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RESEARCH PAPER

Involvement of cytosolic ascorbate peroxidase and Cu/Zn-superoxide dismutase for improved tolerance against drought stress

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Abstract

In order to understand the role of cytosolic antioxidant enzymes in drought stress protection, transgenic tobacco (*Nicotiana tabacum* cv. *Xanthi*) plants overexpressing cytosolic Cu/Zn-superoxide dismutase (cyt*sod*) (EC 1.15.1.1) or ascorbate peroxidase (cyt*apx*) (EC 1.11.1.1) alone, or in combination, were produced and tested for tolerance against mild water stress. The results showed that the simultaneous overexpression of Cu/Zn*sod* and *apx* or at least *apx* in the cytosol of transgenic tobacco plants alleviates, to some extent, the damage produced by water stress conditions. This was correlated with higher water use efficiency and better photosynthetic rates. In general, oxidative stress parameters, such as lipid peroxidation, electrolyte leakage, and H_2O_2 levels, were higher in non-transformed plants than in transgenic lines, suggesting that, at the least, overexpression of cyt*apx* protects tobacco membranes from water stress. In these conditions, the activity of other antioxidant enzymes was induced in transgenic lines at the subcellular level. Moreover, an increase in the activity of some antioxidant enzymes was also observed in the chloroplast of transgenic plants overexpressing cyt*sod* and/or cyt*apx*. These results suggest the positive influence of cytosolic antioxidant metabolism on the chloroplast and underline the complexity of the regulation network of plant antioxidant defences during drought stress.

Key words: Cytosolic ascorbate peroxidase, cytosolic superoxide dismutase, tobacco, water stress.

Introduction

Plants often face the challenge of severe abiotic and biotic stresses in their environment, such as drought, salinity, high light, and pathogen attacks. Most of these stresses exert adverse effects on plant growth and development by inducing many metabolic changes, such as the occurrence of an oxidative stress (Hernández *et al.*, 1993; 2004*a*, *b*; Morán *et al.*, 1994; Karpisnki *et al.*, 1999). Water deficit and salinity, especially under high light intensity or in combination with other stresses, disrupt photosynthesis and increase photorespiration, altering the normal homeostasis of cells and causing an increased production of reactive oxygen species (ROS) such as superoxide ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals (·OH), which are some of the most damaging molecules in plants, capable of inducing changes at a cellular level, leading to membrane damage, protein oxidation, and DNA alteration (Halliwell and Gutteridge, 2000). Even under normal conditions, plants often produce ROS during metabolic processes, via the Mehler reaction in the chloroplasts, electron transport in the mitochondria and peroxisomes, and photorespiration

in the peroxisomes (Asada, 1992; Hernández *et al.*, 1993; del Río *et al.*, 1998).

Water deficit caused by drought or high soil salinity is the most serious environmental stress that limits agricultural production in many regions of the world (Alscher *et al.*, 1997). Numerous studies have clearly shown that water deficit mainly affects photosynthetic CO_2 assimilation and therefore sets a limit to plant growth and productivity (Flexas *et al.*, 2002; Galle *et al.*, 2009). Stomatal closure induced by water deficit has been shown to act as the initial and most prominent limitation to CO_2 assimilation, as diffusion of CO_2 from the atmosphere to the sites of carboxylation in the chloroplast is impaired (Galle *et al.*, 2009). This limitation in the availability of CO_2 favours generation of $O_2^{\cdot-}$ in the chloroplasts (Mehler reaction) (Asada, 1999) as well as activation of glycolate oxidase generating H_2O_2 (Mittler and Zilinskas, 1994). In addition, combination of water stress with high light intensity increases generation of ROS and accelerates the process of senescence development (d'Oro and Trippi, 1987). Also, it has been found that water stress can damage the mechanisms controlling the iron uptake in roots, causing an increase in chloroplastic iron concentration. Superoxide is overproduced in chloroplasts under drought conditions; therefore, drought stress can promote the generation of iron-catalysed ·OH formation which can contribute to the damage produced in water-stressed plants (Price and Hendry, 1991).

To cope with the toxicity of ROS, plants have developed efficient antioxidative mechanisms, involving either the partial suppression of ROS production or the scavenging of ROS which have already been produced. Different non-enzymatic (ascorbate, glutathione, α-tocopherol, and carotenoid) and enzymatic defences, including superoxide dismutase (SOD; EC 1.15.1.1), the ascorbate–glutathione (ASC–GSH) cycle enzymes, catalase (CAT; EC 1.11.1.6), and peroxidases (POX; EC 1.11.1.7), are involved in the scavenging of ROS in plant cells (Noctor and Foyer, 1998; Asada, 1999). SODs are metalloenzymes located in various cell compartments that catalyse the disproportionation of $O_2^{\cdot-}$ to O_2 and H_2O_2 (Fridovich, 1975). There are essentially three types of SODs containing either Mn, Fe, or Cu plus Zn as prosthetic metals (Fridovich, 1975). Ascorbate peroxidases (APXs; EC 1.11.1.1), the main enzymes of the ASC–GSH cycle, also have multiple locations and are among the most important key enzymes that scavenge potentially harmful H_2O_2 from the chloroplasts and cytosol of plant cells (Noctor and Foyer, 1998; Asada, 1999). It is important to control H_2O_2 levels because it can diffuse readily from cell organelles to the cytosol, inducing an oxidative stress in this compartment (del Rio *et al.*, 1998). In addition, chloroplasts are especially sensitive to damage by ROS because electrons that escape from the photosynthetic electron transfer chain are able to react with relatively high concentrations of O_2 in this organelle (Foyer *et al.*, 1994*a*), thus lowering the rate of photosynthesis and affecting the growth of the plant. Although chloroplasts possess a battery of antioxidant defences, this cell compartment is very sensitive to extrachloroplastidic ROS (Asada, 1992). Such high extrachloroplastidic ROS may result from cytosolic SOD activity or proceed from the cell compartment by diffusion, since mitochondrial and peroxisomal membranes are permeable to H_2O_2 (Corpas *et al.*, 1993; Hernandez *et al.*, 1993; del Rio *et al.*, 1998). Hence, the cytosolic antioxidant system is very important in the response to oxidative stress induced by different abiotic and biotic disorders (Mittler and Zilinskas, 1994; Alscher *et al.*, 1997; Hernandez *et al.*, 2000, 2004*a*).

To improve tolerance against oxidative stress and finally to maintain the productivity of plants under these environmental stress conditions, several groups attempted to fortify the antioxidative mechanisms using genetic engineering. Transformation of plants with a single transgene, encoding *sod* or *apx*, has been carried out by several groups with contrasting results. Studies that addressed the overproduction of Cu/ZnSOD (Perl *et al.*, 1993; Sen Gupta *et al.*, 1993), of MnSOD (Foyer *et al.*, 1994*a*; Van Camp *et al.*, 1994; Slooten *et al.*, 1995; McKersie *et al.*, 1996, 2000), or of FeSOD (Van Camp *et al.*, 1996) in the chloroplasts found enhanced tolerance to oxidative stress. In addition, transgenic plants expressing gene constructs for either cytosolic or a chloroplast-targeted cytosolic *apx* have increased tolerance against various abiotic stresses including water stress (Allen *et al.*, 1997; Badawi *et al.*, 2004*b*; Wang *et al.*, 2005). However, many other reports indicated no improvements to oxidative or environmental stress (Pitcher *et al.*, 1991; Payton *et al.*, 1997; Torsethaugen *et al.*, 1997). These divergences have usually been attributed to the complexity of the scavenging pathway, because modification of one enzyme may not affect the pathway as a whole.

As an attempt to overcome this problem, the possibility of pyramiding or stacking genes has emerged (for a review, see Hapelin, 2005). This consists of the co-ordinate manipulation of two or more desirable transgenes of a pathway in the plant. Although this concept is currently very difficult to achieve, several approaches including retransformation, co-transformation, or the use of linked transgenes have been developed (Hapelin *et al.*, 2001). Regarding the antioxidative enzymes, only a few groups tried to use this approach (Aono *et al.*, 1995; Kwon *et al.*, 2002; Rubio *et al.*, 2002; Samis *et al.*, 2002). Results from their studies indicated little to no improvement when pyramiding transgenes encoding two isoenzymes of the same family (Rubio *et al.*, 2002), whereas co-expression of two distinct enzymes allowed a significant enhancement in stress tolerance. Payton *et al.* (2001) showed that co-expression of glutathione reductase (GR) and APX in cotton improved the recovery of photosynthesis, following exposure to low temperature and high light. Kwon *et al.* (2002) obtained transgenic tobacco plants expressing both SOD and APX in the chloroplast with enhanced tolerance to the herbicide methyl viologen (MV). Taken together, all these data indicate that the combination of transgenes encoding ROS-scavenging enzymes could have a synergistic effect on stress tolerance and could be more appropriate to enhance stress tolerance than the use of a single transgene. However,

most authors that used the co-expression methodology have overexpressed the transgenes in the chloroplast and never in the cytosol.

