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## Over-expression of *Trxo1* increases the viability of tobacco BY-2 cells under $H_2O_2$ treatment

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- **Background and Aims** Reactive oxygen species (ROS), especially hydrogen peroxide, play a critical role in the regulation of plant development and in the induction of plant defence responses during stress adaptation, as well as in plant cell death. The antioxidant system is responsible for controlling ROS levels in these processes but redox homeostasis is also a key factor in plant cell metabolism under normal and stress situations. Thioredoxins (Trxs) are ubiquitous small proteins found in different cell compartments, including mitochondria and nuclei (*Trxo1*), and are involved in the regulation of target proteins through reduction of disulphide bonds, although their role under oxidative stress has been less well studied. This study describes over-expression of a *Trxo1* for the first time, using a cell-culture model subjected to an oxidative treatment provoked by  $H_2O_2$ .
- **Methods** Control and over-expressing Ps*Trxo1* tobacco (*Nicotiana tabacum*) BY-2 cells were treated with 35 mM  $H_2O_2$  and the effects were analysed by studying the growth dynamics of the cultures together with oxidative stress parameters, as well as several components of the antioxidant systems involved in the metabolism of  $H_2O_2$ . Analysis of different hallmarks of programmed cell death was also carried out.
- **Key Results** Over-expression of Ps*Trxo1* caused significant differences in the response of TB-2 cells to high concentrations of  $H_2O_2$ , namely higher and maintained viability in over-expressing cells, whilst the control line presented a severe decrease in viability and marked indications of oxidative stress, with generalized cell death after 3 d of treatment. In over-expressing cells, an increase in catalase activity, decreases in  $H_2O_2$  and nitric oxide contents and maintenance of the glutathione redox state were observed.
- **Conclusions** A decreased content of endogenous  $H_2O_2$  may be responsible in part for the delayed cell death found in over-expressing cells, in which changes in oxidative parameters and antioxidants were less extended after the oxidative treatment. It is concluded that Ps*Trxo1* transformation protects TB-2 cells from exogenous  $H_2O_2$ , thus increasing their viability via a process in which not only antioxidants but also *Trxo1* seem to be involved.

**Key words:** Ascorbate, cell death, cell viability, glutathione, hydrogen peroxide, *Nicotiana tabacum*, over-expression, oxidative stress, reactive oxygen species, TB-2 cells, thioredoxin *o*, tobacco.

### INTRODUCTION

Reactive oxygen species (ROS) are continuously produced in the cell as a result of aerobic metabolism, but production increases under biotic and abiotic stress conditions, provoking oxidative stress and activating diverse responses in the plant that are necessary to cope with the situation (Taylor *et al.*, 2009; Martí *et al.*, 2011, 2013). Activation of the antioxidant systems in the cell is one of the mechanisms involved in this response, and plant cells contain a set of non-enzymatic and enzymatic reaction systems responsible for the control of suitable levels of ROS, including the components of the so-called ascorbate–glutathione (ASC–GSH) cycle (Hernández *et al.*, 2000; Caretto *et al.*, 2002; Martí *et al.*, 2009; Lázaro *et al.*, 2013). On the other hand, ROS are also necessary for normal metabolism and are now being considered as second messengers in cellular signal transduction (De Gara *et al.*, 2010; Mullineaux and Baker, 2010; Mittler *et al.*, 2011). Among them,  $H_2O_2$  has been

implicated in the regulation of plant development, the cell cycle and the induction of plant defence responses during stress adaptation, as well as in plant cell death (Dat *et al.*, 2003; Apel and Hirt, 2004; Locato *et al.*, 2008; Díaz-Vivancos *et al.*, 2010; Hakmaoui *et al.*, 2012; Sgobba *et al.*, 2014).

Programmed cell death (PCD) is a highly regulated physiological process that leads to selective elimination of damaged cells. It also plays a key role in plant development and responses to environmental changes (van Doorn and Woltering, 2005; Paradiso *et al.*, 2012; Monetti *et al.*, 2014). In plants, there is not just one type of PCD; there are different regulators and modes of death (Reape *et al.*, 2008). An apoptotic-like PCD (AL-PCD) is described with different morphological features from autophagy and necrosis. Condensation of the protoplast away from the cell wall and DNA cleavage into multimers of around 180 kDa fragments are typical features of AL-PCD but not of necrosis (Burbridge *et al.*, 2007). DNA fragmentation

appears as a smear on agarose gels (McCabe *et al.*, 1997) and seems to occur later than complete DNA degradation. Production of  $H_2O_2$  is considered to be a key event during the action of different stress factors; production increases early in this process of PCD (Locato *et al.*, 2008) and different concentrations of exogenous  $H_2O_2$  induce cell death (Houot *et al.*, 2001; de Pinto *et al.*, 2006). In this process, the control of  $H_2O_2$  by the defence system, including the enzymes ascorbate peroxidase (APX) and catalase, has been demonstrated to play a key role (Murgia *et al.*, 2004; Locato *et al.*, 2008, 2009; de Pinto *et al.*, 2013).

Thioredoxins (Trxs) are ubiquitous small proteins located in different cell compartments. They are involved in the regulation of target proteins through reduction of disulphide bonds. This is a key factor in maintaining protein dithiol/disulphide homeostasis (Balmer *et al.*, 2004; Martí *et al.*, 2009; Meyer *et al.*, 2012; Lázaro *et al.*, 2013). The presence of Trxs in plant mitochondria has been shown in arabidopsis and more recently in pea, being classified as the *Trxo* type (Laloi *et al.*, 2001; Martí *et al.*, 2009). Pea *Trxo1* has been located in both mitochondria and nucleus under normal conditions (Martí *et al.*, 2009), while a nuclear *Trxh* has been found only under oxidative conditions in germinating wheat seeds (Serrato *et al.*, 2001; Serrato and Cejudo, 2003; Pulido *et al.*, 2009). Extensive work has revealed diverse aspects of Trxs in plants, but very little is known about mitochondrial *Trxo1* function, although it has been related to redox regulation of proteins, including the respiratory component alternative oxidase (AOX) (Balmer *et al.*, 2004; Gelhaye *et al.*, 2004; Martí *et al.*, 2009; Yoshida *et al.*, 2013), and to the detoxification of ROS via mitochondrial peroxiredoxin (Prx) IIF (Barranco-Medina *et al.*, 2008). Information about the involvement of this *Trxo1* in the response to abiotic stress is quite scarce, and recent studies have shown its role under salinity (Martí *et al.*, 2011). In mammalian cells, Trx is also involved in the progression of cell death, depending on its redox state, preventing apoptosis as reduced Trx when it binds to apoptosis signal-regulating kinase 1 (ASK1), or inducing it in the oxidized form, in which it is dissociated from the kinase (Lu and Holmgren, 2012). However, in plant PCD the involvement of redox proteins such as Trxs or Prxs is unknown. In this work,  $H_2O_2$  was used as an inducer of oxidative stress and PCD in tobacco (*Nicotiana tabacum*) Bright Yellow-2 (TBY-2) cells that were transformed with *PsTrxo1*. Then, we investigated the effect of this treatment by studying the viability and growth of the cells as well as oxidative stress parameters, such as lipid peroxidation and protein carbonylation, and also the antioxidant status, measuring both the ASC and GSH redox state and  $H_2O_2$  scavenger enzymes. Different hallmarks of PCD were also analysed in a non-over-expressing and a *Trxo1*-over-expressing line of TBY-2 cells in an attempt to analyse the effect of the over-expression of this Trx in the response to  $H_2O_2$  as an inducer of cell death.

