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ORIGINAL ARTICLE

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Antisense reduction of thylakoidal ascorbate peroxidase in *Arabidopsis* enhances Paraquat-induced photooxidative stress and Nitric Oxide-induced cell death

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Abstract The production and characterization of *Arabidopsis* plants containing a transgene in which the *Arabidopsis* tAPX is inserted in antisense orientation, is described. tAPX activity in these transgenic tAPX plants is around 50% of control level. The tAPX antisense plants are phenotypically indistinguishable from control plants under normal growth conditions; they show, however, enhanced sensitivity to the O_2^--generating herbicide, Paraquat. Interestingly, the tAPX antisense plants show enhanced symptoms of damage when cell death is triggered through treatment with the nitric oxide-donor, SNP. These results are in accordance with the ones recently obtained with transgenic plants over-expressing tAPX; altogether, they suggest that tAPX, besides the known ROS scavenging role, is also involved in the fine changes of H_2O_2 concentration during signaling events.

Keywords Antisense · *Arabidopsis thaliana* · Ascorbate peroxidase · Nitric oxide · Oxidative stress · Chloroplasts

Abbreviations APX: Ascorbate peroxidase · ASA: Ascorbic acid · BTP: Bis-tris propane · cAPX: Cytosolic APX · CHAPS: 3-[(3-cholamidopropyl) dimethylammonium]-1-propanesulfonate · HEPES: *N*-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) · HR: Hypersensitive response · kan: Kanamycin · k^r: Resistance to kanamycin · k^s: Sensitivity to kanamycin · MES: 2-(*N*-Morpholino)ethanesulfonic acid · MS medium: Murashige and Skoog medium · NO: Nitric oxide · PCD: Programmed cell death · Pq: Paraquat · ROS: Reactive oxygen species · SNP: Sodiumnitroprusside · tAPX: Thylakoidal APX

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Introduction

Reactive Oxygen Species (ROS), obligate by-products of aerobic metabolism, are produced in excess during various stresses and can damage, through indiscriminate oxidation of all macromolecules, various cellular compartments (Bowler et al. 1992; Allen 1995; Noctor and Foyer 1998; Karpinski et al. 2001). Plant cells have evolved two main strategies to keep ROS concentration under control, the first being the reduction of ROS production through anatomical or physiological adaptations, whereas the second is the scavenging of ROS already produced (Mittler 2002 and references therein).

Plant Ascorbate peroxidases (APX), together with catalases, are the enzymes directly involved in the scavenging of ROS; APX reduce H_2O_2 to water with ascorbic acid (ASA) as specific electron donor (Asada 1999).

Different APX isoforms are localized in the various plant cell compartments, such as chloroplasts (Jespersen et al. 1997), mitochondria (De Leonardis et al. 2000), peroxisomes (Jimenez et al. 1997; Shi et al. 2001) and cytosol (Jespersen et al. 1997; Caldwell et al. 1998). In particular, one of the two chloroplastic isoforms is anchored to the thylakoidal membrane (tAPX), through one major hydrophobic domain responsible for spanning the enzyme to the stroma exposed thylakoid membranes (Jespersen et al. 1997; Shigeoka et al. 2002). Chloroplastic APX (tAPX and the stromatic isoform sAPX) are both involved in the water–water cycle, where the O_2^- produced at the PSI site by the Mehler reaction is reduced to water in a two-step reaction, catalysed by the

SOD and 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).

Different evidences suggest that chloroplastic APX could also be involved in the fine H_2O_2 concentration changes involved in signaling events, as suggested by Mittler (2002). First, tAPX transcript levels are poorly modified by external stimuli leading to oxidative stress (Yoshimura et al. 2000; Panchuck et al. 2002; Shigeoka et al. 2002) suggesting that tAPX are involved in the immediate detoxification of H_2O_2, but not to the protection from ROS produced in excess during environmental changes (Shigeoka et al. 2002). Second, nitric oxide (NO), which regulates the hypersensitive response against pathogen attack with H_2O_2 as killing partner molecule (Delledonne et al. 1998, 2001) inhibits tAPX transcript accumulation and reduces tAPX enzymatic activity (Murgia et al. 2004a). Moreover, transgenic plants overexpressing tAPX show reduced symptoms of cell death when infiltrated with the NO-donor SNP (Murgia et al. 2004a).

Arabidopsis tAPX antisense plants allowed us to reinforce the evidences that tAPX, by affecting the concentration of H_2O_2, is indeed involved in the NO-induced programmed cell death.