Different authors described the importance of the cytosolic antioxidant systems in response to oxidative stress induced by different environmental stresses (Mittler and Zilinskas, 1994; Alscher *et al.*, 1997; Hernández *et al.*, 2000). For this reason, this study was aimed to test whether overexpression of cytosolic *sod* or cytosolic *apx*, alone or in combination, could enhance the tolerance of tobacco to drought stress and if the expression of these transgenes could affect the antioxidant metabolism in the soluble and chloroplastic fractions. To accomplish this goal, transgenic tobacco overexpressing each or both cytosolic transgenes have been generated and tolerance of the plants to a mild water stress has been evaluated by analysing the antioxidant metabolism in the soluble and chloroplastic fractions. The effect of the trangenes on the photosynthesis rate and on chlorophyll fluorescence parameters was also studied. The results showed that the overexpression of at least cytosolic *apx* protects tobacco plants from water stress and affects the antioxidant metabolism in the cytosol as well as in the chloroplast.

Materials and methods

Plant material, plasmid constructions, and transformation

The *Agrobacterium tumefaciens* strain EHA105, carrying the binary vector pBIN+ARS (SOD) or pCGN1578 (APX), was used for inoculation of leaf disc explants. The cytosolic Cu/Zn *sod* (cyt*sod*) from *Spinacia oleracea* (GenBank accession no. X53872) or cytosolic *apx1* (cyt*apx*) cDNA from *Pisum sativum* (GenBank accession no. X62077) was placed in the enhanced portion of the plasmid pRTL2, removed by *Hin*dIII digestion, and cloned in the *Hin*dIII site of either pBIN+ARS or pCGN1578. The resulting constructions also harboured the neomycin phosphotransferase (*npt*II) gene for aminoglycoside selection in their T-DNA (Fig. 1).

The T-DNA region was introduced into tobacco (*Nicotiana tabacum* cv. *Xanthi*) leaf discs according to Horsh *et al.* (1985) using the co-transformation method. Briefly, two strains of *A. tumefaciens* harbouring pBIN+ARS (SOD) or pCGN1578 (APX) were grown in LB medium (OD_{600}=0.5), mixed, and used for inoculation. After 2 d, co-culture discs were cultured on selective MS medium containing 150 mg l^{-1} kanamycin, 300 mg l^{-1} cefotaxime, and 200 mg l^{-1} vancomycin. Regenerated shoots were transferred to the same medium supplemented with 3% (w/v) sucrose and 0.7% (w/v) agar. Kanamycin was reduced to 75 mg l^{-1} for rooting. Rooted plants were transferred to pots with peat substrate and acclimatized to *ex vitro* conditions in the greenhouse by progressively decreasing the relative humidity.

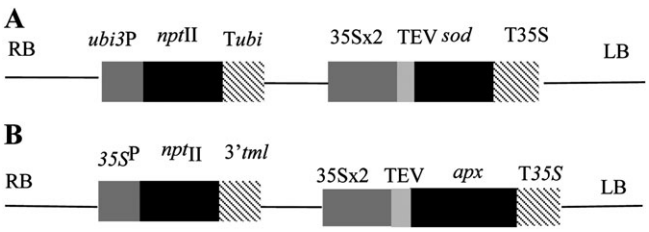


Fig. 1. Schematic representation of T-DNA from (A) pBIN+ARS (SOD) and (B) pCGN1578 (APX) constructs. RB, right border of T-DNA; LB, left border; *35Sx2*, duplicated *CaMV35S* promoter; *T35S*, terminator 35S; *ubi*3P/T, potato ubiquitin-3-promoter/terminator; 3'*tml*, 3' tumour morphology large untranslated region from *Agrobacterium tumefaciens*; *npt*II, neomycin phosphotransferase II; TEV, enhanced 35S; *sod*, cytosolic superoxide dismutase; *apx*, cytosolic ascorbate peroxidase.

PCR and Southern blot analysis of transgenic plants

Genomic DNA was isolated from 50 mg of young tobacco leaves according to the procedure of Doyle and Doyle (1990). The genomic DNA was first subjected to PCR with specific primers for the *npt*II gene (forward 5′-GATTGAACAAGATGGATTGC-3′ and reverse 5′-CCAAGCTCTTCAGCAATATC-3′), followed by duplex PCR using specific primers for the cyt*sod* gene (forward 5′-AAAGGCTGTGGTTGTTCTAA-3′ and reverse 5′-GTCTTGCTGAGTTCATGTCC), and specific primers for the cyt*apx* gene (forward 5′-CTGCTGGTACTTTTGATTCC-3′ and reverse 5′-GAGAGCTTAAGATGTCTTCA-3′). In both simplex and duplex PCR, amplification was performed using the following conditions: 95 °C for 5 min, 35 cycles of 95 °C for 45 s, 54 °C for 45 s, and 72 °C for 1 min, and one cycle of final extension at 72 °C for 10 min. Finally, PCR products (696 bp for *npt*II, 484 bp for cyt*sod*, and 601 bp for cyt*apx*) were separated on a 1.2% agarose gel and visualized by ethidium bromide staining.

For Southern analysis, 10 μg of genomic *Bam*HI-digested DNA samples were separated on 1% (w/v) agarose gels and transferred to positively charged nylon membranes by capillary blotting. The PCR *npt*II, cyt*sod*, and cyt*apx* fragments, amplified using the primers described above, were labelled with digoxigenin (DIG) using the PCR DIG Probe Synthesis Kit (Roche GmbH, Mannheim, Germany). Pre-hybridization and hybridization of filters to labelled probes were performed at 42 °C. Blots were then washed twice at 23 °C in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% (w/v) SDS for 15 min, and twice at 65 °C in 0.5× SSC, 0.1% SDS for 15 min. Hybridizing bands were visualized with anti-DIG antibody–alkaline phosphatase and CDP-Star (Roche) on X-ray films.

Gene expression analysis by qRT-PCR of transgenic plants

Young leaves of transgenic tobacco lines as well as of non-transformed lines were snap-frozen in liquid nitrogen and stored at –80 °C until use. RNA was extracted from each set using an RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The RNA samples were digested with DNase I by using the DNA-free Kit (Ambion, Austin, TX, USA) and quantified using a spectrophotometer Nanodrop ND-1000 (Nanodrop Technologies, Wilmington, NC, USA).

The cDNA was synthesized using a RETROscript cDNA Synthesis Kit (Ambion). Reverse transcription was carried out at 42 °C for 1 h in a 20 μl reaction mixture containing 500 ng of total RNA, 1× reverse transcription buffer, 500 μM of each dNTP, 5 μM of oligo(dT) primers, 0.5 U of RNase inhibitor, and 5 U of Moloney murine leukaemia virus (MMLV) reverse transcriptase.

The expression levels of cyt*sod* and cyt*apx* transgenes and a *β-actin* gene, used as a reference, were determined by real-time RT-PCR using the GeneAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA, USA) in different lines. The cDNA was first synthesized as described above and PCR was carried out on cDNA in triplicate in 96-well plates using the SYBR Green Master Kit (Applied Biosystems).

Primers for the *β-actin* (forward 5′-CTGGCATTGCAGATCG-TATGA-3′ and reverse 5′-GCGCCACCACCTTGATCTT-3′), cyt*sod* (forward 5′-CTTTGCCCAGGAAGGAGATG-3′ and

reverse 5′-TGTGTCACCAAGGGCATGAA), and cyt*apx* genes (forward 5′-GCATGGCACTCTGCTGGTACT-3′ and reverse 5′-CGTTGTTAGCACCATGAGCAA-3′) were designed using the sequence detection system software to amplify an amplicon of 100 bp for cyt*sod* and cyt*apx* and 75 bp for β-actin. Each set of primers was mixed at a final concentration of 300 nM with 2 µl of cDNA and 1× SYBR Green. After denaturation at 95 °C for 10 min, amplification occurred in a two-step procedure: 15 s of denaturation and 1 min of annealing and extension at 60 °C for 40 cycles. These conditions were used for both target and reference genes, and the absence of primer dimers was checked in controls lacking templates. Transcript levels were calculated using relative standard curves for both target and reference genes, which were made after running serial dilutions of specifically purified cDNA of the two genes as described in the user bulletin 2, ABIPRISM 7700. The fold increase of expression levels of each gene was calculated after normalization by the following formula: fold gene expression=(quantity of gene in transgenic line/quantity of reference gene in transgenic line)/(quantity of target gene in non-transformed control/quantity of reference gene in non-transformed control).

Water stress assays

Three-week-old transgenic tobacco lines and non-transformed controls grown in a greenhouse were deprived of irrigation during 3 d (mild stress) or 5 d (severe stress). For control, plants were irrigated with 50 ml of water every day for the duration of the experiment. Symptoms, consisting of collapsed leaves, were photographed at the end of the experiment.