## MATERIALS AND METHODS

### Transformation of tobacco BY-2 cells

The complete cDNA encoding pea *PsTrxo1*, including the original targeting peptide for mitochondria (Martí *et al.*, 2009), was amplified by PCR. The product was ligated into the entry vector

pDONR ZEO (Invitrogen) and recombined with the destination vector pK7WG2D (Karimi *et al.*, 2002), provided by Flanders Interuniversity Institute for Biotechnology (VIB), Gent University, using the Gateway site-specific recombinational cloning technology (Invitrogen). The construct contains the CaMV 35S promoter and also harbours a constitutively expressed GFP gene targeted to the endoplasmic reticulum to facilitate the identification of transgenic calli. The plant expression vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (pMP90RK, GmR; KmR), RifR as described in Schinkel *et al.* (2005), using a Gene Pulser II electroporation system (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Stably transformed *Nicotiana tabacum* 'Bright Yellow-2' (TBY-2) suspension cells were generated by co-culture with recombinant *A. tumefaciens*. Transgenic TBY-2 cells were selected on kanamycin ( $100 \text{ mg L}^{-1}$ ) containing Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) agar plates. Transgenic callus tissue was used to initiate suspension cultures. The TBY-2 suspension cells were maintained in MS medium supplemented with minimal organics [0.47 % (w/v) MS salts,  $0.15 \text{ mg mL}^{-1}$  thiamine,  $0.02 \text{ mg mL}^{-1}$   $\text{KH}_2\text{PO}_4$  and 3 % (w/v) sucrose, pH 5.8] in an orbital shaker at 180 rpm and  $26^\circ\text{C}$  in the dark. Cultures were subcultured every week with a 5 % (v/v) inoculum. Transgenic TBY-2 calli were tested for the presence of recombinant *PsTrxo1* by immunoblot and those with the highest levels of expression were used to establish cell suspension cultures (referred to here as *Trxo1* lines and/or over-expressing lines). The cultures were also checked for the presence of the GFP by fluorescence microscopy and the presence of *Trxo1* protein by western blot, using a *PsTrxo1* antibody as described in Martí *et al.* (2009). A GFP line was also produced in which GFP, but not *Trxo1*, was present in the transformation vector and used as control cells to be compared with the over-expressing *PsTrxo1* cells.

### Cell culture, growth conditions and $H_2O_2$ treatment

The TBY-2 cells were routinely propagated in MS medium supplemented with minimal organics as described above, plus  $100 \text{ mg L}^{-1}$  myoinositol and  $0.2 \text{ mg L}^{-1}$  2,4-dichlorophenoxyacetic acid. The medium was adjusted to pH 5.8 and sterilized by autoclaving at  $121^\circ\text{C}$  for 15 min prior to use. The cells were subcultured to fresh culture medium at weekly intervals and were incubated on a rotary shaker at 130 rpm at  $26^\circ\text{C}$  in darkness.

At the end of the normal 7-day growth period, a 4-mL aliquot of stationary phase cells was transferred to 100 mL of fresh medium and every day an aliquot of cells was taken for analysis.

Cells in the exponential growth phase were exposed to  $H_2O_2$  at final concentrations of 15 and 35 mM. At the indicated time points, cell aliquots were collected for analysis either by filtration on Whatman 3 M paper or by centrifugation at  $10\,000g$  for 10 min at  $25^\circ\text{C}$ .

### Preparation of cell homogenate and subcellular fractions

Mitochondrial, nuclear and cytosolic fractions were isolated from lysed protoplasts essentially as described in de Pinto *et al.*

(2000) but with some modifications. Briefly, TBY-2 cells (10–20 g) were washed with the preplasmolysis buffer consisting of 0.4 M mannitol and 25 mM Tris–MES (2-(*N*-morpholino)ethanesulfonic acid), pH 5.5. The cells were then incubated with 0.25 % (w/v) cellulase (Duchefa), 0.05 % (w/v) pectolyase (Sigma) and 0.1 % (w/v) pectinase (Sigma) in the same medium. Digestion of the cell wall proceeded for 2 h in the dark at 30 °C. The protoplasts were sedimented at 110 g for 5 min at 25 °C and washed twice with preplasmolysis buffer adjusted to pH 6.5. The protoplasts were then suspended in ice-cold lysis buffer (3 mL g<sup>-1</sup> of cells) consisting of 0.4 mM mannitol, 20 mM Tris–HCl, 0.5 mM EDTA, 4 mM cysteine, protease inhibitors 1× (cOmplete, Roche, Germany) and 0.1 % (w/v) bovine serum albumin (BSA), and lysed on ice with a Potter homogenizer (19.5 × 2 cm). An enriched nuclear fraction was obtained by centrifuging the cell homogenate at 1500 g for 5 min at 4 °C. The supernatant was then centrifuged at 15 000g for 15 min at 4 °C to sediment the mitochondria, and the supernatant was centrifuged again at 82 000g for 20 min at 4 °C in order to obtain the cytosolic fraction.

#### Immunoblot analysis

Western blot analysis was performed on the different fractions as described in Vacca *et al.* (2006) using the antibody against PsTrxol and PsPrxIIF as described in Martí *et al.* (2009). Colorimetric detection was performed using alkaline phosphatase and NBT/BCIP reagent (Roche, Mannheim, Germany).

#### Cell viability and nuclear morphology

Cell viability was calculated as the percentage of cells that did not stain with trypan blue. An aliquot of cell suspension was transferred into a test tube with 50 % (v/v) trypan blue solution. After 5–10 min, the trypan blue cell suspension mixture was transferred to a microscope slide and viable (unstained) and non-viable (blue-stained) cells were counted. For each sample 1000 cells were scored.

For the analysis of nuclear morphology, TBY-2 cells were stained with 4,6-diamidino-2-phenylindole (DAPI), as described by de Pinto *et al.* (2002), and visualized using a fluorescence microscope (DMLS, Leica, Wetzlar, Germany) with an excitation filter of 340 nm and a barrier filter of 400 nm.

#### DNA fragmentation analysis

Total genomic DNA was isolated from the control and treated frozen cells (0.3 g), mechanically disrupted with a mortar and pestle in the presence of liquid nitrogen and solubilized in extraction buffer (1 M Tris–HCl pH 8, 0.5 M EDTA, 5 M NaCl). The cellular mixture was lysed at 65 °C for 10 min. After lysis, proteins and polysaccharides were salt-precipitated with 5 M potassium acetate and removed by centrifugation at 13 000g for 15 min at room temperature. Nucleic acids were precipitated with 100 % isopropyl alcohol and 3 M sodium acetate for 20 min on ice and recovered by centrifugation at 13 000g for 3 min at room temperature. The pellet was washed

twice with 70 % cold ethanol. Clean DNA was resuspended in sterile water. The sample was then incubated with RNase H for 1 h at 37 °C in order to digest RNA.

Electrophoresis was carried out on 1 % (w/v) agarose gel with TAE (Tris base, acetic acid and EDTA) buffer. Six micrograms of DNA in Orange G loading buffer [6 % (v/v) glycerol, 0.05 % (v/v) bromophenol blue, 12 mM EDTA pH 8, 0.4 % (w/v) orange G] was transferred to each gel well in order to detect DNA fragmentation.

#### Measurement of $H_2O_2$ and nitric oxide

The  $H_2O_2$  content was measured in the cellular extract using the eFox method (Bellincampi *et al.*, 2000). Briefly, 1 mL of cell culture was harvested by centrifugation at 10 000g for 20 s at 25 °C and the  $H_2O_2$  concentration was measured in the supernatant and pellet. The pellet was homogenized with acid acetone (v/v) and then frozen in liquid nitrogen and unfrozen. Finally, the cell mixture was centrifuged at 10 000g for 10 min at 4 °C, and an aliquot of supernatant (100 µL) was used as the pellet fraction. An aliquot of pellet was added to 500 µL of assay reagent (250 µM ferrous ammonium sulphate, 25 mM  $H_2SO_4$ , 100 µM xylenol orange, 100 mM sorbitol). After 45 min of incubation, peroxide-mediated oxidation of  $Fe^{2+}$  to  $Fe^{3+}$  was determined by measuring the absorbance at 560 nm of the  $Fe^{3+}$ -xylenol orange complex.

Nitric oxide (NO) was measured with 4,5-diaminofluorescein diacetate (DAF2). Briefly, 2 mL of cells was centrifuged at 10 000g for 20 min. The cell pellet was broken in liquid nitrogen and resuspended in 50 mM HEPES buffer pH 7.5 (1:2 w/v). The cell mixture (50 µL) was diluted in 950 µL of 50 mM HEPES buffer and incubated with 0.005 µM of DAF2 for 1 h at 37 °C. Fluorescence intensity was measured using a Shimadzu RF-1501 luminescence spectrophotometer at 495 nm excitation and 515 nm emission (Locato *et al.*, 2008).

