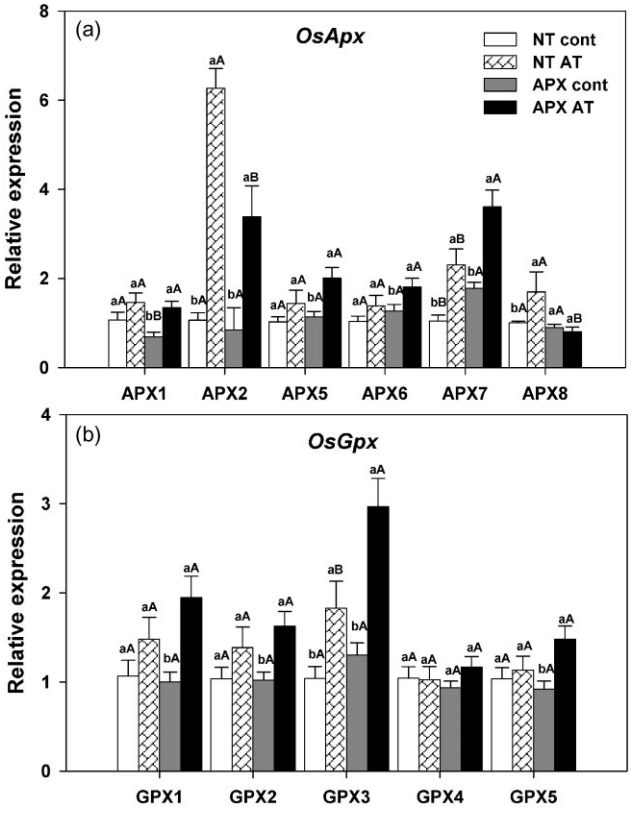


**Figure 2.** Transcript levels of the *OsApx* (a) and *OsGpx* (b) genes in NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; NT, non-transformed.

chloroplastic *OsAPX7* were observed in the pAPX4 mutants, increasing by nearly fourfold compared with the NT control. The *OsAPX5* and *OsAPX6* transcript amounts (both mitochondrial) displayed a slight increase in the AT-treated APX4 plants compared with NT control (Fig. 2a). *OsAPX4* silencing did not alter the expression of the five *OsGPX* genes in the control condition. The AT treatment triggered a slight increase in the amount of *OsGPX1* and *OsGPX2* transcripts (mitochondrial and cytosolic GPXs, respectively), whereas *OsGPX3* (mitochondrial) increased by threefold in the transgenic plants and increased slightly in the NT compared with NT control. CAT inhibition did not alter *OsGPX4* expression (chloroplastic) in either the NT or silenced plants, but induced a slight increase in *OsGPX5* (cytosolic) expression compared with the NT plants (Fig. 2b). Rice has three CAT genes: *OsCATA*, *OsCATB* and *OsCATC*. The silencing of *OsAPX4* did not change the *OsCATA* transcript amount in the mutants, but it was slightly down-regulated in the presence of AT (Supporting Information Fig. S2). The AT treatment down-regulated *OsCATA* more intensely in NT plants. Compared with *OsCATA*, the *OsCATB* gene was slightly up-regulated in the silenced mutants; however, CAT inhibition triggered a strong up-regulation of *OsCATB* in the mutants. The *OsCATB* gene was up-regulated by CAT inhibition in the NT plants to a lower extent compared with the mutants (Supporting Information Fig. S2). The *OsCATC* gene was shown to encode a protein localized in the photosynthetic tissue and related to photorespiration (Iwamoto *et al*. 2000). In this study, the AT completely inhibited the total CAT activity in the leaves of both genotypes, which will be shown later.

## Changes in cell integrity and photosynthesis that indicate that the APX4 mutants exhibited better acclimation to CAT inhibition at the entire-plant level

In this experiment, the entire rice plants (45-day-old) were sprayed with 10 mM AT for 12 h (a short-term exposure) to

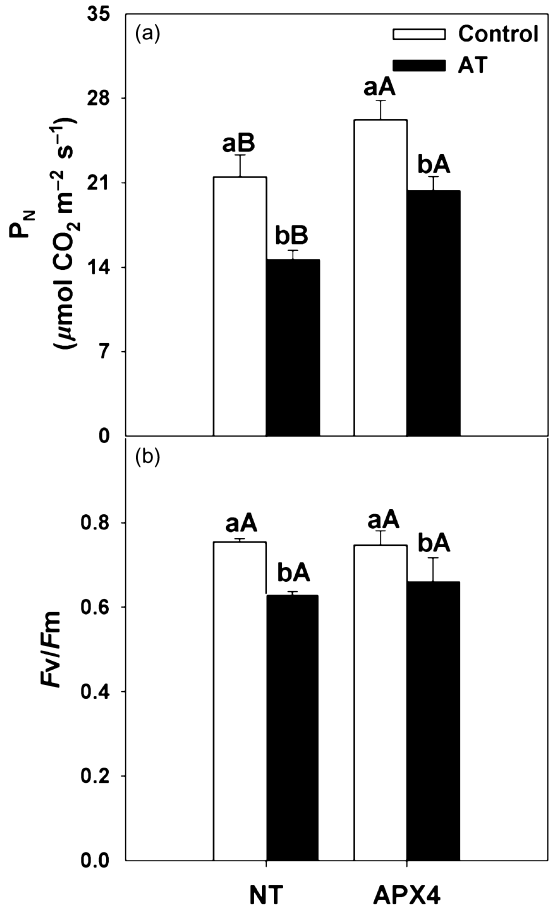


**Figure 3.** (a) Net photosynthesis and (b) maximum quantum yield in NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-aminotriazole; NT, non-transformed.

