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Ectopic expression of cytosolic superoxide dismutase and ascorbate peroxidase leads to salt stress tolerance in transgenic plums

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Summary

To fortify the antioxidant capacity of plum plants, genes encoding cytosolic antioxidants ascorbate peroxidase (cyt*apx*) and Cu/Zn-superoxide dismutase (cyt*sod*) were genetically engineered in these plants. Transgenic plum plants expressing the cyt*sod* and/or cyt*apx* genes in cytosol have been generated under the control of the *CaMV35S* promoter. High levels of cyt*sod* and cyt*apx* gene transcripts suggested that the transgenes were constitutively and functionally expressed. We examined the potential functions of cytSOD and cytAPX in *in vitro* plum plants against salt stress (100 mM NaCl). Several transgenic plantlets expressing cyt*sod* and/or cyt*apx* showed an enhanced tolerance to salt stress, mainly lines C5-5 and J8-1 (expressing several copies of *sod* and *apx*, respectively). Transformation as well as NaCl treatments influenced the antioxidative metabolism of plum plantlets, including enzymatic and nonenzymatic antioxidants. Transgenic plantlets exhibited higher contents of nonenzymatic antioxidants glutathione and ascorbate than nontransformed control, which correlated with lower accumulation of hydrogen peroxide. Overall, our results suggest that transformation of plum plants with genes encoding antioxidant enzymes enhances the tolerance to salinity.

Introduction

Plants are continuously exposed to abiotic stresses, which negatively affect their growth and yield, thereby causing enormous losses in agriculture worldwide. These stressors include drought, salinity and extreme temperatures and lead to the accumulation of excess of reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot-}$), hydroxyl radicals ($^{\cdot}OH$) and hydrogen peroxide (H_2O_2). In this sense, salt stress (NaCl) is known to affect a multitude of physiological and biochemical processes. Although some of the documented changes in salt-stressed plants are adaptative, many may simply be pathological consequences of stress injury (Zhu, 2002). There are two main negative effects of salt stress that influence plant growth and development: water deficit and toxicity associated with excessive Cl^- and Na^+ uptake, leading to Ca^{2+} and K^+ deficiency and to other nutrient imbalances (Marschner, 1995). In addition to osmotic stress and ionic toxicity, salt stress is also manifested as an oxidative stress at subcellular level, mediated by ROS, contributing these three factors to its deleterious effects (Hernández *et al.*, 2001, 2003; López-Gómez *et al.*, 2007).

To cope with the toxicity of ROS, plants have developed efficient antioxidative mechanisms that allow the adaptation to different types of stresses imposed by adverse environments. These mechanisms include nonenzymatic scavengers and low molecular weight compounds, such as ascorbic acid (ASC), glutathione (GSH) and phenols. The mechanisms also involve the enzymatic arsenal of ROS scavengers including enzymes such as superoxide dismutase (SOD), catalase (CAT) and peroxidase (POX) and the ascorbate–glutathione cycle enzymes [ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDHAR) and glutathione reductase (GR)] (Asada, 1999; Noctor and Foyer, 1998). SOD and APX play key roles in ROS detoxification. SODs are metalloenzymes located in various cell compartments that catalyse conversion of O_2^- into oxygen and H_2O_2. There are essentially three types of SODs containing either Mn, Fe or Cu plus Zn as prosthetic metals (Fridovich, 1975). APX, the main enzyme of the ASC–GSH cycle, has multiple locations and is among the most important key enzymes that scavenge potentially harmful H_2O_2 (APX and CuZn-SOD are inhibited by H_2O_2) from different compartments of plant cells such as apoplast (Diaz-Vivancos *et al.*, 2006), chloroplasts and cytosol (Asada, 1999; Noctor and Foyer, 1998). Because ROS, mainly H_2O_2, can diffuse readily from cell organelles to the cytosol, cytSOD and cytAPX play an important role in ROS homoeostasis regulating its levels to avoid an oxidative stress. Hence, the cytosolic antioxidant system is very important in response to oxidative stress induced by different abiotic challenges such as salinity and drought (Mittler and Zilinskas, 1994; Hernández *et al.*, 2000; Faize *et al.*, 2011).