#### Lipid peroxidation and protein oxidation measurements

The level of lipid peroxidation in the cells was measured in terms of malondialdehyde (MDA) content determined by the thiobarbituric acid reaction as described by Zhang and Kirkham (1996). The cells (0.3 g) were homogenized in 0.1 M potassium phosphate buffer pH 7 (1:2 w/v), 0.1 % (w/v) protease inhibitors (cOmplete) and 0.005 % (w/v) cysteine. The homogenate was centrifuged at 10 000g for 10 min and 200 µL of supernatant was collected to react with 1 mL of acid solution containing 15 % (w/v) trichloroacetic acid (TCA), 0.375 % (w/v) thiobarbituric acid, 0.010 % (w/v) butylated hydroxytoluene and 0.77 % (v/v) hydrochloric acid. The mixture was heated at 95 °C for 30 min and cooled in an ice bath, after which it was centrifuged at 3000 g for 10 min. The absorbance of the supernatant was read at 535 nm. The concentration of MDA was calculated using a calibration curve.

Protein oxidation was measured as carbonyl content in oxidatively modified proteins as described by Levine *et al.* (1990). Briefly, 0.3 g of cells were disrupted with liquid nitrogen and resuspended in a buffer containing 0.1 M potassium phosphate buffer, pH 7, plus 0.1 % (w/v) protease inhibitors (cOmplete) and 0.005 % (w/v) cysteine. The cell homogenate was



centrifuged at 15 000g for 15 min at 4 °C and 400 µL of supernatant was recovered. Because DNA can cause interference with the measurement, we precipitated it with streptomycin sulphate 1:10 (v/v) and removed it by centrifugation at 12 000g for 10 min. Two hundred microlitres of supernatant of each sample was incubated with 20 mM 2,4-dinitrophenylhydrazine (DNPH) in 2 M HCl (1:2, v/v), which reacts with carbonyl groups. A parallel incubation with HCl without DNPH was used as a blank for each sample. Incubation was for 1 h at room temperature. Carbonyl proteins were precipitated with 20 % (w/v) TCA and rinsed three times with ethanol:ethylacetate (1:1, v/v). The final pellet was resuspended in 6 M guanidine hydrochloride and incubated for 15 min at 37 °C. Absorbance was measured at 360 nm ( $\epsilon = 22\,000\text{ M}^{-1}\text{ cm}^{-1}$ )

#### Ascorbate and glutathione measurements

Cells (0.3 g) were collected by filtration on Whatman 3MM paper and homogenized with 2 volumes of cold 5 % (w/v) metaphosphoric acid at 4 °C using a vortexer. The homogenate was centrifuged at 20 000g for 15 min at 4 °C and the supernatant was collected for analysis. The ASC and GSH contents were measured by HPLC–electrospray ionization (ESI)/MS trap analysis using an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA) equipped with a thermostated  $\mu$ -well plate autosampler and a capillary pump, and connected to an Agilent Ion Trap XCT Plus Mass Spectrometer (Agilent Technologies) using an ESI interface as described in [Martí et al. \(2013\)](#).

#### Antioxidant enzymatic activities

Cells were ground in liquid  $N_2$  and homogenized at 4 °C in extraction buffer [50 mM Tris–HCl pH 7.8, 0.05 % (w/v) cysteine, 0.1 % (w/v) BSA]. The homogenate was centrifuged at 13 000g for 15 min at 4 °C and the supernatant was used for spectrophotometric analysis.

The activity of APX (L-ascorbate:hydrogen peroxide oxidoreductase, EC 1.11.1.11) was tested as described in [Jiménez et al. \(1997\)](#), by following the  $H_2O_2$ -dependent oxidation of ASC as decreasing absorbance at 290 nm ( $\epsilon = 2.7\text{ mM}^{-1}\text{ cm}^{-1}$ ).

Catalase (EC 1.11.1.6) activity was determined according to [Aebi \(1984\)](#) by measuring the decrease in  $A_{240}$  due to the disappearance of  $H_2O_2$  ( $\epsilon = 39.6\text{ M}^{-1}\text{ cm}^{-1}$ ).

Protein measurement was performed according to [Bradford \(1976\)](#) using BSA as standard.

#### Statistical analysis

Each experiment was performed at least two times with three replicates per treatment. Values presented are mean  $\pm$  s.e. When analysis of variance (ANOVA) showed significant effects, Tukey's *post hoc* test was applied using SPSS software (IBM® SPSS® Statistics 19). Genotype and time were considered the main factors in non-treated conditions, at  $P < 0.05$  (different lower case letters). Treatment with  $H_2O_2$  was considered as the main factor when comparing with control conditions for each line.

## RESULTS

### *Trxol* is over-expressed in TBY-2 cells

The molecular function of the plant *Trxol* gene was investigated using the transgenic TBY-2 system. *PsTrxol* was placed under the control of the CaMV 35 S promoter and inserted in TBY-2 cells by *A. tumefaciens*-mediated transformation. About 100 primary transgenic BY-2 clones were generated and screened using GFP fluorescence as a scorable marker. Ten microcalli exhibiting high fluorescence were selected, expanded and screened for production of PsTrxol using anti-PsTrxol antibody reactivity. Two clones (PsTrxol-1 and PsTrxol-2, [Fig. 1A–D](#)) with the highest PsTrxol protein accumulation (data not shown) were used to establish suspension cultures, which were tested again for anti-PsTrxol reactivity. Fluorescence staining for GFP was consistent with the described endoplasmic reticulum location of the protein ([Karimi et al., 2002](#)). Western blot analysis of protein extracts from control GFP line cell culture and transformed Trxol-1 and Trxol-2 lines revealed a major band with a molecular mass around 15 kDa, which was visualized only in the over-expressing lines ([Fig. 1E](#)). Consistent with previous reports, PsTrxol was efficiently targeted to mitochondria and nuclei, with no detectable cytosolic background staining ([Fig. 1F](#)), as shown by western blot analysis of mitochondrial, nuclear and cytoplasmic fractions from the control GFP (with no signal in these fractions; data not shown) and PsTrxol over-expressing cell cultures, using recombinant PsTrxol protein as positive control.

### Effect of *Trxol* over-expression on cell growth

Growth kinetics were analysed by measuring the optical density (OD) of the cultures at 600 nm. The growth of the GFP cells and *Trxol*-over-expressing cells (lines 1 and 2) was parallel, reaching a peak at day 7 of growth ([Fig. 2](#)).

### Effect of $H_2O_2$ on viability of cultured tobacco BY-2 cells

Tobacco BY-2 cells in the exponential phase were exposed to exogenous 15 and 35 mM  $H_2O_2$ . Cell viability was evaluated for different times in the following 72 h in the GFP line ([Fig. 3A](#)) and *Trxol*-over-expressing lines 1 and 2 ([Fig. 3B](#)). Larger differences were found between GFP and over-expressing lines in the 35 mM than in the 15 mM treatment (data not shown), so we chose 35 mM for further assays. A decrease in viability occurred in all lines but was much higher in the GFP line than in the *Trxol* lines: 35 mM  $H_2O_2$  provoked a decrease in viability of the GFP line of 43 % after 2 h, while in the over-expressing lines, interestingly, the decrease was only around 18 %. Subsequently, in the GFP line viability decreased progressively until becoming negligible at 72 h, while the *Trxol* lines maintained quite high viability (around 60 %) at this time.

The over-expressing line Trxol-1 was chosen for the following assays, in which it was compared with the GFP line as a control line.

### Cytological markers in tobacco BY-2 cells treated with $H_2O_2$

In order to verify whether the over-expression of *Trxol* altered the capability of TBY-2 cells to activate AL-PCD, some