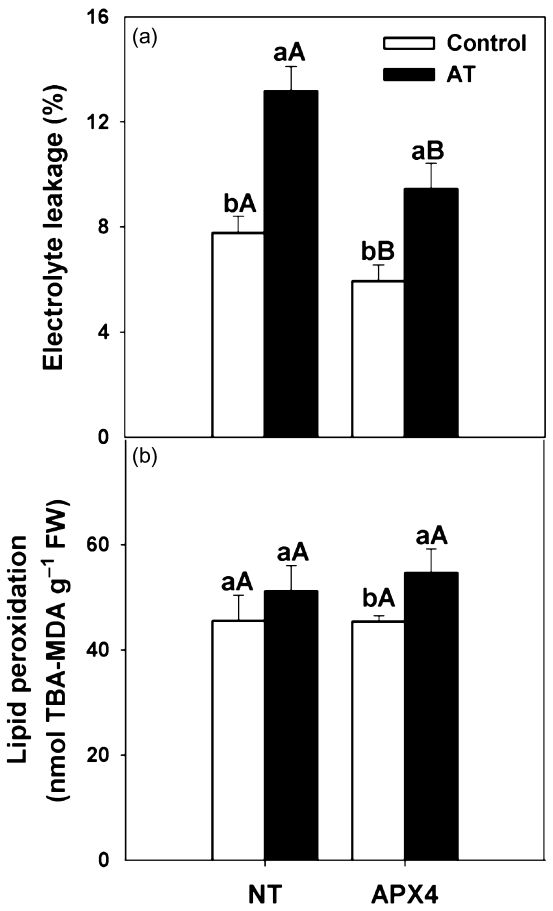


**Figure 4.** Changes in (a) electrolyte leakage percentage and (b) lipid peroxidation in NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; NT, non-transformed.

induce a complete inhibition of CAT activity in leaves while minimizing other physiological side effects caused by excess AT. After the 12 h treatment, both photosynthesis ($CO_2$ assimilation) and cell integrity decreased more intensely in the NT plants compared with the pAPX mutants (Figs 3a and 4a). These effects were proportional to the AT exposure time for both genotypes (data not shown). The changes induced by the transient CAT deficiency suggested that these symptoms reflected a mild physiological stress in both the NT and silenced mutants because the $P_N$ and electrolyte leakage in the leaves changed slightly. The potential quantum yield of PSII and *Fv*/*Fm* decreased slightly in both plants exposed to AT, indicating that the PSII apparatus was essentially unaffected by CAT inhibition (Fig. 3b). The TBARS content, an indicator of lipid peroxidation, was slightly increased in both genotypes; these minor changes apparently did not reflect an oxidative stress, and they corroborated the results that were obtained with other stress indicators and indicated that CAT inhibition for a short-time period induced slight physiological disturbances (Fig. 4b).

## CAT inhibition produced different effects on the activities of antioxidant enzymes and GO in the attached leaves of silenced and NT plants

To analyse the effects of CAT inhibition on the activities of other antioxidant enzymes and an important photorespiration enzyme, the activities of CAT, APX, GPX, GST and GO were measured in the leaves of both genotypes. Despite a short-term exposure to the AT inhibitor (12 h), CAT activity was completely inhibited after 12 h in both of the studied genotypes (Fig. 5a). This AT-induced CAT deficiency was sufficient to induce significant changes in the oxidative and antioxidant enzymatic activities. APX activity was not altered in the NT plants, whereas in the mutants, theactivity was increased in response to