Gas exchange measurements

Gas exchange measurements [maximum net rate of photosynthesis (A_N) and stomatal conductance (g_s)] were taken with a portable photosynthesis system (LI-6400, LICOR Inc., Lincoln, NE, USA) equipped with a LI-6400-40 Leaf Chamber Fluorometer (LI-6400, LICOR Inc.) and a LICOR 6400-01 CO_2 injector. Sixteen measurements per plant of each transgenic line and for the two irrigated conditions were taken late morning (11:00–12:30 h) of the third youngest, fully expanded leaves, which were placed in a 2 cm^2 leaf cuvette under a saturating light of 1500 µmol m^{-2} s^{-1} (provided by the light source of the LI-6400 with 10% blue light). The CO_2 concentration in the LI-6400 leaf chamber (C_a) was set to 400 mol^{-1} air, the leaf chamber temperature was set to 25 °C, and the relative humidity of the incoming air ranged between 40% and 60%. Intrinsic water use efficiency (WUE) was calculated as a result of the A_N/g_s balance registered.

Preliminary experiments for gas exchange measurements were carried out in all lines subjected to severe deficit irrigation (5 d of withholding water), whereas, for the selected lines, data were also recorded after mild water stress (3 d of withholding water).

Measurement of chlorophyll fluorescence

The fluorescence of chlorophyll was measured with a chlorophyll fluorometer (IMAGIM-PAM M-series, Heinz Walz, Effeltrich, Germany) in detached leaves from well-irrigated and water-stressed (3 d) tobacco lines. After dark incubation of plants (5 min), the minimum and the maximal fluorescence yields were monitored. Kinetic analyses were carried out with actinic light (81 µmol quanta m^{-2} s^{-1} PAR) and repeated pulses of saturating light at 2700 µmol quanta m^{-2} s^{-1} PAR for 0.8 s at intervals of 20 s. The effective photosystem II quantum yield [Y(II)], the non-photochemical quenching (NPQ), and the coefficients of non-photochemical quenching (qN) and photochemical quenching (qP) were analysed.

Enzyme extraction and assays of transgenic plants

All operations were performed at 4 °C. For the rest of the biochemical analyses, well-irrigated (controls) and water-stressed plants (3 d of withholding water) were used. Soluble fractions were obtained from the third youngest, fully expanded tobacco leaves (10 g) by differential centrifugation according to published protocols (Hernández *et al.*, 2000; Diaz-Vivancos *et al.*, 2008). For APX activity, 20 mM ascorbate was added. The resulting supernatant was partially purified in Sephadex G-25 NAP columns (GE Healthcare, Madrid, Spain) equilibrated with 50 mM K-phosphate buffer pH 7.0 (with or without 2 mM ascorbate), and was considered as the soluble fraction for use. Chloroplasts were isolated by differential and density gradient centrifugation (Hernández *et al.*, 2004*a*). A resuspension medium containing 20% (v/v) Percoll (GE Healthcare, Madrid, Spain) was layered under the chloroplast suspension by slowly pipetting 10 ml into the bottom of the tube. Tubes were centrifuged at 1700 *g* for 1 min. The pellet of intact chloroplasts was resuspended in 1 ml of washing medium, without bovine serum albumin (BSA), and used for enzyme assays. Chloroplasts were lysed by incubation (v/v) with 10 mM K-phosphate buffer, pH 7.0, containing 0.2% (v/v) Triton X-100, for 1 h. After incubation, the lysed chloroplast preparations were centrifuged at 100 000 *g* for 15 min (Optima Max ultracentrifuge, Beckman, USA), and the supernatants obtained were partially purified in Sephadex G-25 NAP columns (GE Healthcare, Madrid, Spain) equilibrated with 50 mM K-phosphate buffer pH 7.0 (with or without 2 mM ascorbate).

The activities of the ASC–GSH cycle enzymes, POX, CAT, glutathione *S*-transferase (GST), and SOD were assayed as described in Diaz-Vivancos *et al.* (2006, 2008). The glutathione and ascorbate contents were measured according to Zhang and Kirkham (1996) and Foyer *et al.* (1983), respectively. Protein was estimated according to Bradford (1976). All measurements were carried out at least in quadruplicate.

Electrophoretic analysis

To detect APX, non-denaturing PAGE was performed on 10% acrylamide gels, using a Bio-Rad Mini-protean III dual slab cell. A 80 µg aliquot of protein per line was used in native gels. Staining of APX activity was performed as described by Mittler and Zilinskas (1993). Gels were stained in the presence and absence of the specific APX inhibitor *p*-hydroxy-mercury benzoate (pHMB) (0.5 mM final concentration).

Statistical analysis

The effects of the overexpression of cyt*sod* and/or cyt*apx* on the antioxidative metabolism and in the gas exchange measurements in non-transformed and transgenic lines under well-irrigated and deficit irrigation conditions were tested by a two-way analysis of variance (ANOVA). Within each irrigation treatment, lines were compared with non-transformed controls by a Dunnett's test. Additionally, comparisons between transgenic lines, within each treatment, were carried out by using a Tukey's test. Statistical procedures were done using the software package SAS.

Results

Identification of transgenic lines

Transgenic tobacco plants overexpressing cyt*sod* from spinach and/or cyt*apx* from pea were produced using the co-transformation method with the binary vectors pBIN+ARS (SOD) and pCGN1578 (APX). Fifty-six independent transgenic lines cultivated *in vitro* in a kanamycin-containing medium were evaluated by PCR for the presence

of the *npt*II, cyt*sod*, and cyt*apx* genes (data not shown). Out of 56 transformed tobacco lines (*npt*II+), 38.6% were co-transformed with both cyt*sod* and cyt*apx*, 46% contained only cyt*sod*, while only 5.3% contained the cyt*apx* transgene alone, as revealed by duplex PCR (data not shown). These *in vitro* transgenic lines were subjected to molecular (Southern blot and transgenes expression) and biochemical (enzymatic activities) analyses. These preliminary analyses allowed the selection of 15 transgenic lines, with apparently normal vigour and growth, representing each possible transgene combination and a different number of integrations.

In general, Southern blot analysis of transgenic lines using *npt*II, cyt*sod*, and cyt*apx* as probes (Fig. 2) showed a pattern of T-DNA integration ranging from one to five copies.

Expression of cty*sod* and cyt*apx* was confirmed by qRT-PCR and SOD/APX activities (Table 1). The results showed different transcriptional levels of cyt*sod* and cyt*apx* in the 15 transgenic lines compared with the non-transformed control.

All transformed lines, regardless of the integrated transgene, showed higher SOD activity than the non-transformed control (Table 1). On the other hand, lines 11, 16, 17, 27, 39, 43, 45, and 51 showed higher APX activity. Interestingly, some of the plant lines harbouring only the cyt*sod* transgene also had a high APX activity (Table 1). This was the case for lines 11, 17, 27, 43, and 45. Taken together, these results indicate that cyt*sod* and cyt*apx* are constitutively and functionally expressed in the transgenic tobacco lines. Transgenic plants in the greenhouse did not show any apparent phenotype difference compared with non-transformed control plants (data not shown). Subsequently, transgenic lines were acclimatized in the greenhouse under controlled conditions and used for further investigations.

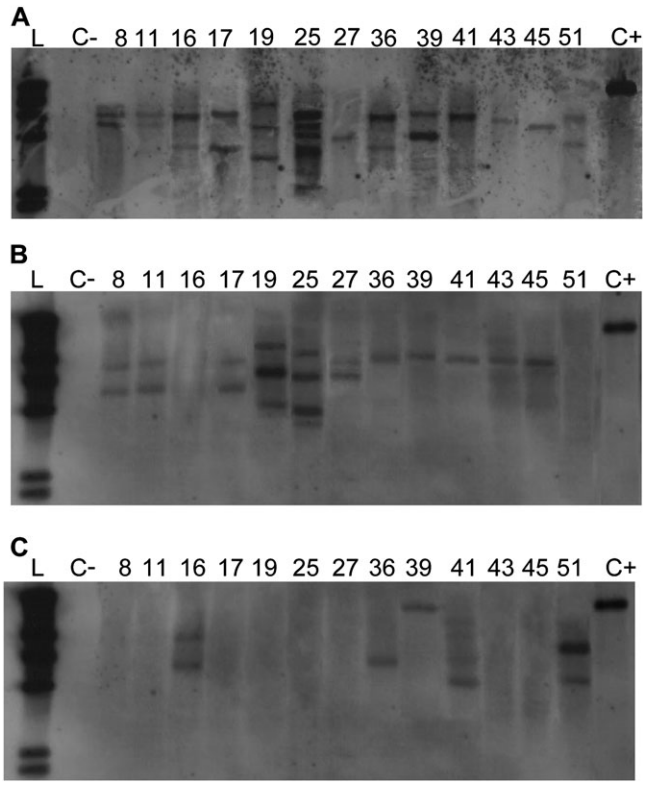


Fig. 2. Southern blot analysis of transgenic tobacco lines, using (A) *npt*II, (B) *sod*, or (C) *apx* probes. A 10 μg aliquot of DNA was digested using *Bam*HI, separated by agarose gel electrophoresis, and subjected to Southern blot analysis. L, λ *Hin*dIII DIG-labelled molecular marker; C–, non-transformed control; C+, positive control [plasmid pBIN+ARS (SOD) or pCGN1578 (APX)]. Numbers indicare different transgenic lines.