Alleviation of oxidative damage using genes encoding antioxidant enzymes such as SOD or APX has been widely used in the development of transgenic plants, and their effect on stress tolerance has been well documented mainly in herbaceous plants such as potato (Perl *et al.*, 1993), tomato (Wang *et al.*, 2005), tobacco (Badawi *et al.*, 2004a,b; Sarowar *et al.*, 2005; Sen Gupta *et al.*, 1993), cotton (Payton *et al.*, 2001), alfalfa (McKersie *et al.*, 2000) and sugar beet (Tertivanidis *et al.*, 2004). The overexpression of antioxidant enzymes has been used before to increase tolerance to salinity (Badawi *et al.*, 2004a,b; Roxas *et al.*, 1997; Zhao and Zhang, 2006). However, to our knowledge, there is no information regarding the effect of these genes in woody plants

or in fruit trees. In addition, most of the antioxidant genes used are from chloroplast or peroxisome (Li *et al.*, 2009), while it became evident that cytosolic antioxidant defence machinery is more appropriate (Alscher *et al.*, 1997; Faize *et al.*, 2011; Hernández *et al.*, 2000; Mittler and Zilinskas, 1994). For instance, cytosolic APX is involved in the protection of the chloroplast under light stress conditions (Pnueli *et al.*, 2003). Overexpression of cytosolic APX in tomato enhanced salt and chilling stress (Wang *et al.*, 2005). In tobacco, overexpression of cytosolic APX alone or in combination with cytosolic SOD resulted in drought stress tolerance by enhancing the antioxidant defence in the cytosol and the chloroplast (Faize *et al.*, 2011). All these data highlight the importance of the cytosolic antioxidant system in the response to oxidative stress induced by abiotic or biotic situations (Mittler and Zilinskas, 1994; Alscher *et al.*, 1997; Hernández *et al.*, 2000; Faize *et al.*, 2011, 2012).

As we described above, most of the studies about the effect of salt stress have been carried out in crop species. However, only scarce results concerning woody plants exist in the literature. The *Prunus* genus includes several species producing edible drupes of great economic importance in terms of fresh fruit, dry fruit and nut consumption (Hummer and Janick, 2009). Stone fruit trees are classified as relatively sensitive to salinity (Bernstein, 1975), being plum rootstocks more tolerant than peach rootstocks (El-Motaium *et al.*, 1994). Rootstock can greatly affect the scion's tolerance to salt or drought stress. For this reason, the use of *Prunus* rootstocks tolerant to salinity could improve fruit production in arid and semi-arid regions (El-Motaium *et al.*, 1994). In this sense, the use of transgenic plants as a rootstock would constitute a future useful tool to improve crop yield of woody plants under the frequently adverse and changing environment.

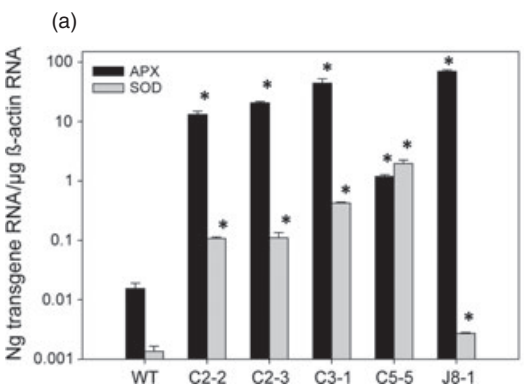
The aim of this work was to investigate the effect of the ectopic expression of these two antioxidant cytosolic defences in the protection of *in vitro* plum plants against salt stress. To achieve this goal, we analysed the effect of NaCl treatments on shoot growth and antioxidative metabolism (antioxidants enzymes, ascorbate and glutathione contents, H_2O_2 levels, lipid peroxidation), in transgenic and wild-type *in vitro* plants.

