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Arabidopsis thaliana plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death

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Summary

Ascorbate peroxidases (APX), localized in the cytosol, peroxisomes, mitochondria and chloroplasts of plant cells, catalyze the reduction of H_2O_2 to water by using ascorbic acid (ASA) as specific electron donor. The chloroplastic isoenzymes of APX are involved in the water–water cycle, which contributes to the photophosphorylation coupled to the photosynthetic electron transport. In order to better clarify the contribution of thylakoidal APX (tAPX) to the reactive oxygen species (ROS) scavenging activity, as well as to the fine modulation of ROS for signaling, we produced *Arabidopsis* lines overexpressing tAPX. These lines show an increased resistance to treatment with the O_2^- generating herbicide Paraquat (Pq). However, when challenged with photoinhibitory treatments at high light or low temperature, or with iron (Fe) or copper (Cu) overload, the tAPX-overexpressing lines show no increased resistance with respect to controls, indicating that in such experimental conditions, tAPX overexpression does not reinforce plant defenses against the oxidative stresses tested. Interestingly, the nitric oxide (NO)–donor sodium nitroprusside (SNP) represses accumulation of tAPX transcript; SNP also partially inhibits tAPX enzymatic activity. After treatment with SNP, the tAPX-overexpressing lines show reduced symptoms of damage with respect to control plants treated with SNP. These transgenic lines confirm that H_2O_2 acts in partnership with NO in causing cell death and highlight the important role of tAPX in the fine modulation of H_2O_2 for signaling.

Keywords: *Arabidopsis thaliana*, ascorbate peroxidase, nitric oxide, oxidative stress, programmed cell death, thylakoids.

Introduction

Reactive oxygen species (ROS) include partially reduced forms of oxygen (H_2O_2, $OH^·$, O_2^-), as well as oxygen in a higher excited state (singlet oxygen $^1O_2$); they are obligate by-products of aerobic metabolism produced in excess during various stresses, including high light, air pollutants, drought, heavy metals, mechanical stress, or pathogen infection (Allen, 1995; Bowler *et al.*, 1992; Karpinski *et al.*, 2001; Noctor and Foyer, 1998).

Plant cells front the noxious effects of ROS by limiting their production and by ROS scavenging (Mittler, 2002 and references therein); such diverse responses are regulated by a complex net of signaling pathways (Knight and Knight, 2001).

Ascorbate peroxidases (APX), by reducing H_2O_2 to water with ascorbic acid (ASA) as specific electron donor, are directly involved in ROS scavenging (Asada, 1999). APX are ubiquitous in the plant cells, being localized in the chloroplasts (Chen *et al.*, 1992; Jespersen *et al.*, 1997), mitochondria (De Leonardis *et al.*, 2000; Jimenez *et al.*, 1997), peroxisomes (Jimenez *et al.*, 1997; Shi *et al.*, 2001; Zhang *et al.*, 1997), and in the cytosol (Caldwell *et al.*, 1998; Jespersen *et al.*, 1997; Kubo *et al.*, 1993; Santos *et al.*, 1996). Chloroplastic APX can be found both anchored to the thylakoidal APX (tAPX), where the enzyme has one major hydrophobic domain responsible for spanning to the stroma-exposed thylakoid membranes, or to the

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stromatic APX (sAPX) (Jespersen *et al.*, 1997; Shigeoka *et al.*, 2002; Yamaguchi *et al.*, 1996). A protein localized in the lumenal side of the thylakoids and with strong homology with other plant APX has also been identified; however, its primary structure lacks a known peroxidase motif, nor has its functionality been tested yet (Kieselbach *et al.*, 2000).

tAPX and sAPX are encoded in spinach by a single gene, which generates both isoenzymes by alternative splicing: the two isoenzymes share the transit peptide and the catalytic domain, but differ in the C-terminal 50 amino acids (Ishikawa *et al.*, 1996, 1997; Yoshimura *et al.*, 1999). Similar observations have been made in pumpkin (Mano *et al.*, 1997) and tobacco (Yoshimura *et al.*, 2002). *Arabidopsis thaliana* represents, at present, the only species containing two different genes encoding for the two different isoforms of chloroplastic APX (Jespersen *et al.*, 1997).

Different stresses induce a rise in total APX activity (Biemelt *et al.*, 1998; Rao *et al.*, 1996; Schutzendubel *et al.*, 2001; Ye and Gressel, 2000). In addition, cytosolic APX (cAPX) transcripts accumulate in response to ozone or sulfur dioxide (Kubo *et al.*, 1995), iron (Fe) or copper (Cu) treatment (Vansuyt *et al.*, 1997), the O_2^--generating herbicide Paraquat (Pq) (Ye and Gressel, 2000; Yoshimura *et al.*, 2000), heat stress (Panchuk *et al.*, 2002), or high light (Fryer *et al.*, 2003; Karpinski *et al.*, 1997; Yoshimura *et al.*, 2000). Also, the transcripts of peroxisomal APX accumulate in response to various oxidative stresses like cold, heat, UV light, H_2O_2, salt, or Pq (Shi *et al.*, 2001; Zhang *et al.*, 1997). On the reverse, levels of chloroplastic APX transcripts are poorly modified by external stimuli (Panchuk *et al.*, 2002; Shigeoka *et al.*, 2002; Yoshimura *et al.*, 2000); nevertheless, an increase in tAPX activity in wheat subjected to water deficit (Sgherri *et al.*, 2000) and the accumulation of tAPX and sAPX transcripts in *Arabidopsis* lines with an antisense suppression of 2-cysteine peroxiredoxin (Beier *et al.*, 2000) have recently been observed.

Besides the poor change in transcript levels upon environmental stresses, chloroplastic APX show other peculiarities with respect to the other APX isoforms. All APX enzymes are labile in ASA-depleted medium, but the half-inactivation time of chloroplastic APX (around 15 sec) is much shorter than that of the other isoforms (Asada, 1992; Ishikawa *et al.*, 1998; Yoshimura *et al.*, 1998).

As far as their function is concerned, chloroplastic APX are involved in the water–water cycle, where the O_2^- produced at the photosystem I (PSI) site by the Melher reaction is reduced to water in a two-step reaction, catalyzed by the SOD and by the chloroplastic APX. The water–water cycle contributes in maintaining a proper ATP/NADPH ratio and in alleviating the over-reduction of photosystems when plants are exposed to photoinhibitory conditions (Asada, 1999).

In order to better clarify the contribution of tAPX in the regulation of ROS levels and in the plant protection against oxidative stress, we produced *Arabidopsis* lines overexpressing tAPX, and tested them under different stress conditions.

Results

Production and isolation of A. thaliana *35S::tAPX lines*

Arabidopsis thaliana tAPX cDNA (X98926 clone; Jespersen *et al.*, 1997) was cloned in the pBE2113-GUS transformation vector (Mitsuhara *et al.*, 1996; Figure 1). *Agrobacterium tumefaciens* GV3101 was transformed by electroporation with such construct or with the empty pBE2113-GUS vector as control. Thirty *A. thaliana*, ecotype Columbia (Col), plants were transformed with *Agrobacterium* by floral dip; each transformed plant was grown and allowed to self-fertilize, and T_1 seeds were collected.

Ninety-one T_1 plants showing resistance to kanamycin (kan; k^r trait) were analyzed by polymerase chain reaction (PCR) for integrity of the inserted transgene, by using the primers annealing on the tAPX and the GUS sequences (Figure 1).

The presence of the intact transgene was confirmed in 20 T_1 lines. These positive lines were transferred on soil and allowed to produce seeds. T_2 seeds were collected and tested for segregation of k^r trait. In eight different T_2 lines, the segregation ratio k^r:sensitivity to kan (k^s) = 3 : 1 confirmed one insertion site (Table 1).

For each of these eight T_2 lines, 20 seedlings were transferred onto the soil, allowed to produce seeds, and the 20 T_3 seed families were tested for segregation of the k^r trait. T_3 lines, homozygous for a single insertion site, were isolated and propagated for further analysis.