*Drought tolerance in transgenic tobacco overexpressing cyt*sod *and/or cyt*apx

To test whether overexpression of cyt*sod* and/or cyt*apx* in tobacco could enhance drought tolerance, the non-transformed control and the 15 transgenic lines were deprived of irrigation for 3 d. There were no visual differences between the non-transformed control and the transgenic lines under control conditions (normal irrigation). However, under water stress, the non-transformed control displayed collapsed leaves while the transgenic lines were less affected. Transgenic lines overexpressing *sod* (8, 11, 17, 19, 25, 27, 30, 43, 44, and 45) or *apx* (16 and 51) alone showed intermediate symptoms. Lines overexpressing both *sod* and *apx* (36, 39, and 41) exhibited the best behaviour (no visible symptoms were observed), regarding drought tolerance. This is illustrated for line 39 in Fig. 3.

Gas exchange measurements

The 15 transgenic lines were also subjected to preliminary analyses for their A_N and g_s under more severe water shortage (5 d of withholding water). Transgenic lines overexpressing both cyt*sod* and cyt*apx* (36, 39, and 41) and one transgenic line overexpressing cyt*apx* alone (51) have higher A_N than the non-transformed control. Stomatal conductance was also remarkably higher in these four lines (data not shown).

Based on these initial data and data of expression, enzymatic activities, and the number of copies, for the remainder of the experiment, lines 17 (with two copies of cyt*sod*), 51 (with two copies of *cyt*apx), and 39 (with a copy of each transgene) were selected, as well as the non-transformed control, and a mild water stress treatment was applied (plants were deprived of irrigation for 3 d) (Table 2). Line and irrigation treatments as well as the interaction between the two had a significant effect on A_N, g_s, and WUE (P <0.001). With respect to gas exchange measurements, the A_N values were significantly increased in the transgenic lines 39 and 51 under irrigated conditions, being 2-fold higher than in the controls and line 17, respectively. The highest g_s values were observed in line 51, and the WUE was significantly lower in lines 17 and 51 and significantly higher in line 39, in relation to non-transformed plants. Under deficit irrigation, the highest A_N values were found in line 51; g_s values were significantly lower in line 39, and the highest values of g_s were in line 51.

Table 1. Characterization of in vitro transgenic lines produced after co-transformation with cytsod and cytapx

In vitro ines were analysed for transgene copy number, mRNA expression (determined by qRT-PCR after 40 cycles), and enzymatic activities. SOD activity is expressed as U mg^{-1} prot, whereas APX is expressed in nmol min^{-1} g^{-1} FW. Data are means ±SE (95%). For determination of mRNA levels, three replicates were used, whereas for SOD and APX activities, six replicates were used.

Line	SOD copy	SOD mRNA accumulation (RQ) (CT_{SOD}/CT_{actin})	SOD activity	APX copy	APX mRNA accumulation (RQ) (CT_{APX}/CT_{actin})	APX activity
Control	0	1.03±0.3 (37.6/21.8)	31.8±1.5	0	1.01±0.17 (30.4/21.7)	1208±33
8	3	$2.74\times10^5\pm5.44\times10^4$ (19.8/22.1)	ND	0	1.3±0.3 (30.5/22.2)	ND
11	2	$2.15\times10^4\pm2.48\times10^3$ (24.1/22.7)	47.3±0.4	0	1.01±0.17 (30.8/22.1)	2072±72
16	0	91±23 (31/21.6)	42.2±2.3	2	$5.7\times10^4\pm2.1\times10^3$ (17.7/21.4)	1609±8
17	2	$4.5\times10^5\pm5.3\times10^4$ (17.7/20.6)	52.3±0.9	0	98±73 (23.0/20.5)	2264±46
19	3	$1.53\times10^6\pm1.5\times10^5$ (18.4/23.1)	54.1±1.6	0	1.5±0.4 (30.1/22.5)	ND
25	4	183±17 (29.7/21.5)	46.4±2.4	0	1.0±0.1 (30.3/21.7)	1330±89
27	2	$8.36\times10^5\pm1.53\times10^5$ (17.7/21.5)	50.5±2.2	0	2.1±1.1 (28.2/20.5)	1937±34
30	4	$2.78\times10^5\pm4.92\times10^4$ (18.1/20.4)	49.6±1.2	0	0.9±0.79 (29.6/20.3)	1169
36	1	$2.39\times10^4\pm2.27\times10^3$ (25.4/24.1)	ND	4	$7.66\times10^2\pm20$ (23.1/24.0)	ND
39	1	$1.10\times10^4\pm2.85\times10^2$ (24.4/21.9)	54.2±1.8	1	$10^4\pm2.3\times10^3$ (16.9/21.4)	1441±82
41	1	$2.39\times10^3\pm2.86\times10^2$ (24.9/20.3)	39.4±1.3	1	3±0.4 (27.3/20.2)	932±4
43	1	$2.87\times10^5\pm3.95\times10^3$ (18.0/20.3)	54.8±1.0	0	2.2±0.1 (29.8/22.5)	1555±58
44	3	$1.55\times10^5\pm4.50\times10^4$ (21.5/22.9)	41.5±0.8	0	0.37±0.19 (32,2/22.9)	1156±23
45	1	$2.66\times10^5\pm2.95\times10^4$ (18.2/20.4)	51.8±1.5	0	1.2±0.1 (30.8/22.2)	1542±40
51	0	$1.06\times10^3\pm95$ (31.2/25.4)	41.0±1.5	2	$4.05\times10^3\pm5.1\times10^2$ (20.2/23.4)	2530±8

RQ, relative quantification. CT transgene/CT actin values are indicted within parentheses, ND, not determined.

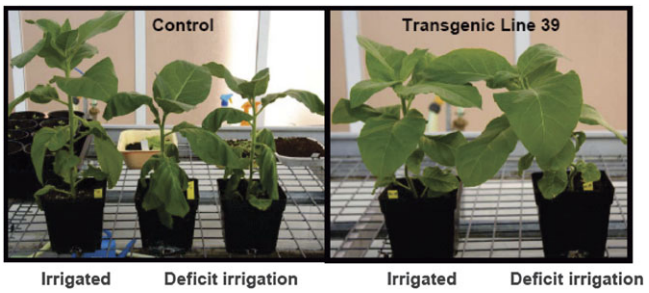


Fig. 3. Drought tolerance in non-transformed tobacco (left) and in line 39 harbouring both *sod* and *apx* transgenes. Photos were taken after 3 d of water shortage.

The WUE was significantly increased in lines 39 and 51, with the highest value found for line 39 (68.2 μmol CO_2 mol^{-1} H_2O) (Table 2).

When plotting the average A_N against leaf mass area (LMA), a highly significant relationship was obtained (R^2=0.75, P <0.01) pooling data together from irrigated and water deficit lines (control and lines 17, 39, and 51). The maximum values of LMA occurred for lines 51 and 39 under irrigated (I) and deficit irrigation (DI) conditions (Fig. 4). Line and irrigation treatments as well as the interaction between the two had a significant effect on A_N and LMA (P <0.001).

Table 2. Net photosynthesis (A_N, μmol CO_2 m^{-2} s^{-1}), stomatal conductance (g_s, mmol H_2O m^{-2} s^{-1}), and water use efficiency (WUE, μmol CO_2 mol^{-1} H_2O) in leaves from the different tobacco genotypes (control, line 17, line 39, and line 51) under irrigated and deficit irrigation conditions (3 d of withholding water)

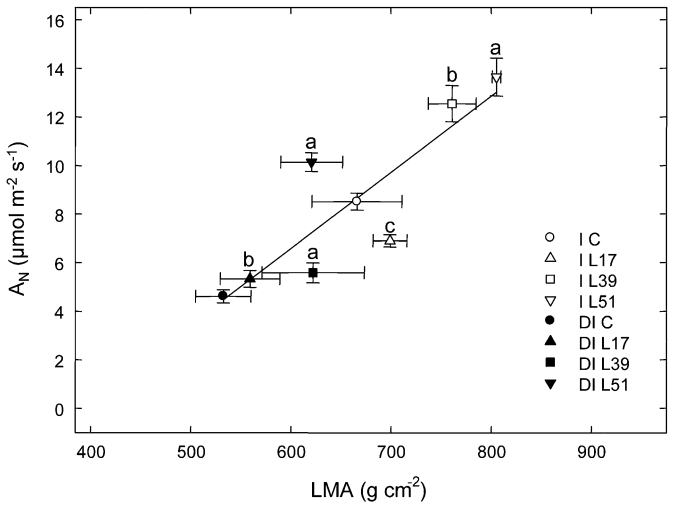
Data represent the mean ±SE from 16 measurements taken from four plants. Lines with an asterisk are statistically different from the non-transformed controls, within each irrigation treatment, according to a Dunnet's test (P <0.05). Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment, according to a Tukey's test.