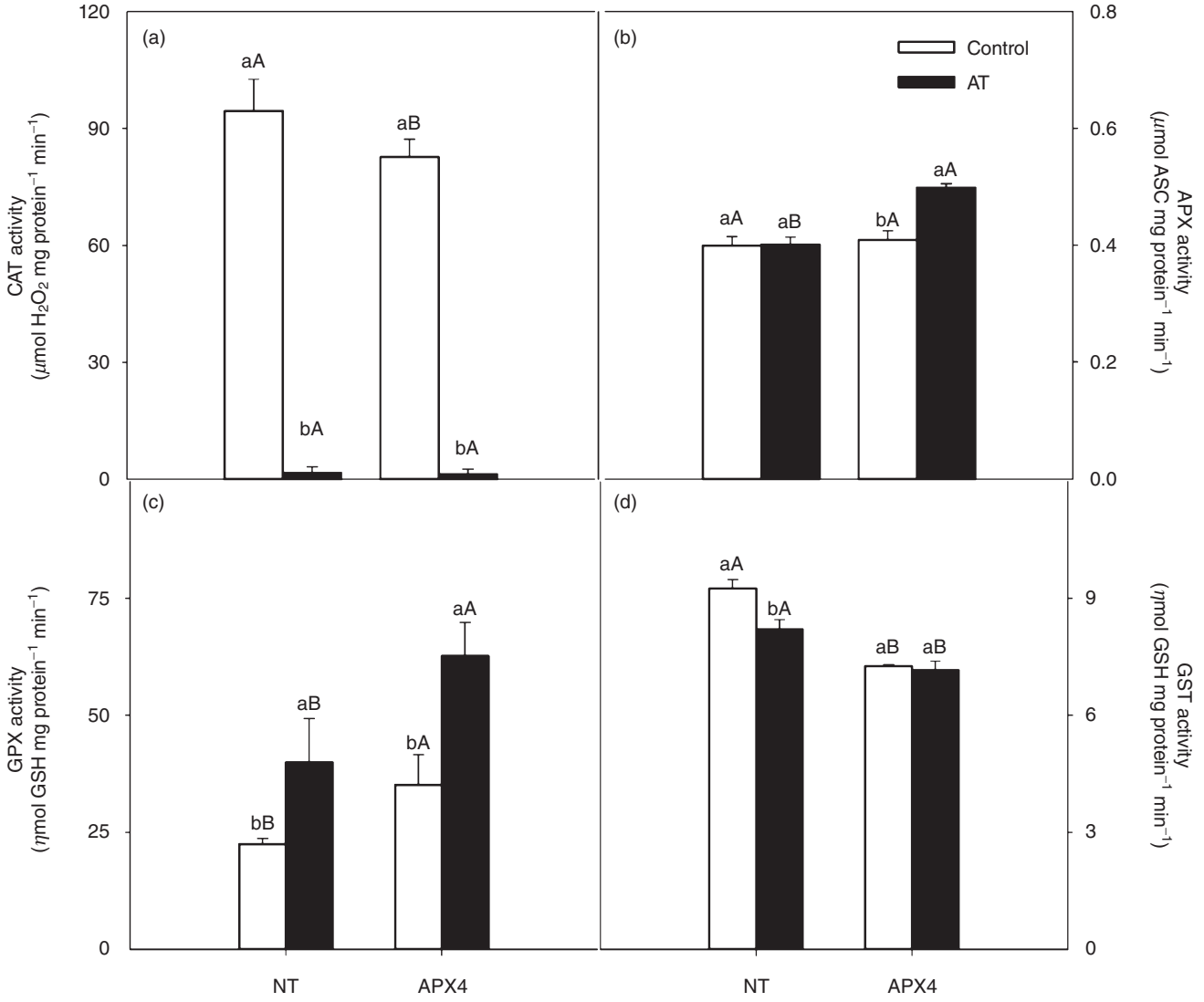


**Figure 5.** (a) CAT, (b) APX, (c) GPX and (d) GST activities in the NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; APX, ascorbate peroxidise; CAT, catalase; GPX, glutathione peroxidase; GST, glutathione-S-transferase; NT, non-transformed.

CAT inhibition (Fig. 5b). GPX activity was increased in response to CAT deficiency in both plants, but this increase was higher in the mutants compared with NT plants (Fig. 5c). GST activity was higher in NT than in mutants under both experimental conditions, but AT induced a slight decrease in GST activity only in the NT plants (Fig. 5d). GO activity remained essentially unchanged in both genotypes treated with AT, but the peroxisomal APX mutants exhibited lower activity under both experimental conditions (Fig. 6a), indicating a potential to display lower photorespiratory $H_2O_2$ production compared with the NT plants. In the presence of HPMS, a specific GO inhibitor, a close correlation between GO activity (Supporting Information Fig. S3) and CAT activity (Fig. 6b) was observed. In addition, in the APX4 mutants, CAT activity was lower than in the NT plants under both experimental conditions. Thus, GO inhibition induced a parallel decrease in CAT activity; however, when CAT was inhibited, the GO activity did not change (Fig. 6a). These results may indicate that GO activity is essential for the control of peroxisomal $H_2O_2$ levels via glycolate oxidation, which is associated with the level of CAT activity; however, reciprocal regulation between these two enzymes could not occur in peroxisomes. This close relationship between these two enzymes was also noted under control conditions jointly with glyoxylate content, the product of the GO reaction (Fig. 6c). Unexpectedly, the glyoxylate content was only strongly decreased in NT plants because of CAT inhibition. The glyoxylate content was unchanged in the mutants in response to AT, but it exhibited levels similar to those noted in the NT plants exposed to CAT inhibition. Interestingly, a close relationship between GO, glyoxylate and CAT activity was observed in the two genotypes