Identification of A. thaliana *transgenic lines with increased tAPX activity in the thylakoidal membranes*

Among the eight 35S::tAPX lines homozygous for a single insertion site, the 14/2, 8/5, 12/3, and 13/2 lines show

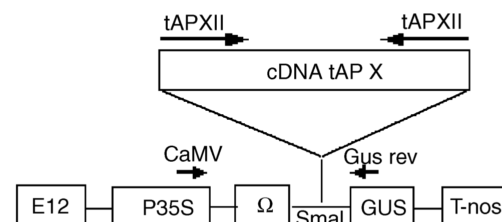

Figure 1. tAPX overexpression vector.
Construction of tAPX overexpression vector used to transform *A. thaliana* var. Col plants. The *Sal*1–*Not*1 fragment of the *A. thaliana* tAPX cDNA (X98926 clone) has been inserted in the *Sma*I pBE2113-GUS vector. The positions of the primers, used for confirming correct orientation of the insert, are indicated by arrows. The drawing is not to scale.

Table 1 Segregation analysis of *A. thaliana*-transformed T_2 seedlings

T_2 lines	Seedlings Total	k^r	k^s	χ^2-values (k^r:k^s = 3 : 1)
2/1	180	131	49	0.47
4/2	169	131	38	0.51
8/5	177	131	46	0.12
11/2	88	64	24	0.24
12/2	308	225	83	0.62
12/3	141	105	36	0.04
13/2	122	91	31	0.01
14/2	187	146	41	1.02
19/2	227	180	47	2.34

Segregation of k^r trait in T_2 35S::tAPX seedlings. For each T_2 line, the total number of seedlings tested and the number of seedlings showing either k^r or k^s trait have been reported. The hypothesis of a segregation ratio k^r:k^s = 3 : 1 has been accepted in all these T_2 lines with the χ^2-test (χ^2-values in the last column) with 0.05 significance level.

increased mRNA accumulation with respect to the wild-type Col and to the control line EV1 (Figure 2a).

These lines also accumulate higher levels of tAPX protein in the thylakoidal membranes (Figure 2b). The chl-mAb6 monoclonal antibody used for the immunodecoration in Figure 2(b) has been raised against the peptide sequence 'DIKEKR' present in spinach sAPX (Yoshimura *et al*., 2001). Because of the presence of the 'DIKEKR' aa sequence also in the *A. thaliana* tAPX (aa 287–292), the antibody recognizes *A. thaliana* tAPX as well. *A. thaliana s*APX possesses a 'EIKEKR' sequence, such antibody could therefore also recognize sAPX; it cannot, however, recognize cAPX as the 'DIKEKR' sequence is not present in the *Arabidopsis* cAPX sequence.

Accumulation of tAPX protein in the thylakoidal membranes of these four tAPX-overexpressing lines is accompanied with a higher tAPX enzymatic activity. Solubilized proteins from isolated thylakoidal membranes have been used for assaying APX enzymatic activity spectrophotometrically. Results obtained confirm that tAPX activity is higher in all the four lines tested (Figure 3a). In particular, the 14/2 line has the highest tAPX activity – around fivefold – with respect to the control line EV1 (Figure 3a).

Analysis of tAPX activity by native PAGE confirmed that the 14/2 line is the strongest tAPX-overexpressing line among the four lines tested (Figure 3b).

Such increase in APX activity in the overexpressing lines is due to an accumulation of the APX enzyme in thylakoidal membranes only as no increased APX activity is detectable by native PAGE analysis in any of the washing fractions obtained during the thylakoidal preparation, starting from whole leaves (data not shown).

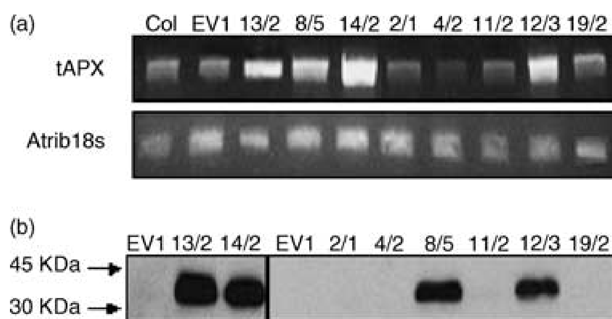

Figure 2. tAPX overexpression in *A. thaliana* 35S::tAPX lines.
(a) RT-PCR amplification of tAPX transcript in the wild-type Col line, EV1 control line transformed with the empty vector, and T_3 lines homozygous for a single insertion event of the transgene. Amplification of the ribosomal Atrib18S has been used as control for the equal loading of RNA (10 ng) in each sample.
(b) Western blot analysis of thylakoidal membrane extracts from mature leaves of the control line EV1 and the eight pBE2113::tAPX lines putatively overexpressing tAPX. Protein extracts corresponding to 2 (left panel) or 5 μg (right panel) chlorophyll content have been loaded onto the urea gel. A monoclonal antibody raised against spinach sAPX, which recognizes *A. thaliana* tAPX, has been used for the immunodecoration.

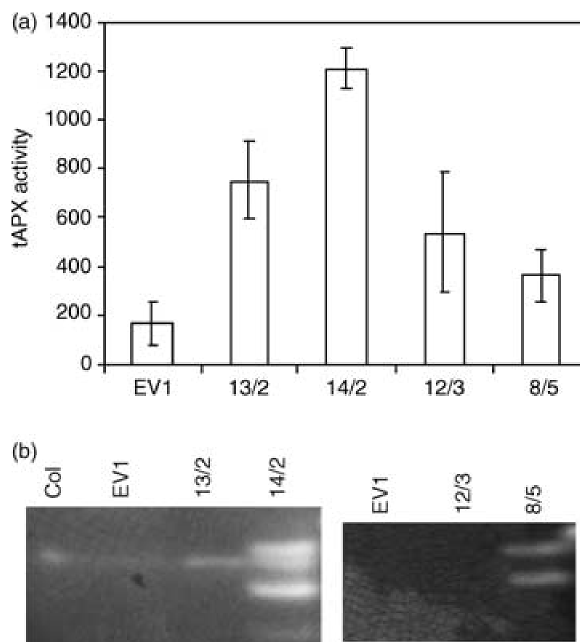

Figure 3. tAPX enzymatic activity in *A. thaliana* 35S::tAPX lines.
(a) Spectrophotometric quantification of tAPX activity. Measurements were performed at 265 nm on 50 μg protein fractions solubilized from thylakoidal extracts. tAPX activity is expressed as nanomoles ASA oxidized per milligram of protein per minute; each reported value is the mean of at least five measurements. Bars correspond to the SD.
(b) Staining of tAPX activity on native PAGE gels. Thylakoidal extracts from fully expanded *A. thaliana* leaves have been solubilized with CHAPS, and 500 μg protein from the solubilized fraction has been loaded in each lane. As controls, the solubilized fractions from thylakoidal extracts of Col and EV1 lines have been loaded. Staining of APX activity has been performed as indicated in Experimental procedures. The two panels correspond to two different gels run and stained in parallel during the same experiment.