Results show that 50% reduction of tAPX in *Arabidopsis*, differently from tobacco, does not affect plant growth under standard conditions. However *Arabidopsis* tAPX antisensing enhances Paraquat-induced photooxidative stress. Most interesting, tAPX antisense plants show enhanced symptoms of NO-induced cell death: these results, together with results in (Murgia et al. 2004a) confirm that tAPX enzymatic activity is involved in the modulation of H_2O_2 fine concentration changes for NO signaling.

Materials and methods

A. thaliana growth

A. thaliana plants of the Columbia (Col) ecotype were grown at 21–25°C, 150 µmoles photons m^{-2} s^{-1} (OSRAM L36 w/11–860 Lumilux PLUS Recyclable-Germany), 14 h/10 h light/dark photoperiod, in Arabaskets (Beta Tech) on sterilized Technic n. 1 DueEmme soil (Netherlands). They were watered with deionized water.

Preparation of the transformation vector containing the *Arabidopsis* tAPX cDNA in antisense orientation

The *Arabidopsis* tAPX cDNA fragment X98926 excised from the pZL1 vector by NotI-SalI digestion (Jespersen et al. 1997), was made blunt-end with Blunting Kit (Amersham) and ligated into the pBE2113-GUS transformation vector (Mitsuhara et al. 1996), previously digested with SmaI. This vector contains a chimeric P35S promoter conferring high level of expression of foreign genes. Antisense orientation of tAPX cDNA in the various *E.coli* colonies transformed with the pBE2113::tAPX vector was checked by PCR. For this purpose the following tAPXII, tAPXIII, CaMV and Gus rev primers have 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 transformation

Agrobacterium tumefaciens strain GV3101 was transformed with pBE2113:: tAPX transformation vector with tAPX cDNA in antisense orientation by electroporation and then used for transforming *Arabidopsis* plants by the "floral dip" technique (Clough and Bent 1998; Desfeux et al. 2000).

T1-transformed *Arabidopsis* seeds were plated on MS plates containing 1% Plant Agar (Duchefa, Amsterdam, Netherlands), 50 µg/ml kanamycin. T1 kan-resistant plants were transferred to the soil when they possess first true leaves and T2 seeds from single T1 plants were collected.

Preparation of thylakoidal membranes

Protocol was modified from (Casazza et al. 2001). Leaves (around 0.4 g) were cut from 3-week-old plants. All the following steps have been performed by using pre-chilled mortars, pestels and buffers. Leaves were homogenized with 2 ml of buffer A. Suspension was passed through eight layers cheesecloth into a corex tube and centrifuged for 3 min at 2,600*g*; the pellet was resuspended in 2 ml of buffer B and centrifuged again for 3 min at 2,600*g*. The last two steps have been repeated and the pellet was resuspended in 5 ml hypotonic B buffer. After 3 min of centrifugation at 2,600*g*, the pellet was resuspended in buffer C, centrifuged at 2,600*g* and finally resuspended again in 80 µl of buffer B. Chlorophyll content was measured by diluting 10 µl thylakoidal extract in 1 ml 80% acetone, which was centrifuged in a microcentrifuge at max speed: chlorophyll content in the supernatant was quantified by measuring the absorbances at 645 nm 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 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 1x final concentration solubilization buffer and then vortexed and centrifuged for 5 min in a microcentrifuge, at max speed. They were then loaded into a urea gel for electrophoresis. Urea gel, gel blotting and immunodecoration were performed according to (Murgia et al. 2004a). For the immunodecoration, the monoclonal antibody chl-mAb6 raised against spinach stromatic APX (sAPX) (Yoshimura et al. 2001) which also recognises *A. thaliana* tAPX was used as primary antibody at 1:1000 dilution. As secondary antibody, an IgG anti-mouse (SIGMA), conjugated with HRP at 1:20000 dilution, was used. Signals were detected with the SuperSignal West Pico Chemiluminescent Kit (Pierce).

3x solubilization buffer: 125 mM TrisHCl pH 6.8, 50 mM DTT (to add just before use), 9% SDS, 20% glycerol, 0.04% bromophenol blue.

Solubilization of proteins from isolated thylakoidal membranes

Leaves (around 1 g) from 3-week-old plants were cut and homogenized (with mortar and pestle) with 4 ml of ice-cold buffer S, by keeping mortar on ice. The suspensions were filtered with eight layers of cheesecloth into a corex tube and centrifuged for 3 min at 2,600*g*, 4 °C, with brake off. The pellets were resuspended in 4 ml of buffer S, centrifuged again for 3 min at 2,600*g*, 4°C, with brake off and resuspended in 100–400 µl of buffer R. After 1 h incubation at 4°C, suspensions were centrifuged at max speed in a minicentrifuge and the supernatants were transferred into a clean Eppendorf tube. Protein content of those samples (kept in the meanwhile at 4°C) were soon after quantified by the Bradford method, using the Bio-Rad Protein Assay Kit; samples were then loaded into a native gel for a PAGE electrophoresis.