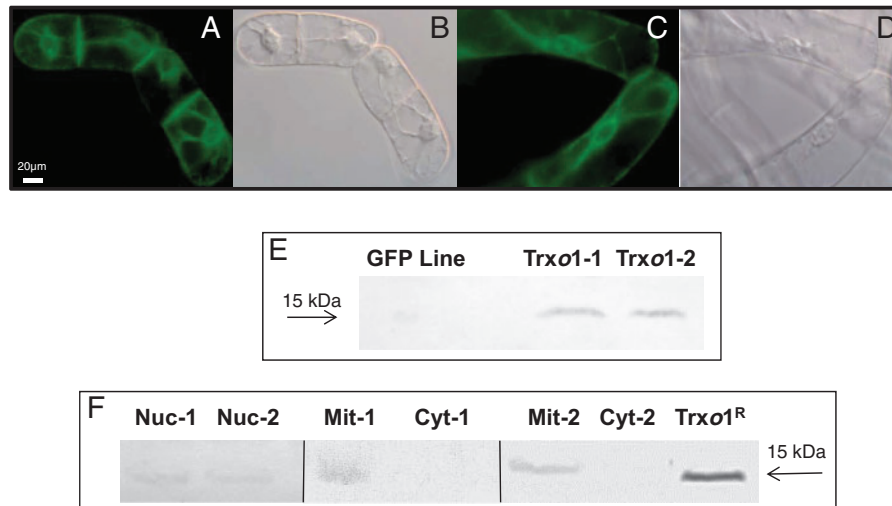


FIG. 1. GFP visualization by fluorescence microscopy and phase contrast imaging in two lines of transformed TB $\gamma$ -2 cells: (A, B) *Trx01*-1; (C, D) *Trx01*-2. Scale bar = 20  $\mu$ m. (E) Western blot analysis of cell extracts from GFP and Ps*Trx01* lines of TB $\gamma$ -2 cells using 50  $\mu$ g of soluble proteins separated by denaturing electrophoresis on a 12 % (w/v) polyacrylamide gel and hybridized with a rabbit anti-Ps*Trx01* antibody in cell extracts of GFP and Ps*Trx01* lines of TB $\gamma$ -2 cells. (F) Western blot analysis of 30  $\mu$ g of soluble proteins from nuclear (Nuc), mitochondrial (Mit) and cytoplasmic (Cyt) fractions of two lines of *Trx01*-over-expressing cells. Ps*Trx01* recombinant protein was used as positive control. The molecular mass standard is shown on the right (kDa). The loading control of the blot in (F) using Ponceau staining is presented as Supplementary Data Fig. S1.

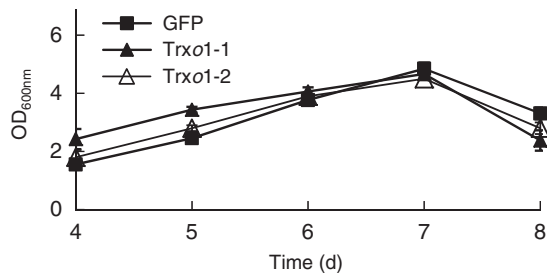


FIG. 2. Optical density of cultures of GFP and *Trx01* lines 1 and 2 of TB $\gamma$ -2 cells. Values represent mean  $\pm$  s.e. of ten experiments.

cytological markers were analysed after the  $H_2O_2$  treatment of the GFP and *Trx01*-1 lines (called the *Trx01* line from now on). The AL-PCD markers appeared in both lines in dead cells. Cell shrinkage, a hallmark of AL-PCD, was patent in both lines after 72 h in a high percentage of dead cells (Fig. 4A), although this marker was more evident in the *Trx01* cells. Staining with DAPI also showed the presence of apoptotic-like nuclei only in cells treated with 35 mM  $H_2O_2$  (Fig. 4B) and not in non-treated cells of either line. The genomic DNA integrity of the cells was also examined. Analysis of DNA fragmentation revealed that in control non-treated cells DNA appeared as a single high-molecular weight band of >10 000 bp (Fig. 5). Treatment of the cells with 35 mM  $H_2O_2$  provoked a different pattern of DNA oligonucleosomal fragment accumulation in the two lines as the cells died. At 72 h in the GFP line, a large band of DNA of low molecular weight appeared, probably because cell death was much more rapid, while in the *Trx01* line DNA laddering, consistent with internucleosomal fragments ( $\sim$ 180 bp multimers), was clearly present at 72 h of treatment.

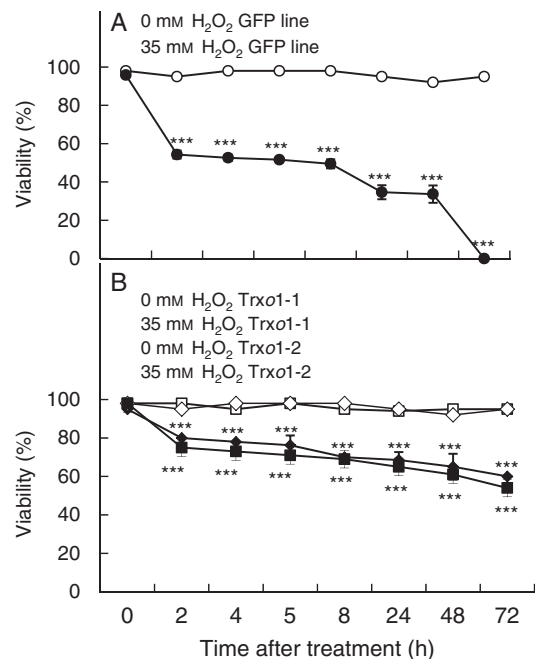


FIG. 3. Effect of 35 mM  $H_2O_2$  on cell viability of (A) GFP and (B) *Trx01* lines 1 and 2 of TB $\gamma$ -2 cells.  $H_2O_2$  was added at day 5 of growth and cell viability was analysed over time. Values represent mean  $\pm$  s.e. of ten experiments. \*\*\*Difference between non-treated and  $H_2O_2$ -treated sample of each line was significant at the 0.1 % level.

#### Oxidative and nitrosative stress markers

To analyse the oxidative stress induced by  $H_2O_2$  treatment, levels of both lipid peroxidation and protein oxidation, as well as  $H_2O_2$  content, were measured in TB $\gamma$ -2 cells of the GFP and

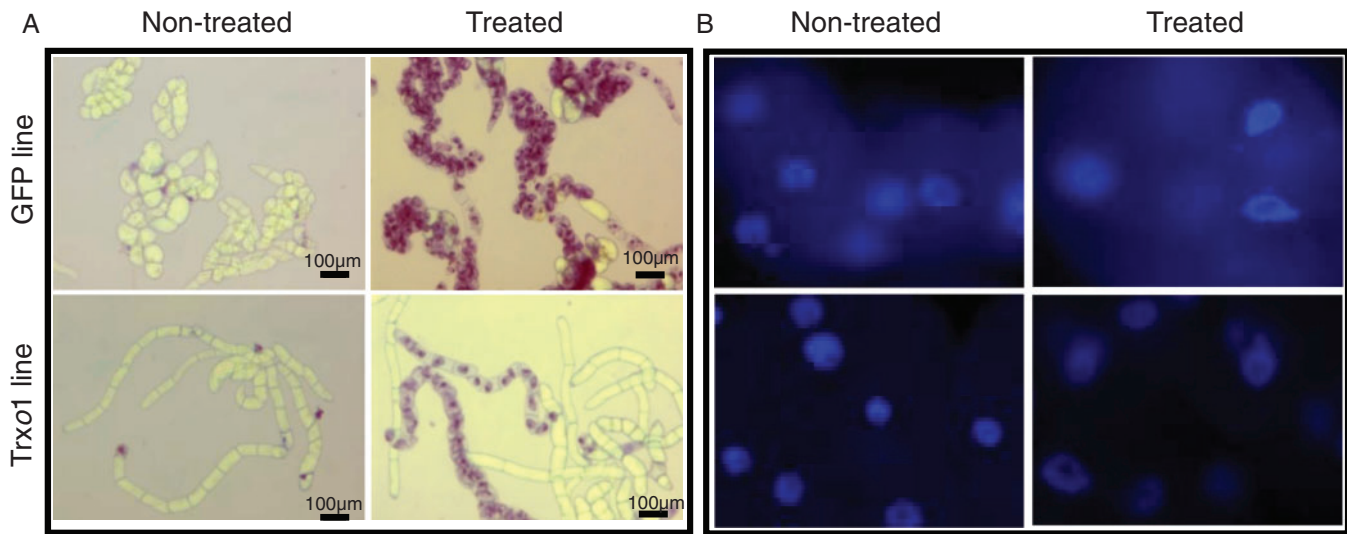


FIG. 4. Cytoplasm shrinkage (A) and nuclear morphology (B) induced by 35 mM  $H_2O_2$  in GFP and *TrxO1* lines of tobacco BY-2 cells after 72 h of treatment. Cells were stained with trypan blue (A) or DAPI (B). Scale bars = 100  $\mu$ m.