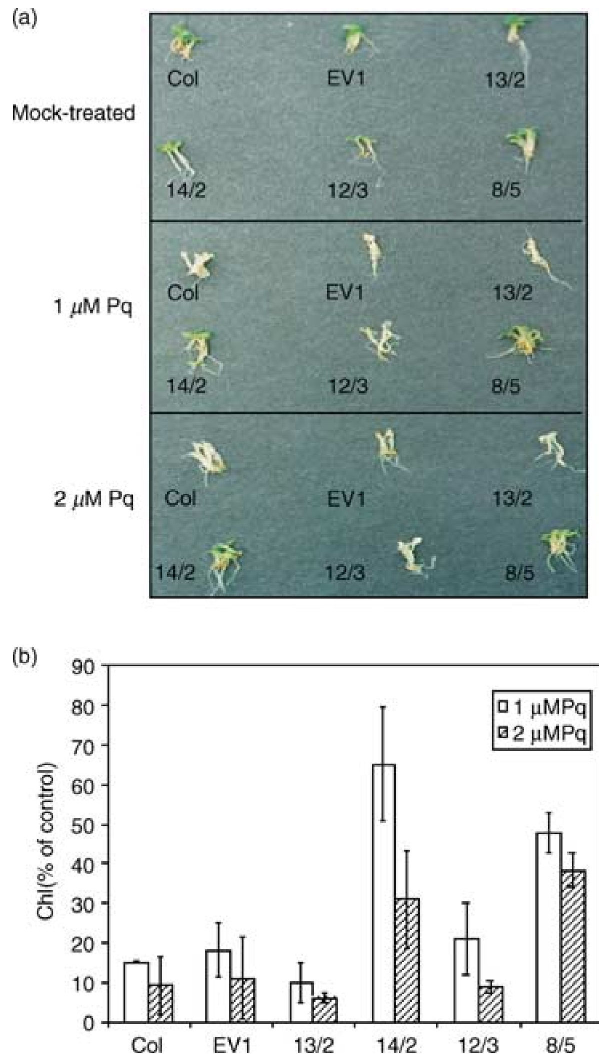

Figure 4. Resistance of tAPX-overexpressing lines to Pq.
(a) Phenotype of 1-week-old seedlings pre-incubated overnight with water (mock-treated), 1, or 2 μM Pq in the dark, and then illuminated at 800 μmol photons m^{-2} sec^{-1} for 12 h at 22°C.
(b) Chlorophyll content of seedlings described in (a). For each line, chlorophyll content (microgram chlorophyll per gram FW) after Pq treatment is given as percentage value with respect to its value in the mock-treated sample. Results represent the mean of two independent experiments. Bars correspond to the SD.

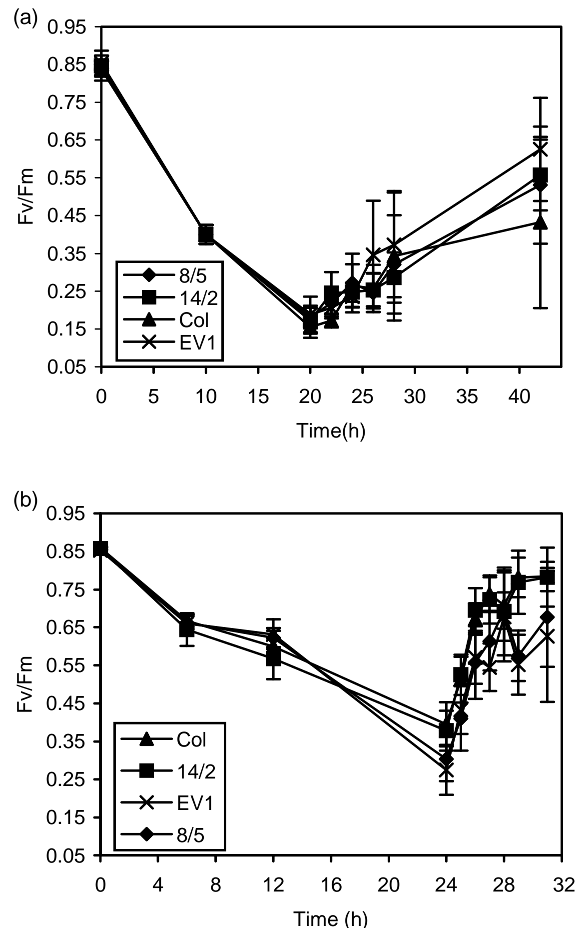

Figure 5. Photoinhibitory treatments on tAPX-overexpressing lines.
(a) Decrease of maximal photochemical efficiency parameter F_v/F_m during 20 h photoinhibition of fully expanded leaves at 22°C, 700 μmol photons m^{-2} sec^{-1}. Recovery during the following hours had been performed at growth conditions. Each point is the result of five different measures.
(b) Decrease of maximal photochemical efficiency parameter F_v/F_m during 24 h photoinhibition of fully expanded leaves at 2°C, 300 μmol photons m^{-2} sec^{-1}. Recovery during the following hours had been performed at growth conditions. Each point is the result of five different measures. Vertical bars correspond to the SDs.

In the 14/2 and 8/5 lines, more than one band could be detected (Figure 3b); possibly, these bands correspond to different post-translational modifications of the same over-expressed tAPX isoform; alternatively, the multiple bands could be due to aggregates of the same protein. Surprisingly, such strong bands could not be detected for the other two overexpressing lines 12/3 and 13/2. The correlation between the data obtained with liquid assay and native PAGE is strong for the 14/2 line, which has the highest tAPX activity levels in both the assays. However, such correlation is weak for the 8/5 line. Maybe because of intrinsic limits of the experimental procedure, 8/5 tAPX activity level has been somehow underestimated in the liquid assay.

A. thaliana *tAPX-overexpressing lines are more resistant to Pq-induced oxidative stress, but not to photoinhibitory treatments nor to Fe/Cu overload*

Seeds of the 14/2 line germinate slower than those of controls and of other tAPX-overexpressing lines. Extent of such delay in growth at very early stages can change from one experiment to another, being sometimes almost unnoticeable. Such phenotype is, however, not correlated to the tAPX overexpression, but is most probably due to the 35S::tAPX insertion site in the 14/2 line, as it could not be

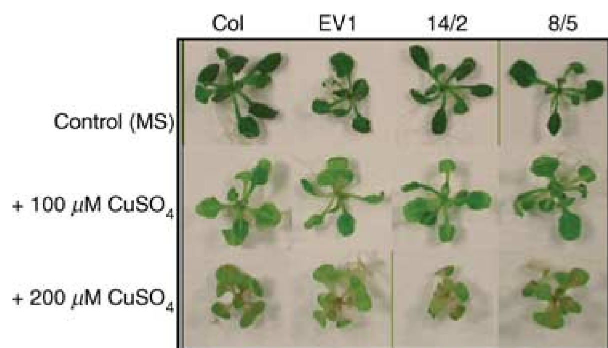

Figure 6. Phenotype of the tAPX-overexpressing lines after treatment with Cu overload.
Seven-day-old Col, EV1, 14/2, and 8/5 seedlings, grown on MS plates, had been then transferred on MS plates supplied with different $CuSO_4$ concentrations. Seedlings had been kept for 7 days under selection and then scored for the phenotype. As control, seedlings of each tested line had been transferred from MS plates to MS plates without $CuSO_4$ extra supply. For each line and concentration tested, eight plates with at least 25 seedlings each were scored.

observed in any of the other tAPX-overexpressing lines. Apart from this, the four *A. thaliana* lines overexpressing tAPX, namely 14/2, 8/5, 13/2, and 12/3, are phenotypically indistinguishable from the controls under normal growth conditions.

Nonetheless, when treated with 1 or 2 μM Pq, an herbicide catalyzing the photoreduction of O_2 to O_2^- around PSI, control seedlings bleach, whereas the 14/2 and 8/5 seedlings remain partially green (Figure 4a). Chlorophyll content of the 14/2 and 8/5 seedlings, after both 1 or 2 μM Pq treatment, is indeed higher than that of the controls (Figure 4b). Notably, the 14/2 line with the highest tAPX activity (Figure 3) retains the highest chlorophyll content after Pq treatment (Figure 4b).

To further assess the tAPX role in the protection against oxidative stresses, mature leaves (21–25 days) of the two tAPX-overexpressing lines 14/2 and 8/5 were photoinhibited at two different conditions: at high intensity of white light (700 μmol photons m^{-2} sec^{-1}) and room temperature (Figure 5a), or at moderate intensity of white light (300 μmol photons m^{-2} sec^{-1}) and chilling temperature (Figure 5b). In both cases, the maximal photochemical efficiencies F_v/F_m of the 14/2 and 8/5 tAPX-overexpressing lines decline like those of the wild-type Col and of the control line EV1 (Figure 5); also, no differences can be observed during the recoveries from the treatments at growth light and room temperature among the different lines tested (Figure 5). The above described photoinhibitory experiments performed on young leaves (12 days) gave the same results (not shown).