Buffer S: 5 mM ASA, 1 mM EDTA, 100 mM Na-phosphate buffer pH 7.0.

Buffer R: 5 mM ASA, 100 mM Na-phosphate buffer pH 7.0, 1% CHAPS.

Detection of tAPX activity by native PAGE and spectrophotometrical quantification of tAPX activity were performed as described in Murgia et al. (2004a).

Quantification of Chl-a and Chl-b content

Six to seven leaves were added to vials containing 1 ml of dimethylformamide for chlorophyll extraction. Vials were closed accurately and kept overnight at 4°C in the dark. The following day, chlorophyll concentrations were quantified as µg chlorophyll/g fresh weight at the spectrophotometer, by using quartz cuvettes, according to the following formula (Porra et al. 1989):

$$\mu g\ \text{chla/ml: } 12.00 \times (Abs_{664} - Abs_{750}) - 3.11 \times (Abs_{647} - Abs_{750})$$

$$\mu g\ \text{chlb/ml: } 20.78 \times (Abs_{664} - Abs_{750}) - 4.88 \times (Abs_{647} - Abs_{750})$$

Paraquat treatment

Sterilized and vernalized seeds were allowed to germinate on standard liquid mineral medium (Gomarasca et al. 1993) into rotating flasks (60 rpm) under continuous light (100 µmoles photons m^{-2} s^{-1}). After 5 days, seedlings were washed twice in buffer E (30 min for each wash); fresh buffer E (as control) or buffer E containing 1 µM Pq were added to the seedlings, which were then kept overnight in the dark, under continuous rotation (60 rpm). Seedlings, together with the buffers they were immersed in, were then transferred into Elisa wells (well diameter:35 mm) and exposed to moderate light intensity (300 µmoles photons m^{-2} s^{-1}). After 24 h seedlings were weighed and added to vials containing 1 ml dimethylformamide for chlorophyll extraction. Vials were closed accurately and kept overnight at 4°C. The following day, chlorophyll concentrations were quantified as µg chlorophyll/g fresh weight according to (Porra et al. 1989).

Buffer E: 0.5 mM $CaSO_4$, 1 mM MES, pH 5.8–6.0 with BTP.

SNP treatment

Leaves of well-watered 21-day-old plants have 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 µmoles photons m^{-2} s^{-1}) unless otherwise specified.

RT-PCR

Total RNA was extracted from *Arabidopsis* leaves with Trizol reagent (Gibco). RT-PCR amplification reactions were performed by using the Access RT-PCR kit (Promega), by following the manufacturer's instructions. sAPX transcript levels were analysed in transformed *Arabidopsis* pBE2113::antisense tAPX plants by amplifying sAPX cDNA fragment (571 bp) with the following primers:

sAPX dir: 5′-AATAGTTGCCTTGTCTGG-3′
sAPX rev: 5′-GGAATATATGATCACCACG-3′

Reactions were performed at 1.5 mM $MgSO_4$, 48°C annealing temperature, 60 ng template RNA, with the following amplification program: first step: 48°C, 45 min. Second step: 94°C, 2 min. Third step: either 15, 25 or 40 cycles at 94°C, 30 s; 48°C, 1 min; 68°C, 2 min. Last step: 68°C, 7 min. Equal loading of RNA quantity in each sample was tested by amplifying the Tubulin4 mRNA according to (Tarantino et al. 2003). Amplification programs for tubulin were the same as for sAPX with the only difference being that the annealing temperature was 60°C.

Results

Production of *Arabidopsis* transgenic plants containing an antisense construct directed against the tAPX gene

Arabidopsis tAPX cDNA (X98926 clone) (Jespersen et al. 1997) was cloned in the antisense orientation in the pBE2113-GUS transformation vector (Mitsuhara et al. 1996) (Fig.1). *Agrobacterium tumefaciens* GV3101 was transformed by electroporation with such construct or with the empty pBE2113-GUS vector as control. Thirty *Arabidopsis* plants were transformed with *Agrobacterium* by floral dip; each transformed plant was grown and allowed to self-fertilize and T1 seeds were collected.