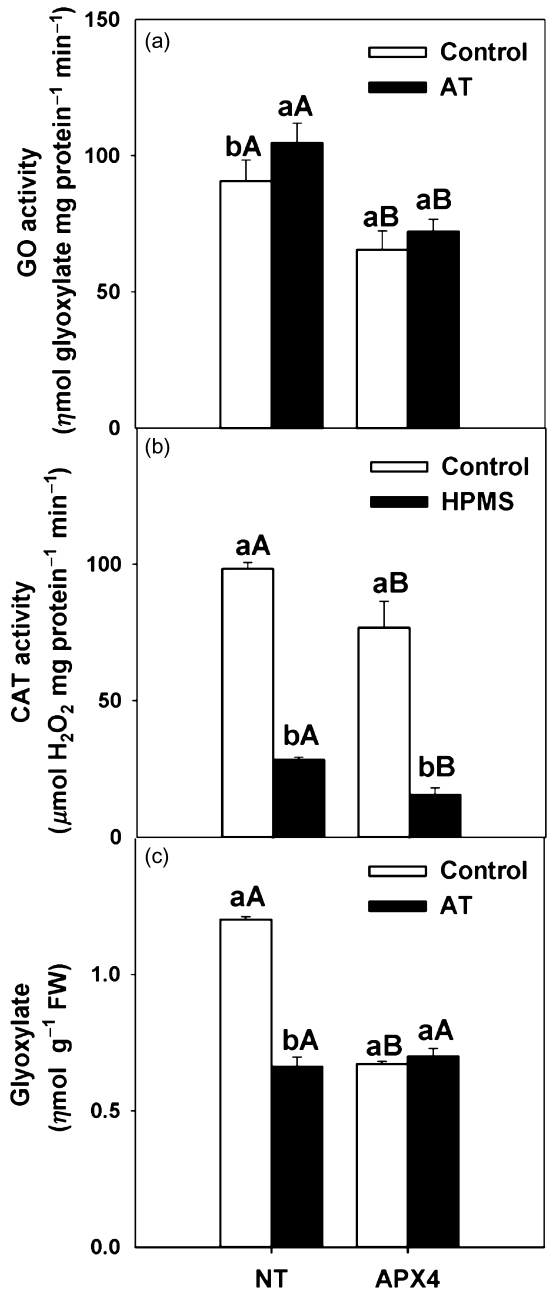


**Figure 6.** (a) GO, (b) CAT activities and (c) glyoxylate content in the NT and APX4 leaves of rice plants exposed to control or 10 mM AT or 10 mM HPMS. Different lowercase letters represent significant differences between the treatments within lines (NT, HPMS and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; APX, ascorbate peroxidise; CAT, catalase; GO, glycolate oxidase; HPMS, α-Hydroxypyridinemethanesulfonic acid; NT, non-transformed.

under control conditions, but not in the presence of AT, indicating that under CAT inhibition, the relationship between the activities of these two enzymes are differently modulated in the two studied genotypes.

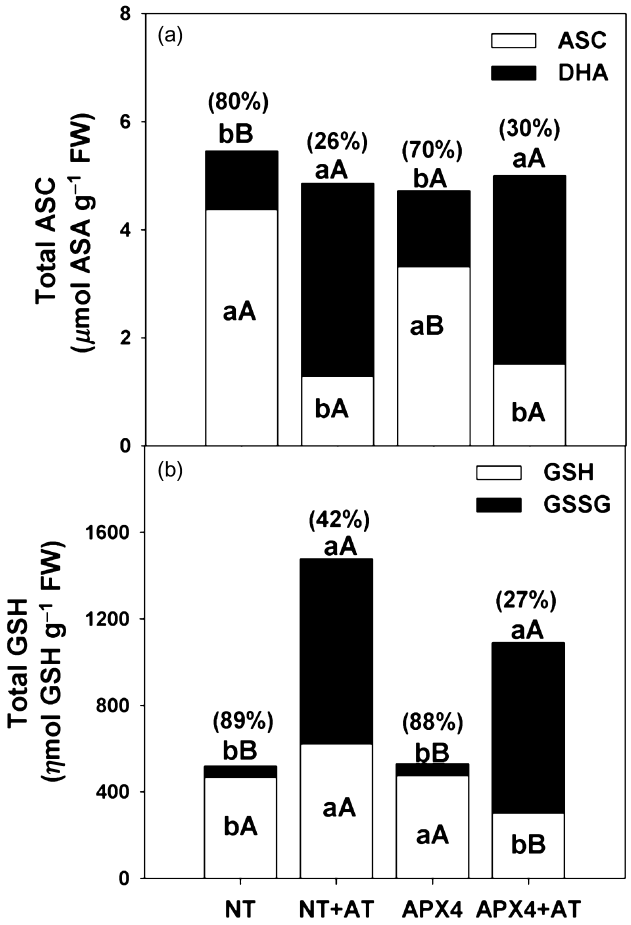


**Figure 7.** (a) ASC and (b) GSH contents in the NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; APX, ascorbate peroxidise; ASC, ascorbate; GSH, glutathione; NT, non-transformed.

## Peroxisomal APX-silenced mutants displayed changes in the oxidation-reduction states of ascorbate and glutathione under CAT inhibition at the entire-plant level

The oxidation-reduction states of ascorbate and glutathione in the presence and absence of CAT inhibition in APX4 mutants was evaluated to investigate whether the silencing could trigger changes in these non-enzymatic antioxidants and their redox states. The total ascorbate content and ASC redox state were slightly lower (70%) in the mutants compared with NT plants (80%), in the control condition (Fig. 7a). CAT inhibition induced a strong decrease in the ASC redox state to approximately 26 and 30% in the NT and mutant plants, respectively, but the total ascorbate was not altered in either genotype. These data may indicate that ASC was intensely oxidized to DHA under CAT inhibition and

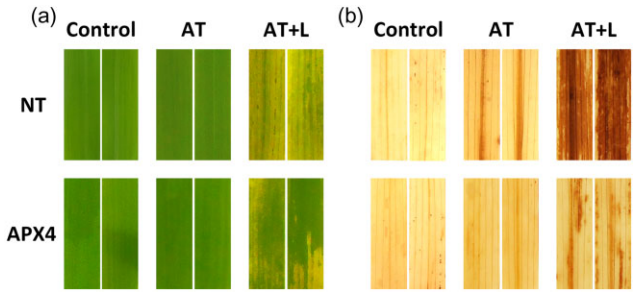


**Figure 8.** Appearance of leaf segments (a) and *in situ* localization of hydrogen peroxide (b) using DAB dye in NT and APX4 leaf segments of rice plants exposed to control or 10 mM AT with or without the increased light regime. The pictures were selected as the most representative from four independent replicates. AT, 3-amino-1,2,4-triazole; APX, ascorbate peroxidise; DAB, diaminobenzidine; NT, non-transformed.