Free Fe ions and, to a lesser extent, Cu ions, catalyze the Haber–Weiss reaction with production of $OH^{\cdot}$ from the less dangerous H_2O_2 and O_2^- (Bowler *et al.*, 1992). Seedlings of the two tAPX-overexpressing lines 14/2 and 8/5 were grown on Murashige and Skoog (MS) plates and then transferred on MS plates supplemented with 0, 100, or 200 μM $CuSO_4$. Seedlings of the 14/2 or 8/5 lines showed the same symptoms of Cu toxicity as their controls (Figure 6). Also, control and tAPX-overexpressing seedlings, grown on MS plates and then transferred on MS plates supplemented with different Fe–EDTA concentrations (0, 50, 100, 200, 250, or 500 μM Fe–EDTA), showed the same symptoms of toxicity (data not shown).

A. thaliana *tAPX-overexpressing lines are more resistant to NO-induced cell death*

H_2O_2 is a key molecule for the onset of the hypersensitive response (HR) against pathogen attack (Mittler *et al.*, 1999b). During viral-induced programmed cell death (PCD) in tobacco, the expression of cAPX is post-transcriptionally inhibited (Mittler *et al.*, 1998, 1999a). It has also been demonstrated by a pharmacological approach that H_2O_2 necessitates nitric oxide (NO) as a partner for causing cell death during HR (Delledonne *et al.*, 1998, 2001). Moreover, the PCD induced by the simultaneous production of H_2O_2 and NO downregulates the cAPX (de Pinto *et al.*, 2002). An *in vitro* NO-mediated inhibition of APX enzymatic activity has also been demonstrated by Clark *et al.* (2000).

In order to assess a possible regulation by NO of the thylakoidal isoform of APX, the tAPX-overexpressing lines, as well as their controls, were infiltrated with the NO–donor sodium nitroprusside (SNP). After 3 h infiltration with 5 mM SNP, tAPX transcript accumulation was drastically reduced (Figure 7a). Such inhibition, observed in the wild-type Col and in the control line EV1, is even more dramatic in the overexpressing lines; however, in this latter case, tAPX transcript does not completely disappear after SNP treatment (Figure 7a).

Transcript levels of different genes involved in the response against oxidative stress or pathogen attack were also analyzed by reverse transcriptase (RT)-PCR. Analysis confirmed specificity of SNP inhibitory effect on tAPX transcript accumulation. In all the lines tested, SNP induces transcript accumulation of a cytosolic isoform of APX, namely APX1 (Figure 7a); *APX1* gene (Storozhenko *et al.*, 1998) was already known to be induced by excess light stress (Karpinski *et al.*, 1997). At the experimental conditions used, SNP does not alter transcript levels of GST1 (Figure 7a), a gene primarily involved in de-toxification processes (Almeras *et al.*, 2003; Conklin and Last, 1995). Interestingly, transcript levels of two pathogenesis-related (PR) genes, *PR1* and *PR2*, were lower in the untreated tAPX-overexpressing lines 14/2 and 8/5 than in the untreated control lines Col and EV1 (Figure 7a).

Besides inhibiting tAPX transcript accumulation, SNP also caused a partial reduction of tAPX enzymatic activity. All the bands of the 14/2 proteic extracts in the native PAGE

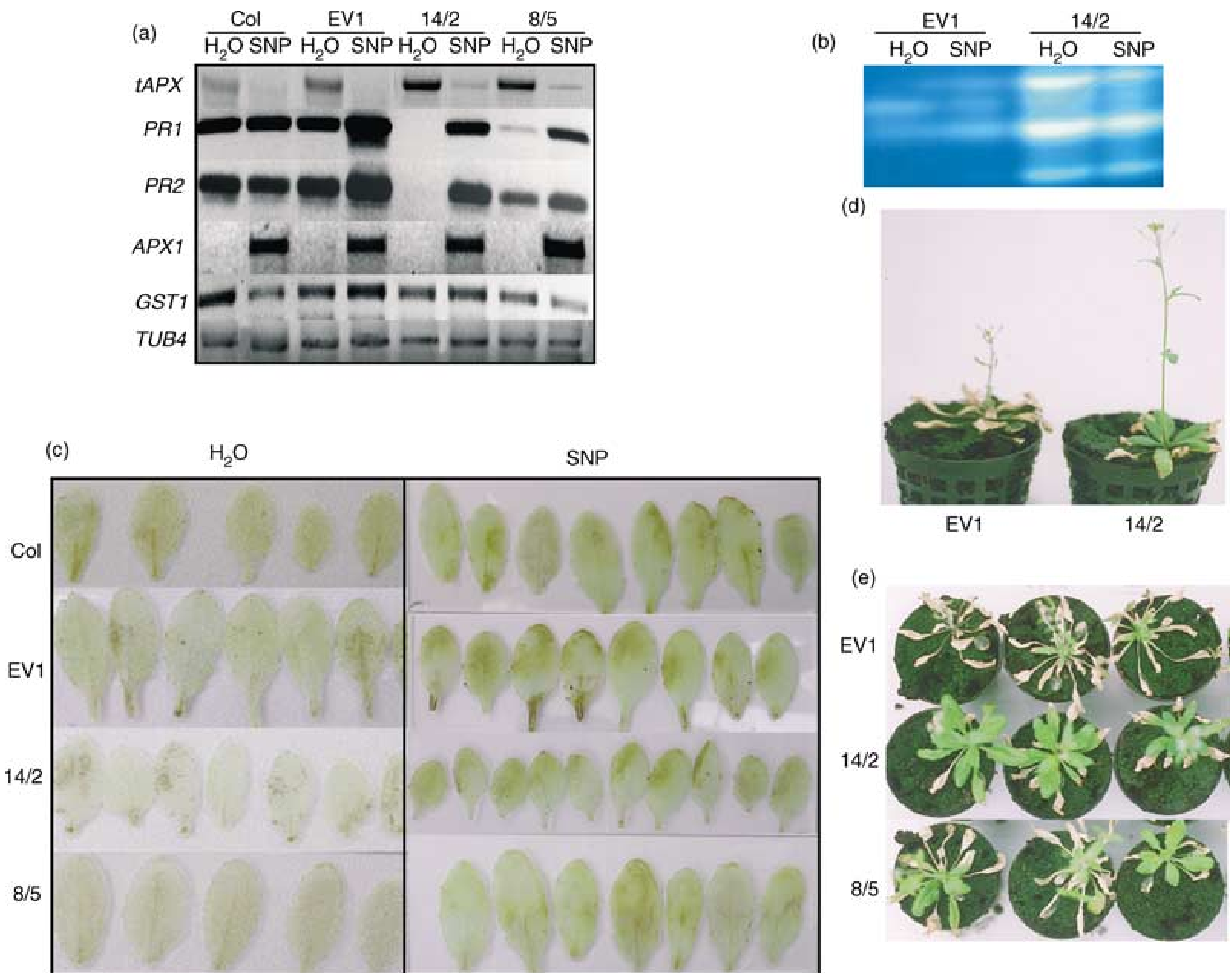

Figure 7. Effects of the NO–donor SNP on the tAPX-overexpressing lines.
(a) RT-PCR analysis of *tAPX*, *PR1*, *PR2*, *APX1*, *GST1*, and *TUB4* transcript levels after infiltration with 5 mM SNP. Leaves of 20-day-old plants (rosette stage) have been infiltrated with 5 mM SNP or water (mock-treated) and kept 3 h at continuous light (120 μmol photons m^{-2} sec^{-1}). Amplification of the *TUB4* gene has been used as control for the equal loading of RNA (6 ng) in each sample.
(b) Staining of APX enzymatic activity in EV1 and 14/2 lines after 3 h infiltration with 5 mM SNP or water as indicated in (a). Thylakoidal extracts from fully expanded leaves have been solubilized with CHAPS, and 500 μg proteins from the solubilized fraction have been loaded in each lane. Staining of APX activity has been performed as indicated in Experimental procedures.
(c) Staining of H_2O_2 with DAB in tAPX-overexpressing lines after 3 h infiltration with 5 mM SNP or water as indicated in (a).
(d, e) Phenotype of EV1, 14/2, and 8/5 plants after infiltration with 5 mM SNP. Plants have been infiltrated with 5 mM SNP and then kept for 7 days at continuous light. Pictures are representative of two independent experiments with at least six to eight plants SNP-infiltrated per line.

gel (stained for tAPX activity), except the lowest one, were affected by SNP treatment (Figure 7b). The SNP inhibitory effect had been partially hidden in the EV1 extracts by overflow of some of the 14/2–H_2O proteic extract into the EV1–SNP lane, as suggested by presence of an upper band in the EV1–SNP sample, which was absent in the EV1–H_2O sample (Figure 7b).