Ninety-four T1 plants showing resistance to kanamycin (k^r trait) were analysed by PCR for integrity of the inserted transgene, by using the primers annealing on the tAPX and on the GUS sequences. Twenty T1 positive lines were transferred on soil and allowed to produce seeds. T2 seeds were collected and tested for segregation of k^r trait. In five different T2 lines, the segregation ratio k^r:k^s = 3:1 confirmed one insertion site (Table 1). For each of these five T2 lines, twenty seedlings were transferred on soil, allowed to produce seeds, and the twenty T3 seed families were tested for segregation of the k^r trait. T3 lines homozygous for a single insertion site were isolated and propagated for further analysis.

Among the five tAPX antisense lines homozygous for a single insertion site, the 2/1 and 16 lines show reduced

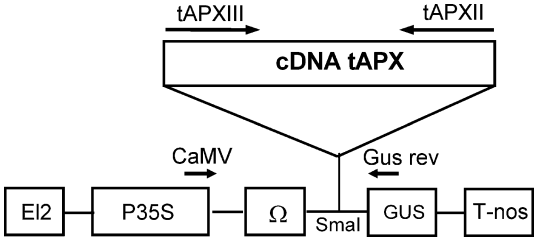

Fig. 1 Construction of tAPX antisense vector used to transform *A. thaliana* var Col plants. The Sal1-Not1 fragment of the *Arabidopsis* tAPX cDNA (X98926 clone) has been inserted in the SmaI pBE2113-GUS vector, in the antisense orientation. The positions of the primers, used for confirming antisense orientation of the insert, are indicated by *arrows*. The drawing is not to scale

Table 1 Segregation analysis of resistance to kanamycin in T_2 *Arabidopsis* seedlings transformed with a transgene containing the *Arabidopsis* tAPX cDNA in antisense orientation

T_2 lines	Total seedl.	k^r seedl.	k^s seedl.	χ^2 values (k^r:k^s = 3:1)
1/14	88	68	20	0.24
2/1	112	88	24	0.76
2/2	432	312	120	1.77
16	225	173	52	0.38
19/1	404	300	104	0.12

For each T_2 line, the total number of seedlings tested, the number of seedlings showing either k^r or k^s trait and the χ^2 values 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 with 0.05 significativity level

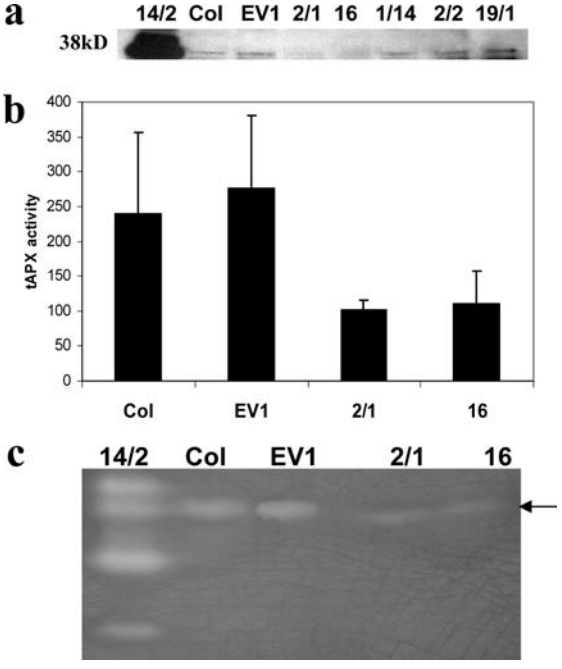

Fig. 2a-c tAPX levels in *Arabidopsis* plants transformed with a transgene in which the *Arabidopsis* tAPX is inserted in antisense orientation. **a** Western blot analysis of thylakoidal membrane extracts from mature leaves of the 14/2 line overexpressing tAPX (positive control), the wt line Col, the control line EV1 transformed with the empty vector and the 5 tAPX antisense lines 2/1, 16, 1/14, 2/2, 19/1. 15 µg protein extracts have been loaded onto the urea gel. A monoclonal antibody raised against spinach stromatic APX which recognises *Arabidopsis* tAPX has been used for the immunodecoration. **b** Spectrophotometric quantification of tAPX activity in the wt line Col, in the control line EV1 and in the tAPX antisense lines 2/1 and 16. Measurements were performed at 265 nm on 100 µg protein fractions solubilized from thylakoid extracts. tAPX activity is expressed as nmol ASA oxidized mg protein^{-1} min^{-1} ; each reported value is the mean of at least five measurements; *bars* correspond to the standard deviation. **c** Staining of tAPX activity on native PAGE-gels in the 14/2 line overexpressing tAPX (positive control), in the control lines Col, EV1 and in the tAPX antisense lines 2/1 and 16. Thylakoidal extracts from *Arabidopsis* full expanded leaves have been solubilized with CHAPS and 700 µg proteins from the solubilized fraction have been loaded in each lane. *Arrow on the right* indicates the band corresponding to tAPX activity in control and antisense plants

tAPX protein accumulation with respect to the wt Col and to the control line EV1 (Fig.2a). As positive control, the thylakoidal membrane extract from *Arabidopsis* 14/2 transgenic line which overexpresses tAPX (Murgia et al. 2004a) was also loaded.