probably high peroxisomal $H_2O_2$ or that secondary oxidizing conditions altered it into peroxisomes. These changes in ASC metabolism occurred to a similar extent in both plant types. The NT and mutant plants presented similar values for total glutathione, GSH and GSSG in the control condition. However, AT treatment differentially and remarkably altered the GSH oxidation-reduction states in these genotypes. In the NT plants, total glutathione was increased by fivefold, whereas in the mutants, total glutathione was increased by 2.5-fold compared with the control (Fig. 7b). The GSH content was not changed in the NT plants, but decreased by 50% in the mutants. In contrast, the GSSG content was increased similarly in both genotypes. The NT plants exhibited higher GSH redox state (42%) compared with mutants (28%). These results indicate that CAT inhibition induced higher GSH synthesis in the NT plants and allowed a more favourable GSH redox state compared with the APX4 mutants. However, these changes in the GSH redox state strongly suggest that CAT inhibition induced high photorespiratory $H_2O_2$ production; this reaction has been widely observed in *Arabidopsis cat2* mutants, which are the major model for studies on the deficiency of CAT and peroxisomal $H_2O_2$ accumulation.

### APX4-silenced rice displayed better acclimation to oxidative stress induced under high photorespiration as modelled by leaf segments

In the experiments with leaf segments, both the NT plants and mutants were exposed to CAT inhibition combined with enhanced photorespiration induced by the high-light condition to compare the differential acclimation between the two genotypes in response to an acute oxidative stress generated by high peroxisomal $H_2O_2$ levels. CAT inhibition, in the presence of high light, initially caused yellow spots in the leaf segments, followed by generalized senescence. These effects were remarkably more accentuated in the NT plants than the APX mutants (Fig. 8a). Interestingly, the single AT treatment under low light or single high-light conditions (data not shown) did not cause any injury symptoms, indicating that senescence was induced by CAT deficiency combined with the enhanced photorespiration induced by high-light conditions. These strong injury symptoms were closely correlated with intense $H_2O_2$ accumulation in the leaf tissue, as revealed by DAB staining (Fig. 8b). The single AT treatment in presence of low light also caused slight $H_2O_2$ accumulation, which was higher in the NT plants than the mutants and $H_2O_2$ accumulated preferentially in the leaf midrib (Fig. 8a,b). The visual indications and $H_2O_2$ accumulation intensity were correlated with important indicators of physiological and oxidative stresses, which are shown in Fig. 9. The electrolyte leakage (an indicator of cellular integrity) and TBARS content (an indicator of lipid peroxidation) in the NT plants were 90 and 100% higher than in the mutants, respectively, whereas the *Fv*/*Fm* (an indicator of PSII integrity), was 130% higher in the mutants than in the NT plants when both were exposed to the combination of AT and high light (Fig. 9). These data strongly indicate that the APX4 mutant was remarkably more acclimated than the NT plants to an acute oxidative stress induced by high photorespiratory $H_2O_2$ caused by CAT deficiency combined with high photorespiration.

## DISCUSSION

The data obtained in this study are intriguing; rice mutants deficient in both pAPX isoforms were less susceptible to mild oxidative stress induced by CAT inhibition under a moderate light regime ($400\ \mu mol\ m^{-2}\ s^{-1}$) and high-light regime ($1000\ \mu mol\ m^{-2}\ s^{-1}$). Our results are surprising because several studies utilizing transgenic plants that overexpress pAPX have demonstrated that these mutants are more resistant to different abiotic stresses (Wang *et al.* 1999; Li *et al.* 2009; Singh *et al.* 2014). Contrary to these reports, Narendra *et al.* (2006) utilized peroxisomal-APX3 KO in *Arabidopsis* and demonstrated that this protein is dispensable for development in normal growth conditions and under abiotic stress. Intriguingly, there are no published studies concerning the performance of pAPX-deficient mutants (using knockdown, KO or inhibitors) in response to high photorespiratory $H_2O_2$ conditions, which was investigated here. Thus, our report is the first to clearly demonstrate that pAPX deficiency in rice plants is likely capable of triggering an effective biochemical/molecular compensatory mechanism for coping with high peroxisomal $H_2O_2$. Paradoxically, the performance exhibited by these plants was inconsistent with expectations because the deficiency in both pAPX and CAT in the mutants should have induced a higher sensitivity to oxidative stress compared with that of the NT plants.