Treatment	Line	A_N	g_s	WUE
Irrigated	Control	8.5±0.6	138.5±11.3	61.8±7.6
	17	6.9±0.5*c	131.1±22.3 c	53.8±7.8*b
	39	12.5± 1.3*b	177.0±22.5*b	70.7±4.6*a
	51	13.6±1.2*a	245.3± 30.7*a	55.5± 2.4*b
Deficit irrigation				
	Control	4.6±0.2	108.1±11.4	43.2±2.5
	17	5.3±0.4*b	108.5±10.0 c	49.4±1.8*c
	39	5.6±0.5*b	82.2±10.9*b	68.2±5.5*a
	51	10.1±1.0*a	168.8±15.6*a	60.0±1.8*b
Source of variation			[a]*F*-values	
Line (A)		341.6***	166.2***	91.7***
Irrigation treatment (B)		761.9***	301.7***	37.5***
A×B		60.0***	29.6***	31.8***

[a] F-values from two-way ANOVA for A_N, g_s, and WUE. *F*-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability.

Chlorophyll fluorescence measurements

Different chlorophyll fluorescence parameters were recorded in tobacco plants under both irrigated and water deficit conditions. Line and irrigation conditions as well as the interaction between the two had a significant effect on qP, NPQ, and Y(II) parameters, whereas qN was only affected by the line studied (P <0.05), and not by the treatment. However, a line×irrigation treatment interaction was observed (Table 3). Data showed that under well-irrigated conditions, lines 39 and 51 showed the highest



Source of variation	A_N	LMA
Line (A)	341.58***	61.23***
Irrigation Treatment (B)	761.92***	484.83***
AxB	60.03***	3.08***

Fig. 4. Relationship between maximum net photosynthesis (A_N) and leaf mass area (LMA) in the irrigated (I, open symbols) and deficit irrigation (DI, filled symbols) (3 d of withholding water) tobacco lines (C, circles; line 17, triangles; line 39, squares; line 51, inverted triangles). Line indicates the linear regression of data: A_N=0.03 LMA–2.25 (r^2=0.75**). Each point is the average of 16 measurements. Horizontal and vertical bars on points are ±SE of the mean (significance levels: **P <0.01). Data represent the mean ±SE from 16 measurements taken from four plants. Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment, according to a Tukey's test.

qP and Y(II) values. Moderate water stress produced changes in some fluorescence parameters. Non-transformed plants showed higher values of qN and NPQ than transformed lines under both conditions (well-irrigated and deficit irrigation). However, under stress conditions, NPQ and qN increased or did not show important variations in tobacco plants, whereas only in non-transformed plants did water stress induce a decrease in Y(II). Finally, analyses also revealed that under water stress, qP values were statistically higher in transgenic lines than in non-transformed plants, especially in line 39 (Table 3).

Oxidative stress parameters

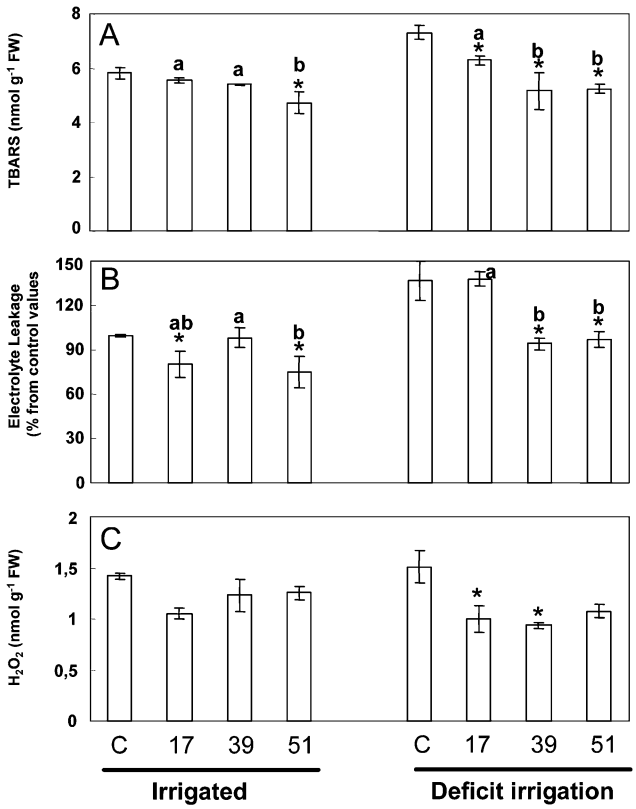
Using the three selected transgenic lines (17, 51, and 39), the antioxidant metabolism in the soluble fraction as well as in chloroplasts was analysed. In order to check the possible protection offered by overexpression of cyt*sod* and/or cyt*apx* under deficit irrigation conditions, different oxidative stress parameters were analysed (Fig. 5). Line and irrigation treatments, as well as the interaction between the two, had a significant effect on lipid peroxidation [measured as thiobarbituric acid-reactive substances (TBARS)] and electrolyte leakage (P <0.001), whereas H_2O_2 contents were only affected by the line studied (P <0.01). Under irrigated conditions, line 51 showed statistically lower TBARS contents than the other studied lines (Fig. 5A). Deficit irrigation only increased lipid peroxidation in non-transformed plants, indicative of membrane damage (Fig. 5A, see also the ANOVA table at the bottom of Fig. 5). Under these conditions, transformed plants showed lower TBARS values than non-transformed plants, the values being statistically lower in lines 39 and 51 than in line 17 (Fig. 5A). This observation indicated that transformed plants displayed better membrane protection than non-transformed plants. In addition, water shortage increased electrolyte leakage in non-transformed plants as well as in line 17

Table 3. Effect of deficit irrigation (3 d of withholding water) on some fluorescence parameters in leaves from different tobacco lines

Data represent the mean ±SE from at least 20 measurements taken from five plants. Lines with an asterisk are statistically different from the non-transformed controls, within each irrigation treatment, according to a Dunnett's test (P <0.05). Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment according to a Tukey's test.

Treatment	Line	qN	qP	NPQ	Y(II)
Irrigated	Control	0.666±0.009	0.753± 0.023	0.284±0.018	0.506±0.009
	17	0.609±0.010*	0.790±0.017 b	0.225±0.013*	0.519±0.005 b
	39	0.600±0.007*	0.839±0.005*a	0.228±0.004*	0.546±0.006*a
	51	0.622±0.006*	0.835±0.011*a	0.248±0.006	0.532±0.003*a,b
Deficit irrigation					
	Control	0.678±0.009	0.693±0.010	0.342±0.008	0.406±0.009
	17	0.648±0.006*a	0.789±0.007*b	0.279±0.007*a	0.507±0.006*b
	39	0.588±0.007*c	0.830±0.006*a	0.231±0.007*b	0.541±0.006*a
	51	0.616±0.008*b	0.759±0.010*c	0.264±0.008*a	0.506±0.010*b
Source of variation			[a]*F*-values		
Line (A)		29.42***	32.10***	25.27***	41.60***
Irrigation treatment (B)		1.78 (ns)	19.59***	22.00***	39.25***
A×B		3.45*	4.90**	3.62*	14.97***

[a] *F*-values from two-way ANOVA for soluble antioxidant enzymes. *F*-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by 'ns'.



Source of variation	Lipid Peroxidation	Electrolyte Leakage	H_2O_2 contents
Line (A)	20.87***	18.79***	6.54**
Irrigation Treatment (B)	80.22***	73.35***	2.44 (ns)
AxB	17.09***	15.32***	1.25 (ns)

Fig. 5. Effect of deficit irrigation (3 d of withholding water) on the oxidative stress parameters lipid peroxidation (measured as TBARS) (A), electrolyte leakage (B), and H_2O_2 contents (C) in leaves from control (C) and transformed tobacco plants (corresponding numbers). I, well irrigated; DI, deficit irrigation. Data represent the mean ±SE from at least four measurements. Lines with an asterisk are statistically different from the non-transformed controls, within each irrigation treatment, according to a Dunnett's test (P <0.05). Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment, according to the Tukey's test. Table: [a]F-values from two-way ANOVA oxidative stress parameters. F-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by 'ns'.

(overexpressing cyt*sod*, Fig. 5B). However, lines 51 (overexpressing cyt*apx*) and 39 (which overexpressed both transgenes) exhibited a significantly lower electrolyte leakage, which correlated with their lower lipid peroxidation. Finally, under well-irrigated conditions, differences between lines were not observed in the H_2O_2 levels. However, under deficit irrigation, transgenic lines accumulated less H_2O_2 than control plants, although differences from the control were only significant for lines 17 and 39. Significant differences between transgenic lines were not observed (Fig. 5C).