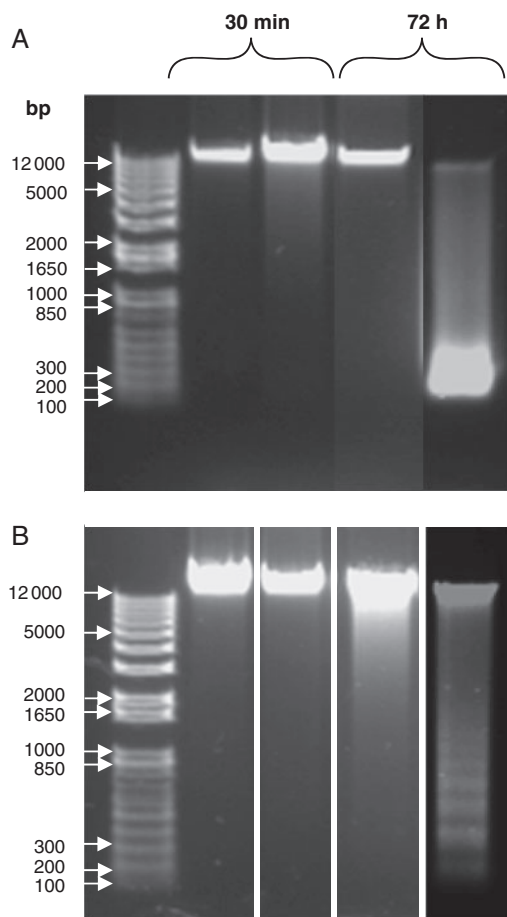


FIG. 5. Analysis of DNA fragmentation. DNA was extracted from (A) the GFP line and (B) the *TrxO1* line of tobacco BY-2 cells, non-treated (NT) and treated (T) with 35 mM  $H_2O_2$  and collected after 30 min and 72 h of treatment. A representative electrophoretic run in which 100  $\mu$ g DNA was loaded for each cell line is presented, and a size standard is shown on the left (bp).

*TrxO1* lines. No differences were found in these parameters between non-treated cells of the two lines except at 48 h, when the *TrxO1* over-expressing line presented higher lipid peroxidation and  $H_2O_2$  content (Fig. 6A–C). The effect of the  $H_2O_2$  treatment revealed that, not only in the GFP line but also in the *TrxO1* line, levels of lipid peroxidation as well as protein oxidation increased over time, although these differences disappeared in protein oxidation at 48 h (Fig. 6A, B). As regards  $H_2O_2$  analysis, the oxidative treatment led to a significant increase in the GFP line. In contrast, the over-expressing line kept a similar content in both treated and non-treated cells, except at 4 h, when a relative increase in  $H_2O_2$  was observed in the treated cells.

In addition, the NO content was measured in the cellular extract of the control GFP and over-expressing cells after the 35 mM  $H_2O_2$  treatment. In non-treated cells, the NO level in the over-expressing line was always significantly higher than in the GFP line, except at 30 min (Fig. 7A). After treatment, differences between treated and non-treated lines were higher in the GFP line than in the over-expressing line over time. The *S*-nitrosoglutathione (GSNO) content of the cells was also analysed, and although no significant differences were found between non-treated lines (except at 48 h), significant differences were found at relatively short periods of time after the oxidative treatment (30 min and 4 h) between non-treated and treated cells. The amount of GSNO was around 35-fold higher in treated than in non-treated cells at 30 min in both lines. However, this difference decreased by a half and a quarter in the GFP line and the over-expressing line, respectively, at 4 h, and showed a further, dramatic decrease at 24 and 48 h. At this time slot no differences were found in both lines, and GSNO was lower in treated than non-treated cells only in the GFP line at 48 h (Fig. 7B).

#### Cellular antioxidant metabolites and enzymes

To analyse the cellular redox state after  $H_2O_2$  treatment of cells of the GFP and *TrxO1* lines, the ASC and GSH pools were

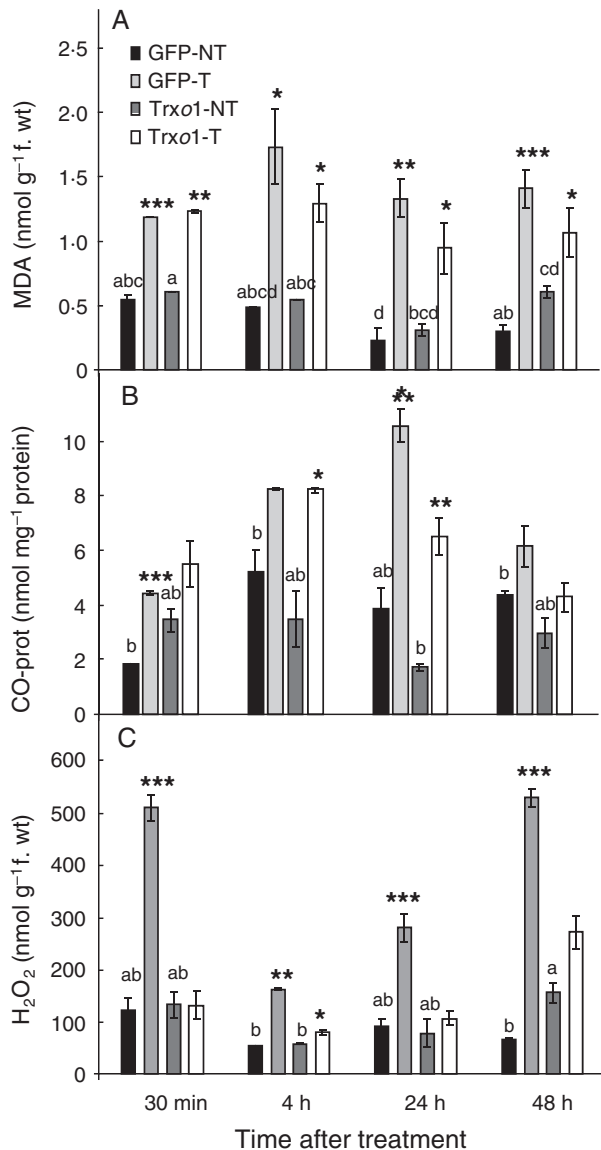


FIG. 6. Changes over time in (A) lipid peroxidation measured as MDA content, (B) carbonyl (CO) proteins and (C)  $H_2O_2$  in GFP and *Trx1* lines of tobacco BY-2 cells, non-treated (NT) and treated (T) with 35 mM  $H_2O_2$ . Values represent mean  $\pm$  s.e. of five experiments. Means for each non-treated sample that do not have common letter are significantly different by Tukey's test at  $P < 0.05$ . \*, \*\*, \*\*\* indicate differences between non-treated and  $H_2O_2$ -treated sample that are significant at the 5, 1 and 0.1 % level, respectively.

measured. Results revealed that, in non-treated conditions, the two lines presented similar total ASC contents [ASC plus dehydroascorbate (DHA)], but DHA was higher in line GFP at 24 h while in the *Trx1* line it was higher at 4 h (Fig. 8A, B). After  $H_2O_2$  exposure, total ASC did not vary in either line (Fig. 8A) while a general increase in DHA was observed in the GFP line over time; this increase was not so evident in the *Trx1* line (Fig. 8B).

In non-treated cells, total GSH content was more than 2-fold higher in the GFP line than in the *Trx1* line (Fig. 8C). This was mainly due to the increase in GSH, the reduced form of glutathione, since the amount of oxidized glutathione (GSSG)

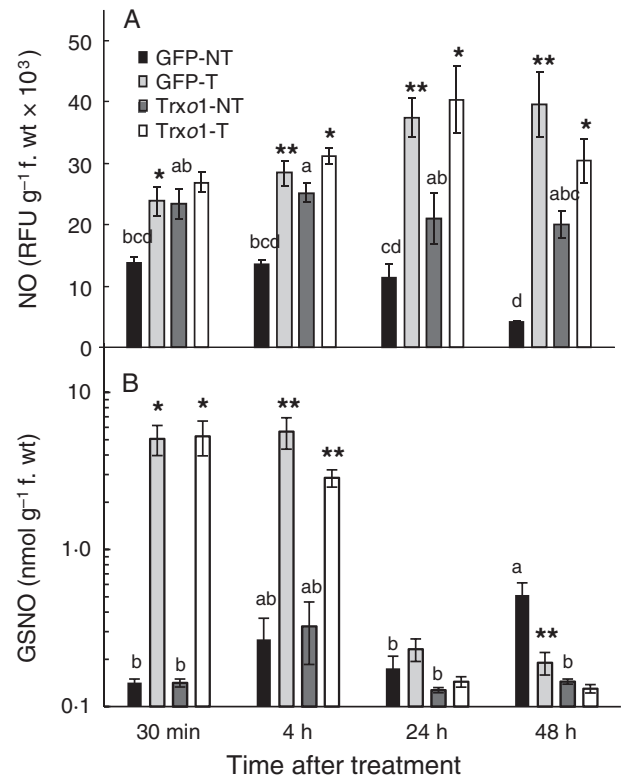


FIG. 7. Content of (A) NO and (B) GSNO of GFP and *Trx1* lines of tobacco BY-2 cells, non-treated (NT) and treated (T) with 35 mM  $H_2O_2$ . Values represent mean  $\pm$  s.e. of three experiments. Note log scale in (B). Means for each non-treated sample that do not have a common letter are significantly different by Tukey's test at  $P < 0.05$ . \*, \*\*, \*\*\* indicate differences between non-treated and  $H_2O_2$ -treated samples that are significant at the 5, 1 and 0.1 % level, respectively. RFU, relative fluorescence units.

was much lower than that of GSH and was similar in the two lines, except at 48 h, when it was around 5-fold less in the *Trx1* line than in the GFP line (Fig. 8D). After  $H_2O_2$  treatment, total GSH decreased to a similar extent in the two treated lines. Under this condition, GSSG content strongly increased in the GFP line at 30 min and 4 h. However, values in the *Trx1* line were similar to those in the GFP line at the later time points.