The complex and unexpected results displayed by pAPX mutants in response to oxidative disturbances are similar to other results involving simultaneous deficiency of cytosolic APX1 and CAT in tobacco and *Arabidopsis* (Rizhsky *et al.* 2002; Vanderauwera *et al.* 2011), cytosolic and thylakoid APX in *Arabidopsis* (Miller *et al.* 2007), cytosolic APX1/APX2 in rice (Rosa *et al.* 2010) and cytosolic APX1/APX2 in *Arabidopsis* (Suzuki *et al.* 2013). Unfortunately, despite of the existence of these data involving simultaneous down-expression of APX and/or CAT in diverse species and

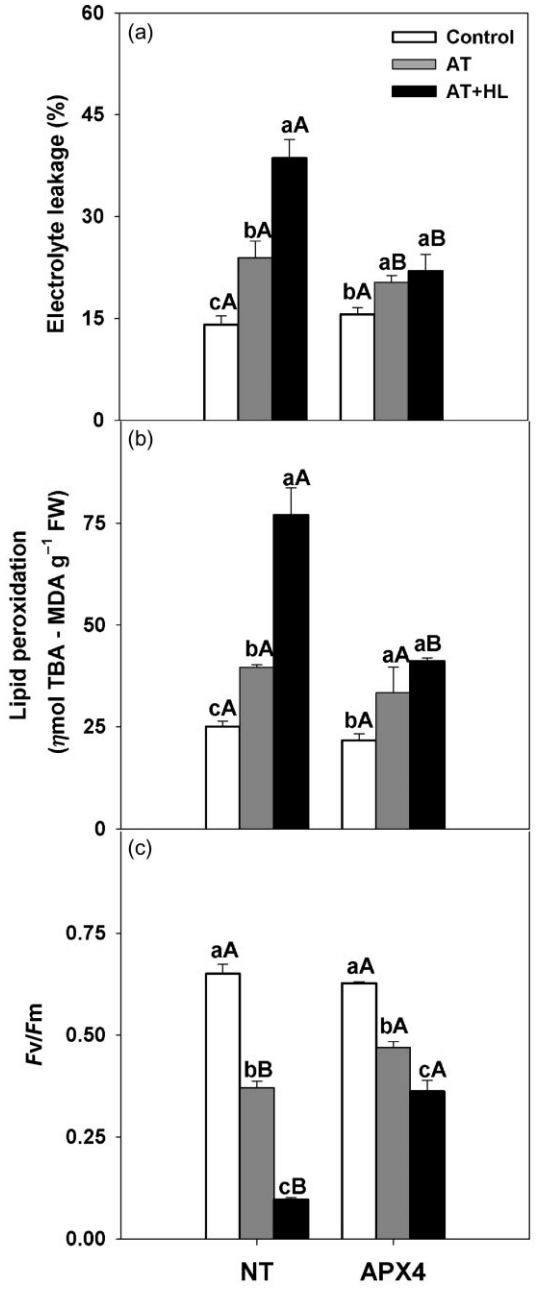


**Figure 9.** Changes in (a) electrolyte leakage, (b) lipid peroxidation and (c) maximum quantum yields of PSII in NT and APX4 leaf segments of rice plants exposed to the control (200 $\mu$mol m$^{-2}$ s$^{-1}$), 10 mM AT or high light (1000 mol m$^{-2}$ s$^{-1}$) + 10 mM AT. Different lowercase letters represent significant differences between the treatments within lines (NT and APX4), and different capital letters represent significant differences between the lines within treatments (control, AT and HL) at a confidence level of 0.05. The data are presented as the means of four replicates (±SD) and were compared by Tukey's test. AT, 3-amino-1,2,4-triazole; APX, ascorbate peroxidise; High Light (HL); PSII, photosystem II; NT, non-transformed.

distinct cellular compartments, these complex responses are still debated, and the elucidation of biochemical mechanisms is lacking. Mittler *et al.* (2011) and Carvalho *et al.* (2014) presented an interesting hypothesis suggesting that for each specific oxidative condition, a singular antioxidant response could occur. In addition, these authors have suggested that the classical antioxidant pathways should be revised and that new metabolic models are needed to explain these complex responses, especially in other model plants in addition to *Arabidopsis*, such as rice.

An overall explanation of the results obtained in this study is that pAPX mutant rice plants could have displayed large epigenetic changes and high phenotypic plasticity. These changes could have been related to efficient compensatory antioxidant mechanisms designed to cope with specific oxidative challenges, which has already been reported for rice plants silenced for cytosolic APX1/2 (Rosa *et al.* 2010; Bonifacio *et al.* 2011; Ribeiro *et al.* 2012; Carvalho *et al.* 2014) and chloroplastic APX (Caverzan *et al.* 2014). In principal, the pAPX silencing should have caused a significant decrease in the peroxisomal APX activity, which should have result in changes in the peroxisomal $H_2O_2$ metabolism. These changes could have triggered additional antioxidant pathways, possibly via $H_2O_2$ and/or GSH signalling (Nyathi & Baker 2006; Munné-Bosch *et al.* 2013). Unfortunately, the literature is very scarce on the cellular mechanisms involved with pAPX in peroxisomes.

The peroxisomal APX isoenzymes are localized in membranes and turned in the direction of the cytosol (Yamaguchi *et al.* 1995; Bunkelmann & Trelease 1996; Mullen *et al.* 1999; del Río *et al.* 2002). Thus, it seems plausible to argue that these enzymes could also act on the cytosolic $H_2O_2$ homeostasis and mediate the peroxisome-cytosol cross-talk. If this condition occurs under *in vivo* conditions, this proposed mechanism could create new paradigms for peroxisomal $H_2O_2$ metabolism and its overall antioxidant role. It has been widely accepted that the $H_2O_2$ produced in peroxisomes may cross the membrane towards the cytosol and/or chloroplasts and mitochondria (del Río *et al.* 2002). This feature could alleviate the oxidative effects on the peroxisomes, especially under CAT deficiency and/or high photorespiration. Moreover, these enzymes could also reduce $H_2O_2$ concentrations in concert with cytosolic APX, which would avoid toxicity and maintain the $H_2O_2$ at levels suitable for signalling. Thus, the deficiency of pAPX could generate specific redox signals, which in turn could trigger signalling for singular redox responses. These mechanisms could be distinct and more effective than those displayed by the NT rice plants, which might only involve classical antioxidant pathways (Mittler *et al.* 2011).