Antioxidant defences

The effect of deficit irrigation in antioxidative metabolism was studied in both transformed and non-transformed tobacco lines at subcellular levels. In soluble fractions, line and irrigation treatments, as well as the interaction between both factors, had a significant effect on APX, monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), and GR activities (P <0.001; except the interaction for GR, P <0.01). However, the other antioxidant activities in soluble fractions, such as GST, POX, CAT, and SOD, were only affected by the lines studied (P <0.001, Table 4).

Under well-irrigated conditions, all transformed lines exhibited significantly higher APX activity than non-transformed controls. In the double transformant (line 39), and in line 51, the soluble APX activity was up to 30% and 50% higher, respectively, than in non-transformed plants (Table 4). Also, these lines (39 and 51) presented a soluble APX activity statistically higher than that of line 17 (Table 4).

Under well-irrigated conditions, soluble CAT values were also much higher in transformed than in non-transformed lines (Table 4). In lines 17 and 39, CAT activity was nearly 90% higher than in the controls, whereas line 51 presented a CAT activity of up to 60% greater than that of the non-transformed plants (Table 4).

Lines 17 and 39 showed higher SOD and POX values than non-transformed plants and line 51. In addition, line 17 showed the highest MDHAR and GST activity values, and line 39 the highest DHAR activity values (Table 4). However, a significant reduction in GR activity was noticed in lines 39 and 51 (both overexpressing cyt*apx*) (Table 4). Under well-irrigated conditions, the two lines harbouring the cyt*sod* gene (lines 17 and 39) displayed the highest SOD activity in soluble fractions, being 2- and 1.6-fold higher than in non-transformed plants, respectively. Line 51 (overexpressing only cyt*apx*) presented a similar SOD activity to that of non-transformed plants (Table 4).

Under conditions of deficit irrigation, an increase in APX from soluble fractions was observed in the transgenic lines in relation to non-transformed plants (Table 4), the soluble APX activity from transgenic lines 39 and 51 being >2-fold the levels shown by non-transformed plants (2.3- and 2.4-fold higher, respectively). These APX values were statistically higher than those presented by line 17. In addition, deficit irrigation increased soluble APX in lines 39 and 51 in relation to their respective well-irrigated controls (by 38% and 25%, respectively) (Table 4). Under the same conditions, lines 17 and 39 exhibited higher DHAR, POX, CAT, and SOD levels than non-transformed plants. However, line 39 presented lower GR activity and line 51 lower GR and GST activities than control plants, respectively (Table 4).

The overexpression of cyt*sod* and cyt*apx* also produced changes in the antioxidative metabolism of chloroplasts. Line and treatment, as well as their interaction, had a significant effect on chloroplastic APX and MDHAR.

The other chloroplastic enzymes analysed, DHAR, GR, POX, and SOD, were affected by the line studied as well as by their interaction with the irrigation treatments. However, no effect of the treatment was observed in these cases.

Under well-irrigated conditions, increased APX, POX, and SOD and reduced MDHAR and GR activities were observed in the chloroplast from line 17 (Table 5). Line 39 showed increased chloroplastic (chl)APX and chlPOX but a drop in chlMDHAR, chlDHAR, and chlGR activities. Finally, line 51 also exhibited an increase in chlPOX activity but a decrease in chlMDHAR, chlDHAR, chlGR, and chlSOD activities. In this case, although changes were not significant, a 1.88-fold increase in chlAPX was also observed (Table 5).

Under conditions of deficit irrigation, transgenic lines also exhibited higher chlAPX activity values than non-transformed plants. In this case, line 51 showed a chlAPX activity 2.65-fold higher than non-transformed plants. The increases observed in lines 17 and 39 were lower, reaching 68% and 41% higher than in non-transformed

Table 4. Effect of deficit irrigation (3 d of withholding water) on the antioxidant enzymes from the soluble fraction of control and transformed tobacco plants

Data represent the mean ±SE from at least four measurements. APX, MDHAR, DHAR, GR, and GST are expressed as nmol min^{-1} mg^{-1} prot. CAT and POX are expressed as μmol min^{-1} mg^{-1} prot, whereas SOD is expressed as U mg^{-1} prot. Lines with an asterisk are statistically different from the non-transformed controls, within each irrigation treatment, according to a Dunnet's test (P <0.05). Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment, according to a Tukey's test.

Treatment	Line	APX	MDHAR	DHAR	GR	GST	POX	CAT	SOD
Irrigated	Control	589.9±18.0	28.6±0.7	116.7±14.5	20.5±1.4	15.7±0.3	58.43±0.9	176.2±0.9	73.4±0.8
	17	733.8±19.5*b	45.8±4.0*a	150.6±0.3b	23.1±1.0 a	22.2±1.5*a	128.9±21.0*a	334.1±30.2*	147.2±2.2*a
	39	765.1±5.6*a	30.7±4.2 b	210.9±1.2*a	11.3±0.6*b	18.2±0.1 a	135.6±4.6*a	325.1±1.6*	116.6±4.6*b
	51	875.6±21.5*a	33.2±2.7 a,b	100.7±0.2 c	14.5±0.3*b	12.1±0.4 b	76.7±0.1 b	274.2±8.1*	71.0±9.8 c
Deficit irrigation									
	Control	453.0±33.8	31.4±2.6	110.7±3.2	17.8±0.5	17.2±1.3	63.8±2.9	221.4±22.6	78.0±6.2
	17	860.1±31.9*b	38.8±3.2 a	177.4±0.2*a	17.4±0.4 a	22.0±0.3*a	119.1±2.4*b	368.7±3.5*a	118.2±4.5*a
	39	1062.7±30.2*a	31.7±2.8 a,b	144.0±0.2*b	10.6±0.5*b	18.8±0.8 b	170.8±3.1*a	306.0±6.7*b	127.7±5.1*a
	51	1097.3±41.4*a	28.2±0.5 b	61.2±1.9*c	13.7±0.5*c	9.4±0.4*c	65.9±0.5 c	176.5±7.7 c	69.5±2.8 b
Source of variation					[a]F-values				
Line (A)		131.56***	74.02***	74.02***	70.98***	52.68***	31.22***	32.34***	81.14***
Irrigation treatment (B)		45.32***	16.42***	16.42***	21.25***	0.12 (ns)	0.41 (ns)	0.53 (ns)	1.09 (ns)
A×B		28.36***	15.94***	15.94***	4.97**	1.65 (ns)	2.04 (ns)	6.58**	6.34**

[a] F-values from two-way ANOVA for soluble antioxidant enzymes. F-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by 'ns'.

Table 5. Effect of deficit irrigation (3 d of withholding water) on the antioxidant enzymes from chloroplastic fractions of control and transformed tobacco plants

Data represent the mean ±SE from at least four measurements. APX, MDHAR, DHAR, and GR are expressed as nmol min^{-1} mg^{-1} prot. POX is expressed as μmol min^{-1} mg^{-1} prot, and SOD is expressed as U mg^{-1} prot. Lines with an asterisk are statistically different from the non-transformed controls, within each irrigation treatment, according to a Dunnett's test (P <0.05). Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment, according to a Tukey's test.

Treatment	Line	APX	MDHAR	DHAR	GR	POX	SOD
Irrigated	Control	40.2±0.8	3.9±0.2	49.4±0.2	7.5±0.4	3.5±0.1	83.8±0.9
	17	90.6±12.7*	2.9±0.1*a	49.3±1.8 a	5.8±0.3*a	13.0±1.9*	91.2±2.6*a
	39	85.9±3.5*	3.6±0.3*b	43.0±0.9*b	2.6±0.3*c	9.7±0.2*	86.4±1.2 a
	51	75.7±2.45	2.9±0.1*a	38.8±0.2*b	3.2±0.1*b	8.3±0.1*	50.4±1.0*b
Deficit irrigation							
	Control	36.5±1.2	3.2±0.3	46.1±2.3	5.9±0.8	6.0±1.3	79.5±3.3
	17	61.4±3.6*b	3.2±0.2 a	43.5±1.1	3.6±0.1*	7.1±0.2 c	69.8±0.1 b
	39	51.6±4.8*b	2.4±0.1*b	46.9±1.2	3.3±0.2*	9.2±0.1*a	101.1±0.1 a
	51	96.5±2.3*a	2.5±0.1b	43.3±0.1	3.1±0.1*	8.2±0.2 b	83.5±9.2 a
Source of variation					[a]F values		
Line (A)		14.98***	12.87***	8.40**	42.04***	8.62**	12.79***
Irrigation treatment (B)		5.32*	25.55***	0.03 (ns)	10.09 (ns)	1.71 (ns)	3.57 (ns)
A×B		6.70**	11.90***	7.28**	7.72**	5.61*	16.43***

[a] F-values from two-way ANOVA for chloroplastic antioxidant enzymes. F-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by 'ns'.

plants, respectively (Table 5). However, under the same conditions, line 39 showed an increase in chlSOD, although changes were not statistically significant (Table 5). Under water stress, line 39 showed statistically higher chlPOX and chlSOD values than line 17, whereas line 51 displayed higher chlAPX, chlPOX, and chlSOD than line 17.