Regarding antioxidant enzymes, APX activity was similar in non-treated cells of the two lines, except at 48 h, when activity in the GFP line was 1.5-fold lower than in the over-expressing line. After 35 mM  $H_2O_2$  treatment, a great decrease in APX activity was observed over the period of study. This decrease was similar in the two lines (Fig. 9A). Catalase activity was around 3-fold higher in the GFP line than in the over-expressing line at 30 min and 4 h in non-treated conditions, whereas it presented a similar value in the two lines at 48 h. The effect of  $H_2O_2$  treatment was opposite in the two lines; whereas in the GFP line catalase activity was always lower throughout the period of study, in the over-expressing line this activity increased (Fig. 9B). Another enzyme involved in the removal of  $H_2O_2$  from cells is mitochondrial PrxIIF, a reported target of *Trx1* (Martí et al., 2009). Its protein level was analysed in both lines under  $H_2O_2$  treatment. It is worth highlighting that over-expression of *Trx1* in cells of the *Trx1* line was not accompanied by a change in the level of its target, PrxIIF, compared with the level in the GFP control line



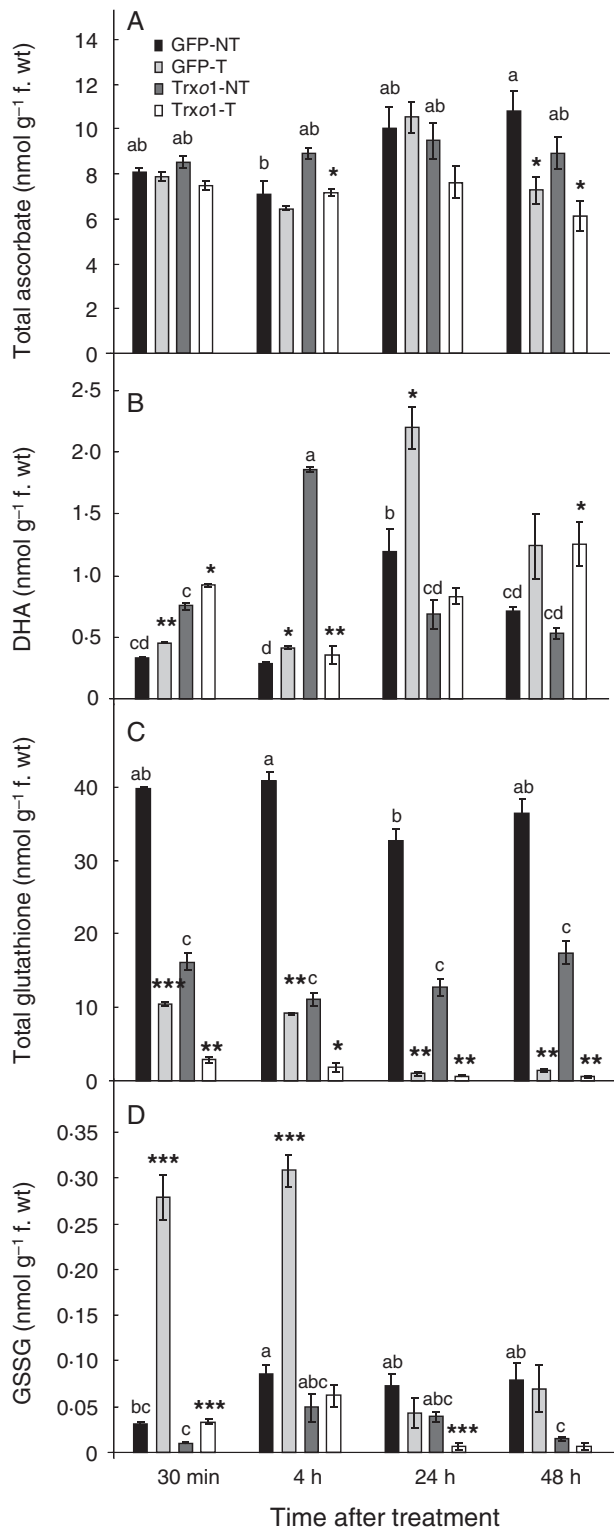


Fig. 8. Content of (A) total ascorbate [ASC + dehydroascorbate (DHA)] and (B) DHA, (C) total glutathione [GSH + oxidized glutathione (GSSG)] and (D) GSSG in GFP and *Trxol* lines of tobacco BY-2 cells, non-treated (NT) and treated (T) with 35 mM  $H_2O_2$ . Values represent mean  $\pm$  s.e. of three experiments. Means for each non-treated sample that do not have common letter are significantly different by Tukey's test at  $P < 0.05$ . \*, \*\*, \*\*\* indicate differences between non-treated and  $H_2O_2$ -treated sample that are significant at the 5, 1 and 0.1 % level, respectively

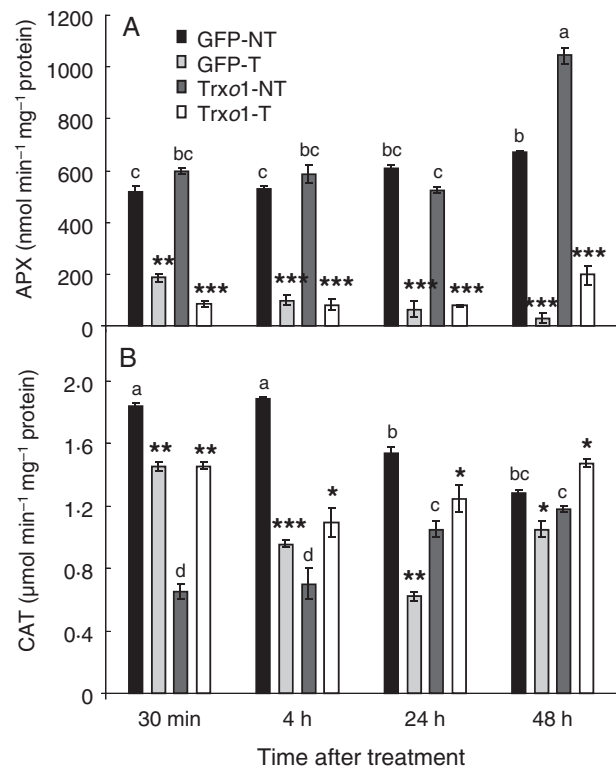


Fig. 9. Changes over time in (A) APX and (B) catalase specific activities of GFP and *Trxol* lines of tobacco BY-2 cells, non-treated (NT) and treated (T) with 35 mM  $H_2O_2$ . Values represent mean  $\pm$  s.e. of three experiments. Means for each non-treated sample that do not have common letter are significantly different by Tukey's test at  $P < 0.05$ . \*, \*\*, \*\*\* indicate differences between non-treated and  $H_2O_2$ -treated samples that are significant at the 5, 1 and 0.1 % levels, respectively

(Fig. 10). However, after 4 h of  $H_2O_2$  treatment, PrxIIF content was reduced by 70 % in the GFP line, while in the *Trxol* line the reduction was less severe, at around 40 %.