Interestingly, the NT plants with CAT deficiency exhibited higher $H_2O_2$ concentrations, evaluated by the semi-quantitative DAB staining method in the leaf segments, which were directly correlated with total glutathione levels and GSH content. Although DAB staining is a semi-quantitative method, in certain situations, it might be suitable to evaluate peroxide accumulation at the tissue level (Queval *et al.* 2008). An accurate quantitative measurement of $H_2O_2$ in leaf tissues by chemical and enzymatic method can be

difficult, and the obtained results are frequently controversial and not easily interpreted physiologically (Queval *et al*. 2011). To overcome this problem, authors have utilized the $H_2O_2$-mediated gene expression as an alternative method for evaluation of $H_2O_2$ accumulation in peroxisomes of plants deficient in CAT (Queval *et al*. 2009; Noctor *et al*. 2013).

Despite the total inhibition of CAT activity in rice leaves, the expression of *OsCATA* and *OsCATB* genes in response to AT application were contradictory. The *OsCATB* transcript amount was strongly up-regulated in silenced mutants, whereas in NT plants, the up-regulation was lower. Although the protein encoded by the *OsCATB* gene is not considered to be located in peroxisomes (Iwamoto *et al*. 2000; Mhamdi *et al*. 2012), our group previously demonstrated that the expression of the *OsCATB* gene was strongly up-regulated in rice leaves after exposure to high salinity (Menezes-Benavente *et al*. 2004). Contrary to what has been reported in the literature, the *OsCATC* gene, which most likely encodes a class I protein for peroxisomes, was not up-regulated by salinity (Menezes-Benavente *et al*. 2004) even after several times of salt exposure. The response presented by the *OsCATB* gene has been recurrent in our lab in the presence of other abiotic stress. However, *OsCATA* expression has been frequently down-regulated in rice leaves in response to abiotic stress. Although these questions are not a central focus of the current work, they require further study to determine the metabolic roles of CAT isoforms in photorespiration activity in rice.

GSH metabolism in peroxisomes under CAT deficiency is important. The GSSG levels in the NT plants were similar to those indicated by pAPX mutants. These results suggest that an intense *de novo* synthesis of GSH was more prominent in NT plants, favouring its redox state. Increases in the total glutathione pool have been frequently found in other species, especially in the *Arabidopsis* mutant *cat2*, which lacks CAT2 (Queval *et al*. 2009; Han *et al*. 2013; Gao *et al*. 2014). The biochemical and molecular mechanisms that control the increase in GSH synthesis in plants in response to increased $H_2O_2$ are still being debated (Queval *et al*. 2011; Han *et al*. 2013; Noctor *et al*. 2013). GSH is the first line of antioxidant defence under high peroxisomal $H_2O_2$ conditions because it is easily regenerated from GR activity and synthesized *de novo* by the rate-limiting γ-glutamylcysteine synthetase (γ-ECS) enzyme (Noctor *et al*. 2012). The GSSG pool is biochemically stable and contributes to the maintenance of adequate GSH regeneration via GR activity (Mhamdi *et al*. 2010; Noctor *et al*. 2012).

The mechanisms associated with increased photorespiratory $H_2O_2$ and GSH oxidation in this study are unclear because the expression of GPX isoforms was up-regulated more intensely in the mutants, in which the $H_2O_2$ levels were lower than in the NT plants. In certain species, GPX may utilize $H_2O_2$ as an electron acceptor and GSH as a reducing agent (Iqbal *et al*. 2006; Chang *et al*. 2009; Bonifacio *et al*. 2011). The GSH oxidation in this reaction might partially explain the lower concentrations of $H_2O_2$ and GSH in the pAPX mutants. However, evidences have suggested that under *in vivo* conditions, thioredoxins and organic hydroperoxide are more important than $H_2O_2$ and GSH for GPX activity (Herbette *et al*. 2002; Iqbal *et al*. 2006). The GST activity apparently was not important for consuming GSH in both genotypes, despite this enzyme/gene family being very large and functionally complex (Dixon *et al*. 2011). However, other reactions dependent on GSH reduction were not measured here, such as monodehydroascorbate reductase (MDHAR), peroxidase-type GST, GSH-dependent thioredoxin reductase and other reactions between GSH and thiol-proteins (Dietz 2014). These reactions could explain the intense GSH oxidation, especially in the NT plants, but further studies are required to elucidate this point in rice.