The reduction of tAPX protein levels in the thylakoidal membranes of the 2/1 and 16 plants matches with a lower tAPX enzymatic activity. Solubilized proteins from isolated thylakoidal membranes have been used for quantifying tAPX enzymatic activity spectrophotometrically (Fig.2b); also, solubilized thylakoidal membranes have been used for assaying tAPX activity on native PAGE (Fig.2c). The positive control 14/2 shows multiple bands as already described in Murgia et al. (2004a). Results obtained confirm that tAPX activity in both 2/1 and 16 plants is around 50% of control levels.

Although the two *Arabidopsis* chloroplastic APX isoforms, tAPX and sAPX, share 72% sequence identity (excluding the C-terminus extension in the tAPX isoform), the antisensing of tAPX in the 16 and 2/1 lines affected the tAPX isoform only: sAPX transcript levels in these antisense plants are indeed the same as those in the control plants (Col and EV1) (Fig. 3). The intensity of the sAPX fragments amplified by RT-PCR in the tAPX antisense lines is indeed the same of the control lines, both after 25 or after 40 amplification cycles. The RT-PCR performed with 15 amplification cycles did not produce any detectable signal of sAPX fragment in any of the four lines tested (data not shown).

Arabidopsis tAPX antisense plants are phenotypically indistinguishable to wt under normal growth conditions

The *Arabidopsis* antisense plants 2/1 and 16 are phenotypically indistinguishable from the controls under normal growth conditions (Fig.4a): 50% reduction of tAPX activity in *Arabidopsis* does not affect the size of the rosette, number of its leaves or leaf chlorophyll content (Fig.4b). This result differs from what has been recently reported for other plant species: tobacco plants transformed with an antisense construct of tobacco tAPX are in fact stunted in growth and never produce seeds (Yabuta et al. 2002).

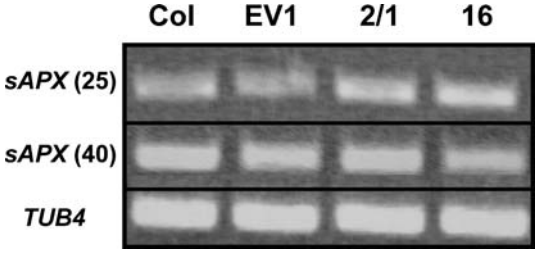

Fig. 3 RT-PCR amplifications of sAPX transcript in the wt line Col, in the control line EV1 and in the tAPX antisense lines 2/1 and 16 with either 25 or 40 amplification cycles. RT-PCR amplifications of TUB4 was performed as control (25 cycles), to check equal loading of RNA template (60 ng) in each sample