It cannot be excluded that the observed partial reduction of tAPX enzymatic activity by SNP treatment could be due to a reduction of total tAPX protein by SNP. For discriminating between the two possibilities, the production of a new antibody against *Arabidopsis* tAPX is required, as no more antibody (chl-mAb6; Yoshimura *et al.*, 2001) was made available for the Western experiment.

Upon treatment with 5 mM SNP, the inhibition of tAPX transcript accumulation and enzymatic activity were accompanied by a rise in H_2O_2 levels in all the tested lines, as observed with 3,3′-diaminobenzidine tetrahydrochloride (DAB) staining (Figure 7c). DAB staining was less intense in the 14/2 and 8/5 lines, indicating that H_2O_2 accumulation upon SNP treatment is lower in the tAPX-overexpressing lines than in the control ones (Figure 7c).

After 2–3 days infiltration with 5 mM SNP, differences between tAPX-overexpressing and control lines became

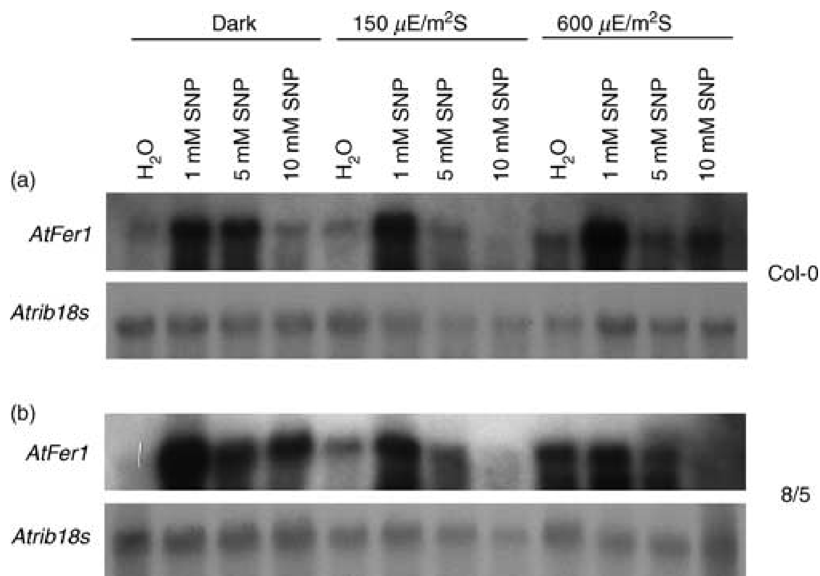

Figure 8. Ferritin transcript levels in Col and 8/5 lines after treatment with different SNP concentrations and light intensities.
Twenty-day-old Col (a) or 8/5 (b) plants have been kept overnight in the dark and then infiltrated, with the aid of a dim green light, with water (mock-treated), 1, 5, and 10 mM SNP. They were then kept for 3 h either in the dark or at two different light intensities, i.e. 150 μmol photons m^{-2} sec^{-1} (150 μE m^{-2} sec^{-1}) or 600 μE m^{-2} sec^{-1}. Leaves were then collected for RNA extraction, and Northern blots were prepared as indicated in Experimental procedures. Five micrograms of total RNA has been loaded in each lane. Filters have been hybridized with the ferritin *AtFer1* probe or with the constitutively expressed *Atrib18s* probe.

visible by the naked eye: plants from the control line EV1 showed symptoms of leaf damage, which became more severe with time. After 7 days, the rosette leaves appeared all damaged with a very short floral stem (Figure 7d,e). On the reverse, tAPX-overexpressing lines showed reduced symptoms of damage: young leaves from the rosette were unaffected and the stem was higher (Figure 7d,e) than that of controls. Once again, the observed phenotype correlates with tAPX activity levels as the 14/2 line, the one with the highest tAPX activity, showed the less severe symptoms of damage. Concentrations lower than 5 mM SNP did not cause severe leaf damage in the Col or EV1 lines; no clear differences between the phenotype of the control and tAPX-overexpressing lines could therefore be observed upon infiltration with 1 mM SNP.

Nitric oxide-mediated ferritin transcript accumulation is not perturbed in the A. thaliana *tAPX-overexpressing lines*

Ferritins are Fe storage proteins that participate in the cellular protective mechanisms against noxious effects of free, not complexed, Fe ions (Curie and Briat, 2003). Plant ferritins, differently from their animal counterparts, are mostly regulated at the transcriptional level (Briat and Lobreaux, 1997; Delledonne *et al.*, 2003 and references therein). Such regulation appears to be a very complex pathway in which many different events come into play, such as various oxidative stresses, external Fe supply, and age (Briat and Lobreaux, 1997; Murgia *et al.*, 2001; Tarantino *et al.*, 2003). In particular, both H_2O_2 and NO can induce transcript accumulation of the ferritin *AtFer1* isoform in *A. thaliana* (Murgia *et al.*, 2002; Petit *et al.*, 2001).

Because of these last evidences, regulation of *AtFer1* transcription at different SNP concentrations and different light conditions was analyzed in the tAPX-overexpressing lines. Such analysis did not reveal any alteration in *AtFer1* transcript regulation in the lines tested: in different conditions, pattern of accumulation was the same in both the 8/5 and Col lines (Figure 8). Same results have been obtained with 14/2 and EV1 (not shown).

Discussion

The protection of the plants against ROS produced in excess during various adverse environmental conditions is achieved by means of different strategies and, in particular, by partial suppression of ROS production and scavenging of the ROS already produced.

Partial suppression of ROS production in the chloroplasts during high-light conditions is achieved, for example, by the ROS-induced degradation of the D1 protein of photosystem II (PSII) (Andersson and Barber, 1996; Miyao *et al.*, 1995; Okada *et al.*, 1996), leading to PSII photoinactivation. Such condition, by reducing O_2^- production at the PSI, alleviates or prevents the irreversible photoinhibition of PSI (Asada, 1999).

Scavenging of ROS is achieved by a complex interplay between different enzymatic and non-enzymatic molecules. In plant cells, APX enzymes are directly involved in the scavenging of H_2O_2, whose steady-state concentration has been shown, by computer simulation, to be mainly affected by APX itself (Polle, 2001). Direct modification of the expression of various APX isoenzymes in different plant systems has assigned a key role in the protection of the plants against various oxidative stresses (Allen *et al.*, 1997; Payton *et al.*, 2001; Rizhsky *et al.*, 2002; Shi *et al.*, 2001; Torsethaugen *et al.*, 1997; Wang *et al.*, 1999).

The present work shows that tAPX overexpression makes plants more resistant to oxidative damage induced by the

herbicide Pq, which generates ion superoxide O_2^- via autooxidation of its radical photoproduced at PSI (Asada, 2000 and references therein). These results are in accordance with those observed in tobacco, where tAPX overexpression also increases tolerance to Pq (Yabuta *et al.*, 2002).