## DISCUSSION

The expression of *PsTrxol* cDNA, a *Pisum sativum* thioredoxin *o1*, in transgenic tobacco cells was associated with the appearance of a peptide of apparent molecular mass close to that predicted for the mature *PsTrxol* protein (Martí *et al.*, 2009), which reacted with an antibody directed against an epitope at the C-terminus of the amino acid sequence of *PsTrxol*. Mitochondrial and nuclear-enriched fractions of cells of the *Trxol* line showed the presence of the *PsTrxol* protein, which was absent in control GFP line cells and in the cytoplasm of *Trxol* line cells. This result confirmed that this protein reached the target organelles in TBY-2-over-expressing cells, in agreement with the subcellular localization described for this *Trxol* in pea (Martí *et al.*, 2009). GFP and GFP variants have been used widely for protein location or over-expression studies in cellular cultures (Sack *et al.*, 2007; Voitsekhojskaja *et al.*, 2014), and they are appropriate markers for positive transformed calli of TBY-2 (Karimi *et al.*, 2002).

The presence of *PsTrxol* provoked some changes in the over-expressing cells, although it did not affect their rate of



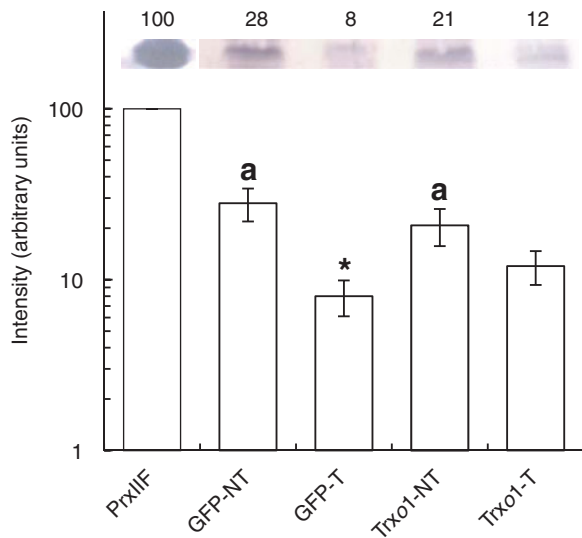


FIG. 10. Western blot analysis of the protein level of PrxIIIF in GFP and Trx1 lines of tobacco BY-2 cells, non-treated (NT) and treated (T) with 35 mM H<sub>2</sub>O<sub>2</sub> after 4 h. A representative blot is shown for recombinant PsPrxIIIF as positive control (100 %). Band intensity (arbitrary units; note log scale) is represented in the graph after quantification using image analysis software (GeneTools, Syngene, Cambridge, UK).

growth, which was similar to that of the control GFP cells. Levels of oxidative stress measured as lipid and protein oxidation were generally similar over time in the two lines, as were also H<sub>2</sub>O<sub>2</sub> content and antioxidants, such as ASC and APX activity. However, in the GFP line the higher NO content and catalase activity and lower GSH content probably pointed to cross-talk between Trx, ROS and reactive nitrogen species, which could influence cellular responses to an oxidative situation such as that provoked by H<sub>2</sub>O<sub>2</sub> treatment. In fact, cells over-expressing Trx1 have been shown to delay and decrease the extent of cell death induced by H<sub>2</sub>O<sub>2</sub>, and in this work we investigated the possible role of Trx1 protein in the response to this oxidative situation. ROS signalling has been described as being related to the chemical characteristics of the species and dose, with H<sub>2</sub>O<sub>2</sub> in particular being a prominent signalling molecule that is relatively stable and mobile among compartments and cells (Vranova et al., 2002; Op den Camp et al., 2003; Gadjev et al., 2008). In general, low concentrations of H<sub>2</sub>O<sub>2</sub> are reported to protect against oxidative and abiotic stress, by activating defence responses aimed at maintaining cellular redox homeostasis; concentrations in a narrow range of concentrations trigger PCD, while higher concentrations can cause necrosis (Van Breusegem and Dat, 2006; Laloi et al., 2006; de Pinto et al., 2006). In fact, previous work has shown that when H<sub>2</sub>O<sub>2</sub> is produced above certain levels, cell death is induced in a concentration-dependent manner (Houot et al., 2001; de Pinto et al., 2002; Dubovskaya et al., 2007). Hydrogen peroxide is known to be able to provoke necrosis or PCD, although both processes are considered to be two extremes of the same phenomenon, namely necroapoptosis (Casolo et al., 2005). In this paper we report for the first time the effect of the over-expression of a Trx1 in an oxidative situation, inducing PCD. In fact, the analysis of the effect of H<sub>2</sub>O<sub>2</sub> on our

TBY-2 cell cultures evidenced the presence of dead cells with some features of AL-PCD, such as cell shrinkage, apoptotic-like nuclei (Houot et al., 2001) and DNA fragmentation, which in the case of the over-expressing Trx1 line was in the form of DNA laddering at the time analysed. Interestingly, over-expressing cells presented very high viability after the treatment in comparison with control cells, being protected from the death inducer H<sub>2</sub>O<sub>2</sub>, and in this work we asked whether Trx1 protein is involved in the response through the antioxidant metabolism.

Analysis of oxidative stress induced by the treatment revealed that 35 mM H<sub>2</sub>O<sub>2</sub> provoked similar oxidative damage in the Trx1-over-expressing and GFP control lines in terms of protein oxidation and lipid peroxidation, although the difference between untreated and treated cells was lower in the over-expressing line at 24 and 48 h of treatment than at other time points. Consistently, differences in intracellular H<sub>2</sub>O<sub>2</sub> content were observed between the two lines when they were subjected to oxidative treatment, which provoked a general increase in the content of H<sub>2</sub>O<sub>2</sub> in cellular extracts of the GFP line, while in Trx1 cells the levels of H<sub>2</sub>O<sub>2</sub> were maintained at similar levels in the treated and untreated cells; this behaviour was consistent with a higher protective capability against oxidative stress in Trx1 cells than in GFP cells, which could be related to higher catalase activity in Trx1 cells than in GFP cells after exposure to H<sub>2</sub>O<sub>2</sub> and the higher viability found in over-expressing cells than in GFP line cells. It has been suggested that exposure of cultured plant cells to H<sub>2</sub>O<sub>2</sub> and accumulation of hydroperoxides results in PCD processes (Houot et al., 2001; de Pinto et al., 2006). The level of lipid peroxidation in TBY-2 cells has been reported to remain constant in PCD induced by narciclasine and heat shock treatment, before the onset of loss of cell viability while increasing in the late stages, implying that MDA accumulation is related to cell death (Locato et al., 2008; Lu et al., 2012).

The different amounts of H<sub>2</sub>O<sub>2</sub> found in the cell contents of the two lines after H<sub>2</sub>O<sub>2</sub> treatment led us to examine some components of the antioxidant system of the cells. Added H<sub>2</sub>O<sub>2</sub> was quickly removed by cell metabolism from the culture medium in which TBY-2 cells were cultured: almost 80 % of added 50 mM H<sub>2</sub>O<sub>2</sub> disappeared within a few minutes (Houot et al., 2001; de Pinto et al., 2006). It has been reported that some of the components of the ROS-scavenging system in plants are involved in PCD. In fact, H<sub>2</sub>O<sub>2</sub>-mediated induced resistance to death in tobacco seems to depend on downregulation of the antioxidant system, and this has been related to a more intense hypersensitive response (or death) during pathogen infection (Király et al., 2002). The ASC–GSH cycle is part of the antioxidant network controlling H<sub>2</sub>O<sub>2</sub> levels and has been implicated in the transduction signal that triggers PCD, in which NO and ROS also play an essential role (de Pinto et al., 2002). Several papers underline that ROS activate NO biosynthesis and vice versa, and that these two classes of reactive species act in synergy to promote PCD (Delledonne et al., 1998; de Pinto et al., 2012). Induction of PCD by NO and H<sub>2</sub>O<sub>2</sub> together requires a decrease in the antioxidants, such as APX, as a first stage and subsequently an alteration in the redox state and levels of ASC and GSH towards the oxidized forms (de Pinto et al., 2002, 2006, 2013). Moreover, an increase in S-nitrosylating agents such as GSNO and NO has recently been reported to occur during PCD induced by H<sub>2</sub>O<sub>2</sub> in TBY-2 cells (de Pinto et al.,