The improved acclimation displayed by silenced plants was associated with lower levels of total glutathione, a GSH redox state and similar GSSG contents and, this profile was associated with lower photorespiratory $H_2O_2$ levels. Thus, these parameters alone are not suitable to explain the effective antioxidant protection against oxidative stress induced by CAT deficiency in rice, which has been reported for certain species (Smith *et al*. 1985; Willekens *et al*. 1997; Han *et al*. 2013). Contrary to what has been widely reported for other species, such as *Arabidopsis* and tobacco (Mhamdi *et al*. 2010; Foyer & Noctor 2013; Han *et al*. 2013), the ASC redox state was greatly altered by high photorespiratory $H_2O_2$ in both genotypes, strongly suggesting that rice intensely utilize this antioxidant during oxidative stress generated by high photorespiratory $H_2O_2$. In addition, GSH, $H_2O_2$ and ASC are considered to be powerful signalling molecules involved with redox homeostasis. The differences found between the two genotypes for acclimatizing to high photorespiratory $H_2O_2$ may be associated with the signalling of these molecules. Alternatively, these differences could have involved only changes in the $H_2O_2$ balance, which is related to the capacity of production and scavenging and involve classical and non-classical antioxidants pathways (rates of GO activity, photorespiration and photosynthesis).

Recent evidence obtained from GR KO *Arabidopsis* mutants and other studies has demonstrated that a set of GSH-dependent gene expression overlaps with the $H_2O_2$-dependent genes and that the signal transduction pathway related to CAT deficiency is very complex and may involve GR expression and phytohormones, such as jasmonic acid, auxins and brassinosteroids (Mhamdi *et al*. 2010; Jiang *et al*. 2012; Gao *et al*. 2014). $H_2O_2$ may be involved in the signalling to express peroxidases (Karpinski *et al*. 1997; Klein *et al*. 2012), which could explain the differential expression of certain APX and GPX isoforms in the mutant and NT plants (Bonifacio *et al*. 2011). In addition, ASC could have acted to signal several processes (Foyer & Noctor 2011), but its role as a signalling molecule in rice is unclear. The signalling mechanisms that involve ASC are not as well understood as those attributed to $H_2O_2$ and GSH (Foyer & Noctor 2011).

Broadly, the metabolic alterations that occurred in both rice plants exposed to high photorespiratory $H_2O_2$ may have utilized cross-talk mechanisms that might have involved peroxisomes chloroplasts and mitochondria because these compartments are localized close to the cytosol and the

coordinated photorespiratory metabolism involves all of these organelles (del Río *et al*. 2002; Peterhansel & Maurino 2011). For example, cytosolic $H_2O_2$ is a powerful signalling mechanism in the expression of several genes involved in photosynthesis (Davletova *et al*. 2005). The pAPX mutants suffered smaller effects related to $CO_2$ assimilation, and this process is the most important electron consumer from the photochemistry phase, restricting $H_2O_2$ production in chloroplasts (Carvalho *et al*. 2014). Moreover, GO is a fundamental enzyme involved in $H_2O_2$ formation in peroxisomes during photorespiration (Zelitch *et al*. 2009).

The lower GO activity observed in the pAPX mutants was correlated with the glyoxylate levels and CAT activity, which suggests lower photorespiratory $H_2O_2$ production (Carvalho *et al*. 2014; Lu *et al*. 2014). Under CAT inhibition, the glyoxylate content was remarkably decreased only in the NT plants, indicating that this change could be related to pAPX silencing or downstream metabolic alterations. Peroxisomal glyoxylate might be consumed in the glyoxylate cycle in glyoxysomes, and during senescence and under certain abiotic stresses, the conversion of leaf peroxisomes into glyoxysomes is possible (del Río *et al*. 2002). The pAPX mutants showed significantly less senescence than the NT plants, and this response was associated with lower $H_2O_2$ levels (measured by DAB staining). It is widely known that $H_2O_2$ is involved in senescence (for a review, see Pintó-Marijuan & Munné-Bosch 2014), and the deficiency of pAPX in rice plants may affect this response by mechanisms that are not clear.

In conclusion, the peroxisomal APX knockdown rice leaves displayed an unexpected physiological acclimation to the oxidative stress generated by a transient CAT deficiency. However, the mutants deficient in pAPX exhibited important alterations in their oxidative and antioxidant metabolism, which could have generated a phenotype more capable of coping with high photorespiratory $H_2O_2$. In this context, the higher $CO_2$ photosynthetic assimilation and lower GO activity in leaves could mitigate the $H_2O_2$ production in peroxisomes. Because the glyoxylate content was lower in the mutants, this metabolite may be involved in photosynthesis and photorespiration modulation with $H_2O_2$ as a signalling mechanism. However, further studies are required to explain the features exhibited by peroxisomal APX-silenced rice mutants in the presence of CAT deficiency.

## ACKNOWLEDGMENTS

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## SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

**Figure S1.** Habitus of representative plants from four replicates (each replicate represented by a pot containing two plants). Non-transformed (NT) plants and three lines of *OsApx4*-silenced plants (Lg, Lh and Lj) after 45 d of growth in a complete nutrient solution under normal growth conditions.

**Figure S2.** Transcript levels of *OsCat* genes in NT and APX4 leaves of rice plants exposed to control or 10 mM AT. Different lowercase letters represent significant differences between the lines within treatments (control and AT), and different capital letters represent significant differences between the treatments within lines (NT and APX4) at a confidence level of 0.05. The data are the means of four replicates (±SD) and were compared using Tukey's test.

**Figure S3.** Time course of GO activity in the leaf segments of NT rice plants exposed to the control or 10 mM HPMS over 12 h. The data are the means of the four replicates (±SD).