However, it must be underlined that Pq traps almost all the electrons generated at PSI (Asada, 2000): in presence of Pq, almost 100% of the linear electron flux goes into O_2^- production. Such pharmacologically induced O_2^- overproduction is far from being comparable to O_2^- production by the water–water cycle in field conditions; also, under adverse environmental conditions, where CO_2 fixation is reduced, the linear electron flux going through the water–water cycle accounts for 10–30% of total linear electron flux (Asada, 1999, 2000; Biehler and Fock, 1996; Canvin *et al.*, 1980; Cheeseman *et al.*, 1997; Miyake and Yokota, 2000). Our results and, in general, all results obtained by using Pq, cannot be considered conclusive for establishing a key role of a given enzyme in the protection against environmentally induced oxidative stresses.

tAPX overexpression, in fact, does not enhance protection against photoinhibition. This is likely because the reduction of maximal photochemical efficiency (measured as F_v/F_m) during photoinhibition is caused by the singlet oxygen ($^1O_2$) produced around PSII. PSII photoinhibition can also be preceded by PSI photoinhibition, for example, during light and chilling treatments (Sonoike, 1996). In this case, however, a reduction of the Mehler's reaction at PSI, with subsequent reduced production of O_2^- and hence of H_2O_2, is expected. It cannot be excluded that the lack of phenotype in the tAPX-overexpressing lines upon photoinhibition, or in the experiments of Fe/Cu overload could be because of the inability of fivefold tAPX overexpression to increase the metabolic flux of the reactions involved, as the theory of metabolic control analysis (MCA) could explain (Morandini and Salamini, 2003 and references therein). Characterization of *A. thaliana* tAPX knockout mutant lines, which has just started in our group, will help clarify this latter point.

In fact, tobacco plants overexpressing tAPX, with around 37-fold higher activity, show enhanced tolerance to photoinhibition at chilling temperatures (Yabuta *et al.*, 2002); however, it must also be underlined that *A. thaliana* tAPX and tobacco tAPX seem to play non-overlapping roles in the plant-defense mechanisms as reduction of tAPX activity is invalidating for tobacco (Yabuta *et al.*, 2002) but not for *A. thaliana* plants. We indeed produced tAPX antisense lines with half residual tAPX activity with respect to wild type: such plants, unless tobacco ones, are phenotypically indistinguishable from wild-type plants when grown under non-selective conditions (Tarantino *et al.*, manuscript in preparation).

Recently, it emerged that ROS can be viewed as cellular indicators of stress and as key secondary messengers involved in the plant response against biotic and abiotic stresses (Delledonne *et al.*, 2001; Knight and Knight, 2001; Mittler, 2002; Pei *et al.*, 2000); it has been proposed that APX are involved in the fine modulation of H_2O_2 levels for such signaling events (Mittler, 2002).

Our results validate, by genetic means, the hypothesis that H_2O_2 mediates the HR upon pathogen attack by acting in partnership with NO as a cell killer (Delledonne *et al.*, 1998, 2001).

In particular, we propose that the higher levels of tAPX enzyme in the tAPX-overexpressing lines are responsible for the reduced symptoms of damage upon SNP treatment. Interestingly, *A. thaliana* transgenic plants, underexpressing tAPX, show, on the reverse, enhanced symptoms of damage upon treatment with SNP (Tarantino *et al.*, manuscript in preparation), i.e. the extent of cell death induced by SNP is inversely correlated to the tAPX-dependent scavenging of H_2O_2. This hypothesis is reinforced by several evidences.

First, 3 h after infiltration with 5 mM SNP, inhibition of both tAPX transcript accumulation and tAPX enzymatic activity can be revealed; the tAPX-overexpressing line 14/2 still maintains a tAPX enzymatic activity, which is higher than that of untreated control lines. Interestingly, the SNP-induced inhibition of *A. thaliana* tAPX, at both mRNA and protein levels, seems peculiar to such chloroplastic isoform. On the reverse, our results with APX1 confirm in *Arabidopsis* what has been already observed in tobacco, i.e. cAPX is instead regulated upon SNP treatment or pathogen attack at two different levels, mRNA and protein, whereby SNP/pathogens induce cAPX transcript accumulation, but repress protein accumulation and inhibit cAPX activity (Clark *et al.*, 2000; Mittler *et al.*, 1998, 1999a; Murgia *et al.*, 2004).

Second, besides the inhibition of tAPX transcript accumulation, SNP induces accumulation of different transcripts tightly correlated with HR, such as PR1 and PR2. This result suggests that cell death induced by SNP in the infiltrated leaves, as already shown in the literature (de Pinto *et al.*, 2002), is of apoptotic type. Notably, in the tAPX-overexpressing lines, the basal levels of PR1 and PR2 transcripts are much lower than in the control lines, indicating that differences between control and tAPX-overxpressing lines at molecular level can be also detected in untreated plants.

Third, leaf infiltration with SNP triggers a rise in H_2O_2 levels (detected through DAB staining), which is higher in the control than in the tAPX-overexpressing lines. This latter result confirms once more that the concentration of one of the two killing partners causing PCD, i.e. H_2O_2 (see above), is truly altered.

Besides the PCD, NO and H_2O_2 are also involved in the complex signaling pathway, leading to accumulation of *A. thaliana AtFer1* ferritin isoform (Delledonne *et al.*,

2003; Murgia *et al.*, 2002; Petit *et al.*, 2001). However, in the latter case, tAPX overexpression does not perturb the pathway. The pattern of *AtFer1* transcript accumulation upon SNP infiltration is the same in both control and tAPX-overexpressing lines, regardless of the different redox states of the plants (modulated through exposure to different light conditions). Several hypotheses can be formulated; for example, NO acts downstream of the oxidative step in the signaling pathway, leading to *AtFer1* transcript accumulation, but it can, at least, be excluded that H_2O_2 and NO partnership, leading to ferritin regulation, is of the same type as the one leading to PCD.

In conclusion, our results highlight the important role of tAPX in the fine modulation of H_2O_2 for signaling, and open new perspectives on the cross-talks between chloroplasts and the other cell compartments during both biotic and abiotic stresses.

Experimental procedures

Arabidopsis thaliana *growth*

Arabidopsis thaliana plants were grown at 21–25°C, 150 μmol photons m^{-2} sec^{-1} (OSRAM L36 w/11–860 Lumilux PLUS Recyclable, Germany), 14-h light/10-h dark photoperiod, into Arabaskets (Beta Tech, Gent, Belgium) on sterilized Technic n. 1 DueEmme soil (the Netherlands). They were watered with de-ionized water.

Preparation of the transformation vector containing the A. thaliana *tAPX cDNA*

The tAPX cDNA fragment X98926 (Jespersen *et al.*, 1997) was received as *Escherichia coli* DH10B strain, transformed with the amp-resistant pZL1 cloning vector containing the X98926 cDNA. The cDNA was excised from the pZL1 vector by *Not*I–*Sal*I digestion, made blunt-end with Blunting Kit (Amersham Pharmacia Biotech, Bucks, UK), and ligated into the pBE2113-GUS transformation vector (Mitsuhara *et al.*, 1996) previously digested with *Sma*I. This vector contains a chimeric P35S promoter conferring high level of expression of foreign genes.

Correct orientation of tAPX cDNA in the various *E. coli* colonies transformed with the pBE2113::tAPX vector was checked by PCR. For this purpose, the tAPXII, tAPXIII, CaMV, and Gus rev primers had been used.
tAPXII: 5′-TCTCTTTCCCCCGCCGTCACCTC-3′ (annealing 50 bp downstream tAPX starting ATG);
tAPXIII: 5′-TGTTAGGATACTTGTCTTTGAGAGG-3′ (annealing 490 bp downstream tAPX starting ATG);
CaMV: 5′-CTCGGATTCCATTGCCCAGCTAT-3′ (annealing 520 bp upstream 3′ end);
Gus rev: 5′-CACCACCTGCCAGTCAACAGACG-3′ (annealing 610 bp downstream Gus starting ATG).

Arabidopsis thaliana *transformation*

Agrobacterium tumefaciens strain GV3101, containing the helper plasmid pMP90, which confers resistance to gentamycin, was transformed with pBE2113::tAPX transformation vector by electroporation.