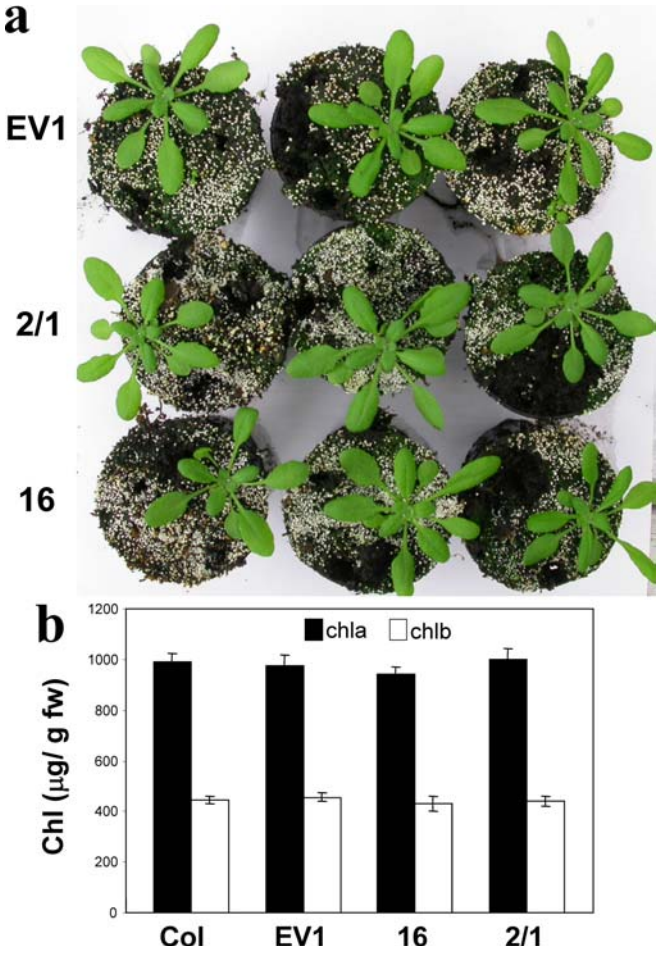

Fig. 4 a,b Phenotype of tAPX antisense plants under standard growth conditions. **a** Phenotype of EV1 control plants transformed with the empty vector, and of the tAPX antisense plants 2/1 and 16. **b** chlorophyll a (chla) and chlorophyll b (chlb) contents in leaves of 3-week-old Col, EV1, 16, 2/1 plants, expressed as µg chlorophyll/g fresh weight. *Bars* correspond to the standard deviation

Arabidopsis tAPX antisense plants are more sensitive to Paraquat-induced photooxidative stress but not to photoinhibitory treatments nor to Fe/Cu overload.

The tAPX antisense plants 2/1 and 16 show enhanced sensitivity to the treatment with Paraquat (Pq), an herbicide catalysing the photoreduction of O_2 to O_2^- around PSI. Upon treatment with 1 µM Pq, by the time the seedlings of the 2/1 and 16 lines completely bleach, control seedlings are still partially green, as chlorophyll contents after Pq treatment show (Fig.5). The 14/2 transgenic line overexpressing tAPX (Murgia et al. 2004a) has been used as positive control: as expected, it retains the highest chlorophyll content after Pq treatment (Fig.5). Leaf disks from mature leaves of 2/1 and 16 tAPX antisense plants, when exposed to different light intensities (150, 300, 600 µmoles photons m^{-2} s^{-1}) and different Pq concentrations (0.1, 0.5, 1 µM Pq) show, however, no enhanced sensitivity with respect to the controls (data not shown); these results indicate that

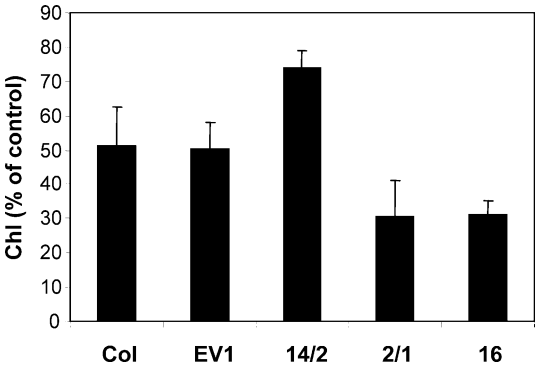

Fig. 5 Resistance of tAPX antisense lines to Pq. Chlorophyll content of one-week-old Col, EV1, 14/2 (used as positive control), 2/1 and 16 seedlings, pre-incubated overnight with water (mock-treated) or 1 μM Pq in the dark, and then illuminated at 300 μmol photons m^{-2} s^{-1} for 24 h at 22°C. For each line, chlorophyll content (μg chlorophyll/g fresh weight) after Pq treatment is given as % value with respect to its value in the mock-treated sample. Results represent the mean of two independent experiments; *bars* correspond to the standard deviation

the enhanced sensitivity of the tAPX antisense plants to Pq is, at least in the experimental conditions applied, an age-dependent phenotype.

To further assess tAPX role in protection against oxidative stresses, mature leaves (21–25 day) of the two tAPX antisense lines 2/1 and 16 were photoinhibited at two different conditions: at high intensity of white light (800 μmoles photons m^{-2} s^{-1}) and room temperature (Fig.6a) or at moderate intensity of white light (300 μmoles photons m^{-2} s^{-1}) and chilling temperature (Fig.6b). In both cases, the maximal photochemical efficiencies (Fv/Fm) of the 2/1 and 16 lines decline like those of the wt Col and of the control line EV1; also, no differences can be observed during the recoveries from the treatments at growth light and room temperature among the different lines tested (Fig.6a, b).