By native-PAGE only one band with APX activity was observed, mainly in lines 39 and 51 (Fig. 6), whose activity was inhibited by pHMB, a specific inhibitor of APX activity (data not shown). The APX band in non-transformed plants and in line 17 was barely observed. The increase in APX activity observed by kinetic analysis in lines 39 and 51 was also perceived in gels (Fig. 6).

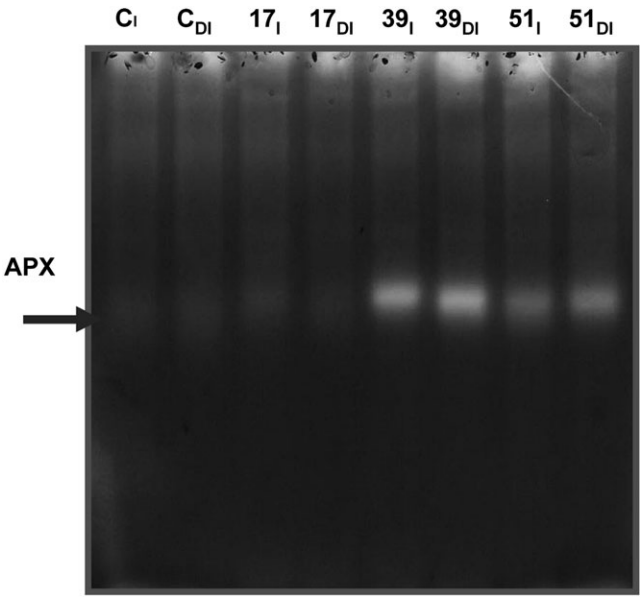


Fig. 6. Effect of deficit irrigation (3 d of withholding water) on total APX as revealed on a native acrylamide gel of non-transformed and transgenic tobacco lines. I, well irrigated; DI, deficit irrigation.

The effect of the overexpression of cyt*sod* and/or cyt*apx* on the ascorbate and glutathione contents in tobacco leaves was also analysed. Line and treatment, as well as their interaction, had a significant effect on reduced ascorbate (ASC) (P <0.05) and on reduced and oxidized glutathione (GSH and GSSG) (P <0.001; except GSH for line source, P < 0.05). For oxidized ascorbate (DHA), although a significant effect was produced by line and treatments (P <0.01), no interaction was recorded (Table 6). The overexpression of cyt*sod* increased ASC contents (line 17). No significant changes were observed in lines 39 and 51 (Table 6). In the last case, DHA was not detected and, accordingly, those plants presented the highest reduced state of ascorbate (Table 6). Under deficit irrigation, ASC contents showed statistical changes in relation to well-irrigated plants. Line 51 (overexpressing cyt*apx*) experienced an increase in ASC as well as in DHA and a decrease in the redox state of ascorbate (Table 6). GSH content was higher in lines 17 and 39 than in controls and line 51, but no changes were monitored in the GSSG content (Table 6). Under conditions of deficit irrigation, line 51 displayed increased GSH levels as well as ASC contents, whereas, in all the transformed lines, an accumulation of GSSG was observed. Accordingly, a decrease in the redox state of glutathione was monitored in lines 17 and 39 (Table 6).

Table 6. Effect of deficit irrigation (3 d of withholding water) on the levels of ascorbate and glutathione in leaves from different tobacco lines

Data represent the mean ±SE from at least four measurements. ASC and DHA are expressed as nmol g^{-1} FW. GSG and GSSG are expressed as μmol g^{-1} FW. Lines with an asterisk are statistically different from the non-transformed controls, within each irrigation treatment, according to a Dunnett's test (P <0.05). Different letters indicate significant differences (P <0.05) between transgenic lines, within each treatment according to a Tukey's test.

Treatment	Line	ASC	DHA	ASC/ASC +DHA	GSH	GSSG	GSH/GSH +GSSG
Irrigated	Control	35.7±0.5	1.48±0.35	0.960	57.8±2.5	6.34±0.40	0.901
	17	60.4±2.5*a	2.01±0.23	0.967	80.2±2.9*a	6.69±0.33	0.923
	39	40.73±3.6b	2.64±0.7	0.939	82.2±0.5*a	7.08±0.54	0.920
	51	25.3±1.7 c	ND	1	62.8±1.1 b	6.23±0.22	0.909
Deficit irrigation							
	Control	44.6±2.5	3.18±0.40	0.933	81.2±2.9	4.64±0.21	0.945
	17	50.7±5.5	4,37±0.12	0.920	78.6±2.8 b	11.56±0.42*a	0.872
	39	38,6±4.8	5.15±0.12	0.891	72.14±6.3 b	7.75±0.51*b	0.902
	51	59.4±9.3*	8.03±1.86*	0.880	119.5±7.4*a	8.01±0.83*b	0.937
Source of variation					[a]*F* values		
Line (A)		33.1*	5.58**		3.88*	22.54***	
Irrigation treatment (B)		5.21*	15.32**		16.17***	18.13***	
A×B		6.90*	0.31		11.77***	17.41***	

ND, not detected.
[a] *F*-values from two-way ANOVA for non-enzymatic antioxidant. *F*-values significant at 99.9% (***), 99% (**), or 95% (*) levels of probability. Non-significant values are indicated by 'ns'.

Discussion

Cytosolic antioxidant defences and response to stress

The importance of the cytosolic antioxidant system in the response to oxidative stress induced by different abiotic and biotic stress situations has been described (Mittler and Zilinskas, 1994; Alscher *et al.*, 1997; Hernández *et al.*, 2000, 2004*a*). Several studies show that environmental stresses, such as salinity, drought, or paraquat and SO_2 exposure, which initially affect the chloroplasts, also induce changes in the cytosolic antioxidant system (Mittler and Zilinskas, 1994; Alscher *et al.*, 1997; Hernández *et al.*, 2000). However, to our knowledge, there are no reports that changes in the cytosolic antioxidant defence may affect the chloroplast.

In order to determine the role of cytCu/ZnSOD and cytAPX enzymes in response to abiotic stress, and to see how the cytosolic antioxidant system interacts with the chloroplast, *in vitro* transgenic tobacco plants expressing the cyt*sod* and cyt*apx* genes in the cytosol under the control of the *CaMV35S* promoter were generated. High levels of cyt*sod* and cyt*apx* gene transcripts as well of their respective activities suggested that the transgenes were constitutively and functionally expressed. Interestingly, some of the plant lines harbouring only the cyt*sod* transgene also had high APX activity. This was the case for lines 11, 17, 27, 43, and 45. This result is not surprising since the reaction product of the SOD activity (H_2O_2) is the substrate for APX activity. A possible role for H_2O_2 in the signalling for APX induction in plants has been described by different authors (Karpinski *et al.*, 1999; Morita *et al.*, 1999; Yoshimura *et al.*, 2000; Hernandez *et al.*, 2004*b*). Recently, it was shown that pre-treatments of pea seeds with 20 mM H_2O_2 induced cytosolic and stromal APX and increased total APX activity in pea seedlings (Barba-Espin *et al.*, 2010). On the other hand, Sen Gupta *et al.* (1993) reported a 3-fold increase in APX activity and mRNA in transgenic tobacco plants that overexpressed chloroplastic Cu/Zn*sod*. The observation that APX activity can increase in SOD transformants suggests that control of antioxidative metabolism is complex, possibly involving post-transcriptional regulation and co-regulation.

The co-expression of two different antioxidant enzymes has also been used by other authors to increase tolerance against oxidative stress conditions. Aono *et al.* (1995) showed that co-expression of *gr* and Cu/Zn*sod* in the cytosol of transgenic tobacco plants increased tolerance to MV exposure. However, most authors who have used the co-expression methodology have overexpressed the transgenes in the chloroplast. Moreover, and to our knowledge, there are no data dealing with the effect of combination of cytosolic *sod* and cytosolic *apx* on drought tolerance. For example, Kwon *et al.* (2002) described how the overexpression of Cu/Zn*sod* or Mn*sod* along with *apx* in the chloroplast led to increased protection against MV. The overexpression of Cu/Zn*sod* and *apx* in tobacco chloroplast improved the photosynthetic performance of the plant during photo-oxidative stresses such as high salinity, drought, and polyethylene glycol treatments when compared with non-transformed plants (Badawi *et al.*, 2004*a*, *b*). These results indicated that expression of two transgenes encoding antioxidant enzymes in transgenic plants could have synergistic effects on stress tolerance (Kwon *et al.*, 2002). The present results showed that the overexpression of at least *apx* in the cytosol of transgenic tobacco plants alleviates the damage produced by water stress conditions. In addition, overexpression of Cu/Zn*sod* and/or *apx* in the cytosol induces an increase in the activity of some antioxidant enzymes in the chloroplast.