2013). In our cells, the difference in  $H_2O_2$  content, rather than NO and GSNO, seemed to influence the difference in progression of PCD after oxidative treatment in GFP and *Trxol* cells. Together with peroxidases, in particular APX, another important  $H_2O_2$  scavenger is the enzyme catalase. Both APX and catalase activities have been reported to decrease during treatments that induce PCD, with an earlier response of APX (de Pinto *et al.*, 2006; Locato *et al.*, 2008, 2009; Lu *et al.*, 2012). Consistently, even under our conditions, APX decreased very rapidly after PCD induction in both cell lines, thus confirming APX involvement in the redox impairment occurring during PCD. On the other hand, the behaviour of catalase was different in the two cell lines both in the control and the treated condition. Indeed, under the control condition, catalase had lower activity in *Trxol*-over-expressing cells than in GFP line cells, whereas when cells were exposed to  $H_2O_2$  catalase activity decreased in GFP cells undergoing PCD, which is consistent with literature data (Locato *et al.*, 2008), but increased in *Trxol*-over-expressing cells. This could suggest that, under control conditions, the decrease in catalase activity was balanced in *Trxol* over-expressing cells by the presence of *Trxol* protein. The behaviour of catalase under  $H_2O_2$  treatment was also consistent with the alteration in endogenous  $H_2O_2$  content. Indeed,  $H_2O_2$  increased in GFP cells but remained at control levels in *Trxol*-over-expressing cells, pointing to catalase as mainly influencing the endogenous level of  $H_2O_2$  in these cells. Peroxiredoxin IIF, one of the *Trxol* targets, is also involved in the control of hydroperoxides, including  $H_2O_2$  (Dietz *et al.*, 2006; Martí *et al.*, 2009). In spite of the lower PrxIIF content in the line over-expressing *Trxol* than in the GFP line, when cells were exposed to  $H_2O_2$  treatment this protein decreased markedly in the GFP line but to a much lesser extent in the *Trxol* line. In previous work in which a proteomic study was performed in TBY-2 cells exposed to  $H_2O_2$  treatment, Prxs as well as *Trxh* were found to be differentially expressed (Vannini *et al.*, 2012), with a general decrease in the amounts of the proteins. This was related to increased cellular oxidation and decreased protein stability due to inhibition of the Prx-chaperone function in this situation, in which PCD hallmarks were also evidenced. Over-expression of *Trxol* would lead to the opposite situation, delaying the cell death induced by  $H_2O_2$ , probably by a regulatory action on *Trxol* target proteins. Among these, mitochondrial PrxIIF, at least, seems to decrease under  $H_2O_2$  treatment, but to a lesser extent in *Trxol*-over-expressing cells than in GFP line cells. Due to the peroxidase activity of PrxIIF, this difference could contribute to the higher tolerance of  $H_2O_2$  treatment characterizing the *Trxol*-over-expressing cells. However, we cannot exclude the possibility that levels of other  $H_2O_2$ -scavenging enzymes must be increased in *Trxol*-over-expressing cells.

The cellular redox balance is controlled by the Trx and ASC–GSH systems, which regulate the ROS content in the cell and, directly or indirectly, the redox status of a plethora of metabolites. Cysteine oxidation is one of the post-translational modifications due to oxidative stress. It is known that the oxidation of cysteine affects enzyme activity when the oxidized amino acid is localized in the active site of the protein (Camejo *et al.*, 2013), but alteration of enzyme activity could also occur when the oxidized cysteine is localized in a structural part of the protein (Holmgren and Bjornstedt, 1995). Plant *Trxol* lacks

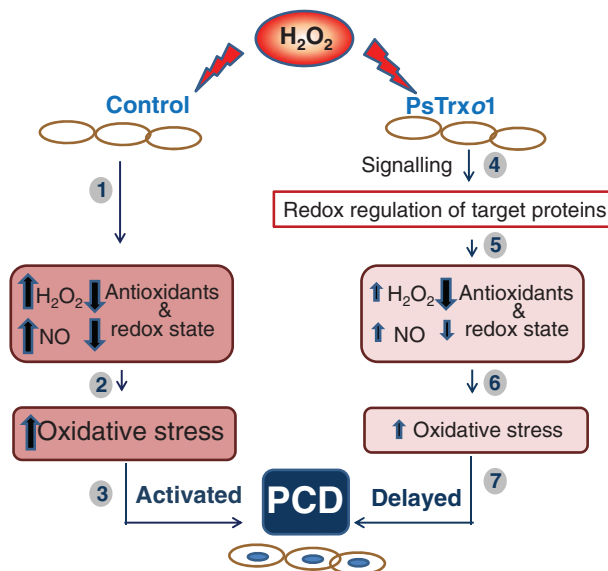


Fig. 11. Role of PsTrxol in TBY-2 cell death after treatment with 35 mM  $H_2O_2$ . A differential response in control cells and cells over-expressing PsTrxol was observed after this treatment. In control cells a decrease in the levels of antioxidants (APX and catalase) and in the redox state of ASC and GSH is accompanied by an increase in  $H_2O_2$  and NO. This situation causes oxidative stress in the cell, with increased lipid peroxidation (2) and a PCD process (3). In over-expressing PsTrxol cells,  $H_2O_2$  treatment induces a signalling response that implies the redox regulation of target proteins (4). This situation causes a decrease in antioxidant components, such as APX and PrxIIF, and an increase in catalase, accompanied by a decrease in the redox state of ASC and GSH (5). These changes contribute to less oxidative stress than in control cells, evidenced by smaller changes in NO and  $H_2O_2$  and decreased lipid peroxidation (6). Nevertheless, as a consequence of this situation, PCD occurs but is delayed (7).

structural cysteine, and for this reason it has been considered more resistant than other proteins to oxidative stress, as described for prokaryotic Trx and mammalian mitochondrial Trx-2 (Miranda-Vizuete *et al.*, 1997; Spyrou *et al.*, 1997). An increase in intracellular ROS has been described in Trx-2-deficient cells: the cells entered apoptosis while total GSH decreased. These deficient cells, when treated with buthionine sulphoximine (BSO), augmented cell death, possibly by increasing the number of necrotic cells (Tanaka *et al.*, 2002). In the present work, the over-expressing *Trxol* cells had lower amounts of GSH than GFP control cells, but they did not show higher levels of GSH oxidation or conjugation to NO as GSNO. This could indicate that GSH biosynthesis may be affected in over-expressing cells. It has been reported that the reduced cytoplasmic Trx1 and Trx2 in yeast double mutants produces an increase in total GSH content, mainly in the oxidized form, by an unknown underlying mechanism of adjusting GSH levels in response to Trx deficiency (Muller, 1996). Also, an inverse relation has been reported between GSH level and Trx expression: mRNAs encoding two *Trxh* types were found to be increased by GSH depletion in a GSH-deficient root *meristemless* mutant (Schnaubelt *et al.*, 2015). In agreement with this, as mentioned above, our *Trxol* over-expressing cells presented lower levels of GSH than the control cells. They showed less change in this antioxidant after  $H_2O_2$  treatment, suggesting that over-expression was able to compensate for the decreased GSH

content and to reduce its oxidation. It was previously reported that elevated transcription of yeast *Trx2*, mediated by YAP1, conferred resistance to  $H_2O_2$ , pointing to this Trx as a crucial part of the oxidative stress response (Kuge and Jones, 1994), similar to the feasible role found for *Trx1* in this work.

In conclusion, over-expression of Ps*Trx1* caused significant differences in the response of TBVY-2 cells to high concentrations of  $H_2O_2$ , consisting of higher and maintained viability in over-expressing cells, while the control line presented a severe decrease in viability and marked oxidative stress, with generalized cell death after 3 d of  $H_2O_2$  treatment (Fig. 11). In over-expressing cells, the increase in catalase activity, the decreased  $H_2O_2$  and NO contents and maintenance of the GSH redox state, as well as the levels of PrxIIF and *Trx1*, might sustain the delay in cell death (AL-PCD) in relation to control cells. The similar changes in the contents of ASC, APX, GSNO and the oxidative parameters measured point mainly to *Trx1* as a pivotal factor responsible for the delay in  $H_2O_2$ -induced PCD, not only as an antioxidant. Further work in this direction would give us the opportunity to elucidate new functional roles of *Trx1* in plant development and responses to stress leading to cell death.

#### SUPPLEMENTARY DATA

Supplementary data are available online at [www.aob.oxford-journals.org](http://www.aob.oxford-journals.org) and consist of Figure S1: loading control of the western blot in Fig. 1F using Ponceau staining of the membrane.

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