Free Fe ions and, to a lesser extent, Cu ions, catalyse 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 antisense lines 2/1 and 16 were grown on MS plates and then transferred on MS plates supplemented with either different Fe-EDTA concentrations (0, 50, 100, 200, 250, 500 μM Fe-EDTA) or $CuSO_4$ concentrations (0,100, 200 μM $CuSO_4$). Seedlings of the tAPX antisense lines show the same intensity levels of of Fe/Cu toxicity symptoms as their controls (not shown).

Antisense reduction of tAPX in *Arabidopsis* enhances nitric oxide-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 infection, H_2O_2scavenging is reduced through inhibition of cAPX protein synthesis (Mittler et al. 1998, 1999a). Nitric oxide (NO) is the killing partner of H_2O_2during HR (Delledonne et al. 2001, 2002): notably, the programmed cell death induced by the simultaneous production of H_2O_2 and NO, in the absence of pathogens, also down regulates cAPX (de Pinto et al. 2002; Murgia et al. 2004b).

Recently, we showed that the NO-donor SNP inhibits both accumulation of tAPX transcripts and tAPX enzymatic activity (Murgia et al. 2004a): accordingly, symptoms of damage in the tAPX overexpressing plants upon treatment with the NO-donor SNP are reduced with respect to wt ones (Murgia et al. 2004a). The transgenic lines with reduced tAPX levels show, on the reverse, enhanced symptoms of damage upon treatment with 5 mM SNP (Fig.7).

Discussion

APX enzymes are directly involved, in plant cells, in the scavenging of H_2O_2. The importance of APX in the

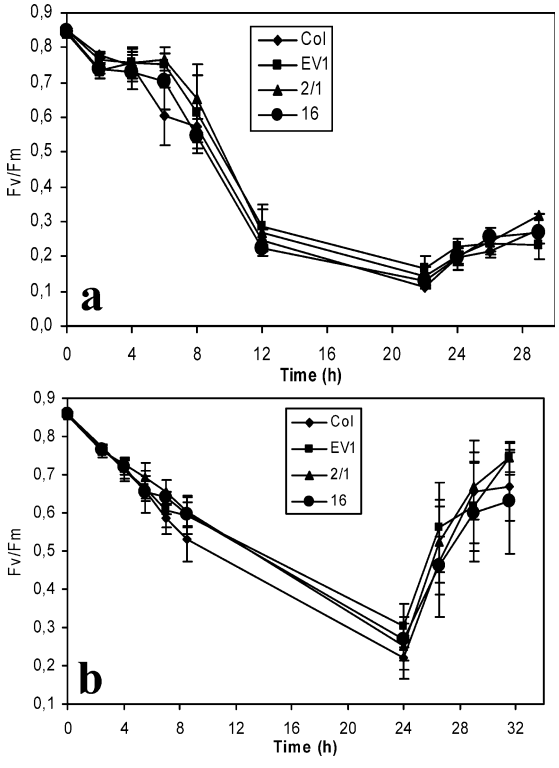

Fig. 6 a,b Photoinhibition of tAPX antisense plants at two different conditions. **a** Decrease of photochemical efficiency Fv/Fm during 22 h photoinhibition of 22-day-old leaves at 22°C, 800 μmol photons m^{-2} s^{-1}. Recovery during the following hours has been performed at growth conditions. Each point is the result of five different measures; vertical bars correspond to the standard deviation. **b** Decrease of photochemical efficiency Fv/Fm during 24 h photoinhibition of 22-day-old leaves at 2°C, 300 μmol photons m^{-2} s^{-1}. Recovery during the following hours has been performed at growth conditions. Each point is the result of five different measures; *vertical bars* correspond to the standard deviations

protection against noxious effects of ROS has been established through transgenic modification of the expression of various APX isoenzymes in different plant systems (Allen et al. 1997; Torsethaugen et al. 1997; Wang et al. 1999; Shi et al. 2001; Payton et al. 2001; Rizhsky et al. 2002;Yabuta et al. 2002; Murgia et al. 2004a).

In the present work, the production and characterization of *Arabidopsis* transgenic lines transformed with a construct in which *Arabidopsis* tAPX cDNA is inserted in antisense orientation, is reported. Results show that antisense tAPX plants with 50% residual tAPX activity are, unlike their tobacco counterparts (Yabuta et al. 2002), phenotypically indistinguishable from wt plants. However, in tobacco, a single gene codes for both tAPX and sAPX isoforms by alternative splicing (Shigeoka et al. 2002); it cannot be therefore excluded a priori that tAPX antisensing in tobacco (Yabuta et al. 2002) also antisensed the sAPX isoform, drastically reducing overall chloroplastic APX content. This is however not the case for the tAPX mutants described in the present work; in *Arabidopsis* two different genes code indeed for the sAPX and tAPX isoforms, which share (excluding the C-terminus extension in the tAPX isoform) 72% sequence identity; in the Results it was shown that the antisense tAPX plants have wt levels of sAPX transcript.