Agrobacterium, transformed with the pBE2113::tAPX vector, was grown overnight at 28°C on 500 ml Luria broth (LB) medium containing 50 μg ml^{-1} kan and 25 μg ml^{-1} gentamycin; bacterial culture was then centrifuged at 4000 ***g*** for 10 min, and the pellet was re-suspended in sterilized infiltration buffer up to a final OD_{600} of 0.8; bacteria were then used for transforming *A. thaliana* var. Col by the 'floral dip' technique (Clough and Bent, 1998; Desfeux *et al.*, 2000).

T_1-transformed *A. thaliana* seeds were plated on MS plates containing 1% Plant Agar (Duchefa, Amsterdam, the Netherlands) and 50 μg ml^{-1} kan. T_1 kan-resistant plants were transferred onto the soil when possessing first true leaves, and T_2 seeds from single T_1 plants were collected.

RT-PCR

Total RNA was extracted from *A. thaliana* leaves with Trizol® reagent (Gibco Invitrogen, Milan, Italy). RT-PCR amplification reactions were performed by using the Access RT-PCR kit (Promega Biosciences, San Luis, CA, USA), following the manufacturer's instructions. tAPX transcript levels were analyzed in transformed *A. thaliana* pBE2113::tAPX plants by amplifying tAPX cDNA fragment (≈1300 bp) with the following primers:
tAPXd: 5′-TTTCGCCACCGTACGTGACAATGTC-3′;
tAPXr: 5′-AAACCAGAGAAATCGGAGTTG-3′.

Equal amount of RNA in each sample (10 ng) was tested by RT-PCR amplification of Atrib18s cDNA fragment (≈400 bp) with the following primers (Cho and Cosgrove, 2000):
Atrib18sd: 5′-TTGTGTTGGCTTCGGGATCGGAGTAAT-3′;
Atrib18s r: 5′-TGCACCACCACCCATAGAATCAAGAA-3′.

tAPX, PR1, PR2, APX1, GST1 mRNA expression profiles after SNP treatment were evaluated by RT-PCR amplifications of tAPX (≈651 bp), PR1 (≈400 bp), PR2 (≈425 bp), APX1 (≈720 bp), GST1 (≈610 bp) cDNA fragments with the following primers:
tAPXfor: 5′-AATAGTTGCCTTGTCTGG-3′;
tAPXrev: 5′-GGAATATATGATCACCACG-3′;
PR1for: 5′-AAGCTCAAGATAGCCCACAAG-3′;
PR1rev: 5′-CGTTCACATAATTCCCACGAG-3′ (Pastori *et al.*, 2003);
PR2for: 5′-TCTGAATCAAGGAGCTTAGCC-3′;
PR2rev: 5′-CCAGAAACCGCGTTCTCGATG-3′;
APX1for: 5′-TGACGAAGAACTACCCAACCG-3′;
APX1rev: 5′-AAAGCTTCATGTGGGCCTCAG-3′;
GST1for: 5′-AGTTTTCGGTCACCCAGCTTC-3′;
GST1rev: 5′-AGAACCTTCTGAGCAGAAGGC-3′;

Equal amount of RNA in each sample (6 ng) was tested by RT-PCR amplification of β-tubulin4 (*TUB4*; Accession no. AtG44340) cDNA fragment (≈350 bp) with the following primers:
TUB4for: 5′-AGAGGTTGACGAGCAGATGA-3′;
TUB4rev: 5′-CCTCTTCTTCCTCCTCGTAC-3′.

Preparation of thylakoidal membranes

Protocol is a modified version of that one in Casazza *et al.* (2001). Leaves (around 0.4 g) were cut from 3-week-old plants, and were kept in cold distilled water at 4°C for 30 min in the dark. All the following steps had been performed at 4°C, by using pre-chilled mortars, pestles, and buffers. Leaves were homogenized with 2 ml buffer A. Suspension was passed through eight layers cheesecloth into a corex tube and was centrifuged for 3 min at 2600 ***g***; the pellet was re-suspended in 2 ml buffer B and centrifuged again for 3 min at 2600 ***g***. The last two steps had been repeated and the pellet was re-suspended in 5 ml hypotonic B buffer. After 3 min centrifugation at 2600 ***g***, the pellet was

re-suspended in buffer C, centrifuged at 2600 ***g***, and finally re-suspended again in 80 μl buffer B. Chlorophyll content was measured by diluting 10 μl thylakoidal extract in 1 ml 80% acetone, which was centrifuged in a microcentrifuge at maximum speed: chlorophyll content in the supernatant was quantified by measuring the absorbances at 645 and 663 nm by the Arnon formula (Arnon, 1949).

Buffer A: 0.4 M sorbitol, 5 mM EDTA, 5 mM EGTA, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 20 mM tricine (pH 8.4); buffer B: 0.3 M sorbitol, 2.5 mM EDTA, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 20 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES; pH 7.6); buffer hypotonic B: as buffer B without sorbitol; buffer C: 0.1 M sorbitol, 0.15 M NaCl, 2.5 mM EDTA, 10 mM $NaHCO_3$, 5 mM $MgCl_2$, 20 mM HEPES (pH 7.6).

Western blotting

Thylakoidal membranes, prepared as indicated above, were solubilized by adding 1× final concentration solubilization buffer, and were then vortexed and centrifuged for 5 min in a microcentrifuge at maximum speed. They were then loaded into a urea gel for electrophoresis. Protein marker was prepared by adding 3 μl 2× Laemmli buffer to 3 μl low rainbow marker (Amersham); the mixture was boiled for 5 min, centrifuged, and loaded into the gel.

3× solubilization buffer: 125 mM Tris–HCl (pH 6.8), 50 mM DL-dithiothreitol (DTT; to add just before use), 9% SDS, 20% glycerol, 0.04% bromophenol blue; 2× Laemmli buffer: 100 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS, 3% bromophenol blue, 350 mM β-mercaptoethanol was added just before use.

Urea gel was prepared essentially according to Barbato *et al.* (1991):

- Running gel: 6 M urea; 15% acrylamide mix (from 30% acrylamide mix stock); 0.8 M Tris–HCl (pH 8.8), all reagents were mixed by continuous stirring until urea was completely dissolved; 0.075% ammonium persulfate (APS; from 10% stock solution) and 0.075% *N,N,N',N'*-tetramethylethylendiamine (TEMED) were then added.
- Stacking gel: 6 M urea; 6% acrylamide mix (from 30% acrylamide mix stock); 0.125 M Tris–HCl (pH 6.8), all reagents were mixed by continuous stirring until urea was complete dissolved; 0.075% APS (from 10% stock solution) and 0.075% TEMED were then added.
- Internal running buffer (between the two gels): 25 mM Tris–HCl (pH 8.6), 192 mM glycin, 0.1% SDS.
- External running buffer: 25 mM Tris–HCl (pH 8.6), 192 mM glycin.
- 30% acrylamide mix: 29.2% acrylamide, 0.8% *N,N'*-methylene-bis-acrylamide.

Electrophoresis was performed for 30 min at constant 100 V, and then for 3 h at constant 150 V. After electrophoresis, proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Pall Corp., Bio Trace TM 0.45 μm, Gelmal Laboratory, Ann Arbour, USA). Transfer was performed for 35 min at 350 mA.

- Transfer buffer: 3 mM Na_2CO_3, 10 mM $NaHCO_3$, 10% methanol.

For the immunodecoration, the monoclonal antibody chl-mAb6 raised against spinach sAPX (Yoshimura *et al.*, 2001), which also recognizes *A. thaliana* tAPX, was used as primary antibody at 1 : 1000 dilution. As secondary antibody, an IgG antimouse (Sigma Aldrich, Milwaukee, WI, USA), conjugated with horseradish peroxidase (HRP) was used, at 1 : 20 000 dilution. Signals were detected with the SuperSignal West Pico Chemiluminescent Kit (Pierce Biotechnology, Rockford, IL, USA).