Effect of water stress on antioxidative metabolism

Under conditions of deficit irrigation, an oxidative stress was produced in non-transformed tobacco plants, observed in the increased lipid peroxidation data. Cell membranes are the primary site of injury caused by drought, and ROS are the main mediators of peroxidation damage to various cellular components (Zhang and Kirkham, 1996). In control plants, the increased lipid peroxidation values were accompanied by increased leaf electrolyte leakage, indicating damage to membranes. A similar increase in lipid peroxidation and relative electrolyte leakage was described in two different *Populus* populations subjected to drought stress (Xiao *et al.*, 2009). In addition, under water shortage conditions, non-transformed plants accumulated more H_2O_2 in leaves than line 17 (overexpressing cyt*sod*) and line 39 (harbouring both genes), and this response correlated with the increase in antioxidant enzymes as well as the higher constitutive levels of POX and CAT and the maintenance of other antioxidant enzymes such as MDHAR and DHAR in the soluble fraction and chloroplast. Under water stress, although no statistical differences were observed, line 51 also accumulated 30% less H_2O_2 than non-transformed controls. Excessive H_2O_2 accumulation is one of the mechanisms by which plants are damaged under environmental stress, including salt and drought stress (Hernández *et al.*, 1993; Selote *et al.*, 2006). Drought-acclimated wheat seedlings were able to limit H_2O_2 accumulation and membrane damage as compared with non-acclimated plants during severe water stress conditions. This response was correlated with the up-regulation of APX activity and by maintaining ASC–GSH redox pools in acclimated plants (Selote *et al.*, 2006).

To obtain more information on different functions of cyt*sod* and cyt*apx* and their relationship with antioxidative systems and in all cell compartments, analysis was also expanded to the other ASC–GSH cycle enzymes (MDHAR, DHAR, and GR). POX and CAT (other H_2O_2-eliminating enzymes) were also determined. Line 17, expressing cyt*sod,* showed the highest MDHAR activity in soluble fractions, and these data correlated with their ASC contents. Similarly, under conditions of deficit irrigation, lines 39 and 51 showed the lowest GR activity but, in contrast, line 51 exhibited the highest GSH contents, although an accumulation of GSSG also occurred. In this case, an increase in

GSH synthesis or transport to the leaves cannot be ruled out.

Line 39 showed the highest DHAR activity in soluble fractions, but a correlation with ASC contents was not observed. However, in line 51, DHA accumulated under conditions of water irrigation, which correlated with a low DHAR activity as well as an increased APX activity. The overexpression of DHAR in the cytosol of transgenic tobacco plants provides a significantly enhanced tolerance to oxidative stress imposed by drought and salt stresses (Eltayeb *et al.*, 2006). The net photosynthesis rate of DHAR-overexpressing plants was significantly higher than that of the control plants under drought stress. This response was attributed to the higher ASC levels in these transgenic plants (Eltayeb *et al.*, 2006).

The overexpression of cyt*apx* and/or cyt*sod* produced an increase in the activity of other antioxidant enzymes, such as POX and CAT, in soluble fractions as well as in some chloroplastic enzymes, such as APX and POX. Under stress conditions, a higher POX and CAT activity was noticed in lines 17 and 39, and an increase in POX and SOD activities was also observed in the chloroplastic fraction from line 39. In pea plants, CAT activity increased with drought stress. This increase seems to be involved in the removal of H_2O_2 that is produced in peroxisomes during photorespiration (Mittler and Zilinskas, 1994). During drought, an increase in photorespiration has been described that can increase H_2O_2 production in the peroxisome due to the increased activity of glycolate oxidase (Zelitch, 1973; Mittler and Zilinskas, 1994). H_2O_2 could also diffuse through the peroxisomal membrane into the cytosol (Del Río *et al.*, 1998), thus increasing the risk of oxidative damage in this compartment. However, lines 17 and 39 showed higher CAT levels, under both control and water shortage conditions, which could control H_2O_2 levels in the peroxisome during the water stress period and prevent its accumulation in the cytosol. In addition, in the three transgenic lines, higher levels of soluble APX were observed, which could contribute to controlling H_2O_2 levels in the cytosol under water shortage conditions, in the case of H_2O_2 leakage from other cell compartments to the cytosol. Moreover, under water stress conditions, transgenic lines showed higher chlAPX and chlPOX activities than the controls, and line 39 presented higher chlSOD and chlPOX activities, and line 51 higher chlAPX and chlSOD activities than line 17. The increase in the activity of chloroplastic antioxidant enzymes can provide protection against water stress-induced oxidative stress in the chloroplasts. Our results agrees with data presented by Selote *et al.* (2006), which showed that the exposure of drought-acclimated wheat plants to severe water stress resulted in an increase in APX, CAT, and POX activities. This response was not observed in non-acclimated plants, which correlated with higher membrane damage (Selote *et al.*, 2006).

Effect of water stress on photosynthesis

In this work, the up-regulation of different antioxidant enzymes in transformed tobacco plants was reflected in increased WUE and in maximum net photosynthesis (A_N) by LMA in lines 39 and 51 under water shortage conditions. This can be linked to better protection of the chloroplast, allowing it to cope with the ROS generation that can take place in the chloroplastic electron chain under water stress conditions (Tambussi *et al.*, 2000; Selote *et al.*, 2006). Transgenic lines showed significantly higher qP and Y(II) values than non-transformed plants under moderate water stress. Moreover, transgenic lines maintained or even increased the qN and NPQ parameter. The maintenance of or increase in qN values in stress situations has been associated with a protective response in order to avoid photoinhibitory damage to the reaction centres (Rahoutei *et al.*, 2000). It has been observed that a loss in qP is accompanied by an increase in the lifetime of the exciton in PSII, which can increase the probability of chlorophyll triplet formation and the associated formation of singlet oxygen ($^1O_2$) (Foyer *et al.*, 1994*b*). Under the same conditions, Y(II) only decreased in non-transformed plants. This parameter represents the proportion of the light absorbed by chlorophyll associated with PSII that is used for photochemistry (Maxwell and Johnson, 2000). These results correlated with the higher photosynthetic rate data obtained in transgenic lines, in relation to non-transformed plants, under deficit irrigation conditions, especially in line 51. The co-expression of Cu/Zn*sod* and *apx* (line 39) produces relatively small effects on photosynthesis in comparison with plants overexpressing *apx* (line 51).

ChlAPX and chlSOD take part in the so-called water–water cycle (Asada, 1999). The water–water cycle acts in the protection of its scavenging enzymes (chlAPX and chlSOD are inhibited by H_2O_2); the stromal enzymes; the PSI complex from oxidative damage by the $O_2^{\cdot -}$ photoproduced in PSI; and other ROS derived from $O_2^{\cdot -}$. Other physiological functions include reinforcement of CO_2 assimilation (by protection of the Calvin cycle enzymes that can be inhibited by H_2O_2), and the dissipation of excess photon energy (Asada, 1999). These results agree with those of different authors showing that the overexpression of *apx* and/or *sod* protects photosynthesis under different stress situations, including drought, salinity, or MV exposure. In all cases, although the stress situation decreased net photosynthesis, values were always much higher in the transformed plants than in non-transformed plants, indicating some degree of acclimation in the transformed plants (Kwon *et al.*, 2002; Badawi *et al.*, 2004*a*; Eltayeb *et al.*, 2006).

Globally, the data showed that overexpression of both cyt*sod* and cyt*apx*, or at least of cyt*apx*, enhances, to some extent, tolerance to mild water stress. The best behaviour of line 39, expressing both cyt*sod* and cyt*apx*, and line 51, expressing cyt*apx*, under deficit irrigation, is correlated with an increase in APX activity in soluble and chloroplastic fractions as well as high constitutive POX activity in soluble fractions and maintenance of this activity in chloroplasts. In addition, line 39 showed high DHAR and CAT activities in soluble fractions, and SOD and POX in both compartments. Moreover, line 51 showed the highest ASC and GSH levels in leaves under deficit irrigation, which can also

account for its good behaviour in relation to A_N. In this sense, it is important to note that ASC is involved in photoprotection and the regulation of photosynthesis and that GSH has a role in the recycling of ASC (Noctor and Foyer, 1998).

In conclusion, the data showed that co-expression of cytosolic antioxidant genes (*sod* and *apx*) has only minor effects during drought, similar to those exhibited when expressing cytosolic APX alone. However, a partial protection of photosynthesis and membrane integrity was observed. This was monitored by better A_N, lipid peroxidation, and electrolyte leakage data in these plants (overexpressing *sod* and *apx* or at least *apx*) under moderate drought stress than in non-transformed plants. In addition, these results outlined the importance of the cytosolic antioxidant machinery in the protection of the chloroplasts and the complexity of regulation of antioxidant defences during drought stress.

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