It will be interesting to verify whether the reduction of tAPX activity drastically affects normal growth in plant species other than tobacco.

Reduction of tAPX activity in *Arabidopsis* does not alter sensitivity to photoinhibition. These results are in accordance with what is observed in tAPX overexpressing plants (Murgia et al. 2004a): the reduction of maximal photochemical efficiency (measured as Fv/Fm) during photoinhibition is in fact 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 treatment (Sonoike 1996). In this latter 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.

Iron and copper catalyze, in the presence of H_2O_2 and O_2^-, the production of hydroxyl radicals $OH^{\cdot}$, the most reactive chemical species known, through the Fenton reaction (Bowler et al. 1992). An impaired H_2O_2 scavenging could therefore enhance toxicity symptoms upon Fe or Cu overload. However, such symptoms of toxicity upon Fe or Cu overload in tAPX antisense seedlings are the same as in control ones. In future, we intend to analyse antisense sAPX plants, which have been already produced in our group and which will be characterized in terms of sAPX enzymes activity levels, and also double mutants impaired in both tAPX and sAPX activity, to understand in greater detail the contributes of the two chloroplastic isoforms in the protection against Fe/Cu toxicity.

The *Arabidopsis* tAPX antisense seedlings are more sensitive to oxidative damage induced by the herbicide Pq, which generates ion superoxide O_2^- via auto-oxidation of its radical photoproduced at PSI (Asada 2000 and references therein). Also these results are in accordance with those observed in tobacco and in *Arabidopsis*, where tAPX overexpression increases tolerance to Pq (Yabuta et al. 2002; Murgia et al. 2004a).

Recent findings highlighted the importance of ROS as cellular indicators of stress and as key secondary messengers involved in the plant response against biotic and abiotic stresses (Pei et al. 2000; Knight and Knight 2001; Delledonne et al. 2001; Mittler 2002). In particular, a cooperation between NO and H_2O_2, with a defined concentration balance between the two molecules, is required during the plant hypersensitive disease-resistance response (Delledonne et al. 2001, 2002, 2003).

Results presented in this work confirm what we already observed with plants overexpressing tAPX (Murgia et al.2004a): perturbation of tAPX activity in *Arabidopsis*, by affecting H_2O_2 concentration levels, alters the NO-induced cell death.

We therefore propose that tAPX enzyme is involved in the fine regulation of H_2O_2 levels involved in the cell death triggered by NO. When tAPX levels are perturbed, treatment with NO indeed causes an alteration in the extent of leaf damage: the extent of cell death induced by SNP is inversely correlated to the tAPX-dependent scavenging of H_2O_2, as results presented in this work and previous observations (Murgia et al. 2004a) confirm.

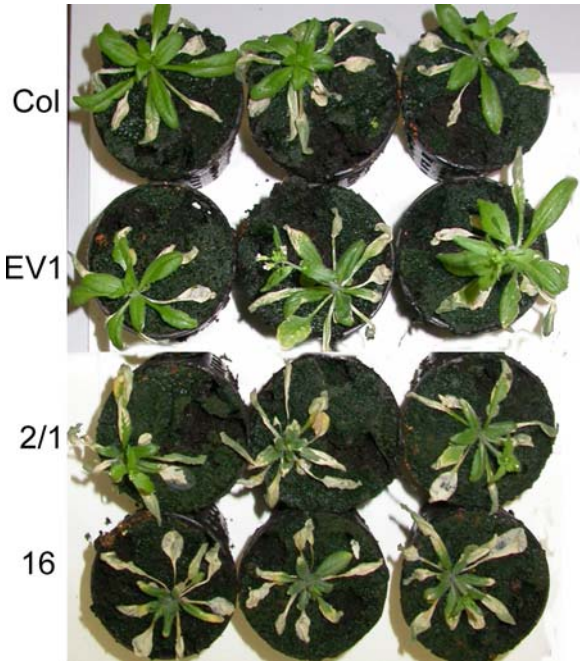

Fig. 7 Effects of the NO-donor SNP on the tAPX antisense lines. Phenotypes of 21-day-old plants Col, EV1, 2/1 and 16 after infiltration with 5 mM SNP and 7 days exposure at continuous light (120 μmol photons m^{-2} s^{-1}). Pictures are representative of two independent experiments with 6–8 plants SNP-infiltrated /line

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