Solubilization of proteins from isolated thylakoidal membranes

Leaves (around 1 g) were cut from 3-week-old plants and kept in cold distilled water for 30 min in the dark at 4°C. All the following steps were performed at 4°C, by using pre-chilled mortars, pestles, and buffers, unless otherwise specified. Leaves were homogenized (with mortar and pestle) with 4 ml buffer S. The suspensions were filtered with eight layers cheesecloth into a corex tube and centrifuged for 3 min at 2600 ***g*** with brake-off. The pellets were re-suspended in 4 ml buffer S, centrifuged again for 3 min at 2600 ***g*** with brake-off, and re-suspended in 400 μl buffer R. After 1 h incubation at 4°C, the suspensions were centrifuged at maximum speed in a minicentrifuge, and the supernatants were transferred into a clean Eppendorf tube. Soon after, protein contents of the samples (kept, in the meanwhile, at 4°C) were quantified with the Bradford method, by using the Bio-Rad Protein Assay Kit; the samples were then loaded into a native gel for a PAGE. Buffer S: 5 mM ASA, 1 mM EDTA, 100 mM sodium phosphate buffer (pH 7.0); buffer R: 5 mM ASA, 100 mM sodium phosphate buffer (pH 7.0), 1% 3-[(3-cholamidopropyl)dimethylammonio] -1-propanesulfonate (CHAPS).

Detection of tAPX activity by native PAGE

Running gel (7.3%): 8 ml buffer A, 16 ml 28% acrylamide mix, 8 ml H_2O, 32 ml 6 mM APS, 20 μl TEMED. Before TEMED addition, the solution had been de-gassed for 5 min at 800 mbar. Such solution is sufficient for preparation of two gels (16 cm × 18 cm).

Stacking gel (6%): 4.5 ml buffer B, 9 ml 10% acrylamide mix, 5 ml H_2O, 7.5 ml 26.5 mM riboflavin. The solution had been de-gassed for 5 min at 800 mbar, poured, and allowed to polymerize under white light.

Buffer A: 3 M Tris–HCl (pH 8.9); buffer B: 0.46% (v/v) TEMED, 13.66% (v/v) buffer A (pH 6.9) with HCl; 28% acrylamide mix: 28% (w/v) acrylamide, 0.735% (w/v) *N,N'*-methylene-bis-acrylamide; 10% acrylamide mix: 10% (w/v) acrylamide, 2.5% (w/v) *N,N'*-methylene-bis-acrylamide. Buffers A, B, and acrylamide mixes were filtered and stored at 4°C. 26.5 mM riboflavin stock was also filtered and stored in the dark at 4°C no longer than 1 month as it is photolabile.

Gel electrophoresis (Bio-Rad dual vertical slab gel electrophoresis cell) was performed for 2 h at constant 32 mA, 4 h at constant 50 mA, at 2–4°C, with continuous stirring of the running buffer. Running buffer (10× Davis): 0.04 M Tris, 0.38 M glycin (pH 8.3). A final concentration of 1 mM ASA was added just before use.

After electrophoresis, the gel had been pre-incubated for 5 min in Davis 1× buffer containing 1 mM ASA in the light and then incubated for 15 min in the light, on continuous agitation, in the following solution: 0.1 M sodium phosphate buffer (pH 6.4), 4 mM ASA, 4 mM H_2O_2. The gel had been washed throughout with H_2O and incubated for 15 min in the dark in the following solution: 0.125N HCl, 0.1% $K_3Fe(CN)_6$, 0.1% $FeCl_3$ (de Pinto *et al.*, 2000).

Spectrophotometric quantification of tAPX activity

Proteins were solubilized from thylakoidal extracts as described above. Spectrophotometric measurements were performed in a 1 ml final volume, containing 50 μg protein extract and 0.1 M sodium phosphate buffer (pH 7.0). Just before each measure, 2 μl 50 mM ASA and 10 μl 17 mM H_2O_2 were added at the same time to the cuvette, and mixed with two to three rapid stirrings with a plastic spatula. tAPX activity was measured as change of

absorbance at 265 nm because of decrease in ASA concentration, with the spectrophotometer JASCO V-530 by recording, for each measure, Abs (absorbance) values during the first 20 sec with a 0.1-sec data pitch. dAbs per minute values had been extrapolated from values recorded between 5 and 15 sec. APX activity was obtained by subtracting the aspecific ascorbate oxidase activity measured without addition of protein extracts to the mix to values obtained by adding the protein extracts ($\varepsilon_{265\ nm}$ ASA: 14 000).

Pq treatment

Sterilized and vernalized seeds were allowed to germinate on standard liquid mineral medium (Gomarasca *et al.*, 1993) into rotating flasks (60 r.p.m.) under continuous light (100 μmol photons m^{-2} sec^{-1}). After 5 days, seedlings were then washed twice in buffer E (30 min each wash); fresh buffer E (as control) or buffer E containing 1–2 μM Pq was added to the seedlings, which were then kept overnight in the dark under continuous rotation (60 r.p.m.). Seedlings, together with the buffers they were immersed in, were then transferred into Elisa wells (well diameter 35 mm) and exposed to high light (800 μmol photons m^{-2} sec^{-1}). After 12 h, seedlings were weighed and added to vials containing 1 ml dimethylformamide for chlorophyll extraction. Vials were closed accurately and kept overnight in the dark at 4°C. The following day, chlorophyll concentrations were quantified as microgram chlorophyll per gram FW according to Porra *et al.* (1989). Buffer E: 0.5 mM $CaSO_4$, 1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; pH 5.8–6.0) with bis–tris propane (BTP).

Photoinhibitory treatments

Leaves of 21-day-old plants were cut at the petiole level and laid on phosphate agar plates (1 mM K_2SO_4, 0.1 mM $CaSO_4$, 10 mM sodium phosphate buffer (pH 7.0), 0.75% Plant Agar (Duchefa, the Netherlands)). Photoinhibitory treatments were performed by using the facility described by Tarantino *et al.* (1999). Recovery was performed in continuous light in the growth chamber. Photochemical efficiency of PSII, expressed as F_v/F_m, was calculated by evaluating emission of chlorophyll fluorescence with a portable Plant Efficiency Analyzer (Hansatech Instruments, England).

SNP treatment

Leaves of well-watered 21-day-old plants had been infiltrated with 5 mM SNP or water (control), with a 1-ml syringe without needle at the abaxial leaf page. Plants were then kept under continuous light (100 μmol photons m^{-2} sec^{-1}) unless otherwise specified.

$CuSO_4$ and Fe–EDTA treatments

Sterilized and vernalized seeds were plated on square Petri dishes containing 35 ml MS medium each. Plates, in an upright position, were left for 6 days in the growth chamber (100 μmol photons m^{-2} sec^{-1}) at continuous light and room temperature. Seedlings were then transferred onto the selected Petri dishes containing MS medium with increasing $CuSO_4$ or Fe–EDTA concentrations: 0, 100, 200 μM $CuSO_4$ or 0, 50, 100, 200, 250, 500 μM Fe–EDTA. Plates, kept in a horizontal position, were left in the growth chamber at standard conditions. The seedling phenotypes were scored during the following days. MS medium: 4.4 g l^{-1} MS (Duchefa M0222), 1% Plant Agar (Duchefa P1001), sucrose 1% (pH 6.0) with BTP.

H_2O_2 staining with DAB

Arabidopsis thaliana leaves were cut and submerged into wells (Transwell, 24 mm diameter; Corning Costar Corp., Corning, NY, USA) containing 6–8 ml DAB solution (1 mg ml^{-1} DAB (Sigma; pH 3.8) with NaOH (Thordal-Christensen *et al.*, 1997; Zhou *et al.*, 2000). Petioles were cut again when fully immersed in the solution in order to prevent cavitation. Leaves were then left into the solution overnight in the dark. The following day, leaves were briefly rinsed in distilled water twice and transferred into Falcon tubes containing EtOH 96% (with holes in the caps). Falcon tubes were allowed to float onto boiling water until chlorophyll was completely removed from leaves (15–20 min). Leaves were then transferred in fresh EtOH (96%).

Northern blots

Northern blots, *AtFer1*, and *Atrib18s* cDNA probes were prepared as described by Murgia *et al.* (2002).

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