Results

Recovery of transgenic lines harbouring cyt*sod* and cyt*apx* transgenes

Transgenic plum plants overexpressing cyt*sod* from spinach and/or cyt*apx* from pea were produced cotransforming hypocotyl slices (Petri *et al.*, 2008) with the binary vectors pBin+ARS (SOD) and pCGN1586 (APX). Transformation rate was very high and averaged 39% for seed explants of the plum cv. Claudia Verde. Our transgenic plum lines harbouring cyt*sod* and/or cyt*apx* did not show morphological abnormalities. During the molecular evaluation of the transgenic lines (Figure 1), one copy of *cytapx* was also detected in the nontransformed control corresponding with the endogenous *apx* gene (Figure 1b), probably due to the high homology between the endogenous APX and the APX that we used for transformation. However, in the case of cyt*sod* from spinach, the sequence has low homology with the endogenous gene and was not detected in nontransformed plants (Figure 1b). Based on Southern blot analyses, two transgenic lines harbouring both transgenes (C2-2 and C3-1), one line overexpressing only cyt*sod* (C5-5) and two lines harbouring cyt*apx* (C2-3 and J8-1) were selected for further studies. Expression of cyt*sod* and cyt*apx* was confirmed by qRT-PCR analysis (Figure 1a).

Results showed different transcriptional levels of cyt*sod* and cyt*apx* in the analysed transgenic lines compared with the nontransformed plants ($P < 0.01$ in both cases). All the transgenic lines, including line C5-5 harbouring only cyt*sod*, showed significantly higher *apx* transcript levels than nontransformed plants ($P < 0.001$), line J8-1 (harbouring four copies of cyt*apx*) displaying the highest *apx* transcript levels (Figure 1a). Lines harbouring cyt*sod* showed significantly higher *sod* transcript levels when compared with nontransformed plants (Figure 1a). These significantly higher *sod* transcript levels were also found in lines C2-3 and J8-1 despite the absence of integration of cyt*sod* transgene (Figure 1). We do not know the exact mechanism of this response; probably, a retroregulation mechanism exerted by *apx* could be involved. Because APX works in conjunction with other antioxidants enzymes to scavenge ROS, it is possible that



(b)

Line	SOD	APX	NPTII
WT	0	(1)	0
C2-2	2	1 (1)	3-4
C2-3	0	2 (1)	3
C3-1	2	1 (1)	3-4
C5-5	3	(1)	4
J8-1	0	4 (1)	4

Figure 1 (a) Transcripts abundance (data in a logarithmic scale represent the mean ± SE of at least five RNA extractions) and (b) Southern blot analysis of transgenic plum lines. Genomic DNA from transgenic plants was digested and blotted with probes for three transgenes, *cytsod*, *cytapx* and *npt*II. The *npt*II transgene is a selection marker whose expression confers resistance to aminoglycoside antibiotics and was common to both constructions. Numbers represent the bands detected in each transgenic line after the hybridizations with probes for each transgene and are related to the number of insertions of the transgene. An additional APX (number between parentheses) copy was detected by Southern blot probably due to the high homology between the endogenous APX and the APX that was used for transformation. WT, nontransformed plants. Asterisks represent significant differences ($P < 0.001$) between each transgenic line and the wild-type control for SOD and APX transgenes according to a Dunnett's test.

the overexpression of cyt*apx* may also induce the expression and/or activity of other antioxidants enzymes (Wang *et al.*, 2005; Faize *et al.*, 2011).

Effect of cytsod and cytapx transgenes on growth and salt stress tolerance

The effect of salt stress on transgenic shoots was initially assessed by visual evaluation of *in vitro* growing shoots. In the presence of 50 mM NaCl, transgenic lines as well as nontransformed plants showed only moderated stress symptoms (data not shown). However, severe and extremely severe symptoms of stress were produced in nontransformed plants when cultured in media supplemented with 100 and 150 mM NaCl, respectively (Figure 2). Significant differences in the score of salinity injury were found between these two treatments ($P < 0.05$) and between different lines ($P < 0.001$).

When transgenic lines were compared with the wild-type nontransgenic control, within each NaCl treatment, all transgenic lines had significantly lower score of salinity injury in the 100 mM treatment ($P < 0.05$), whereas only C5-5 and J8-1 had significantly lower scores ($P < 0.05$) than the wild-type control in the 150 mM treatment (Figure 2a). Nontransgenic control showed yellowing and necrosis of the midrib (Figure 2c) at 100 mM NaCl, but most of the transformed plants remained unaffected as illustrated in the picture of line J8-1 (Figure 2d,e).

In the presence of 200 mM NaCl, all transgenic and wild-type plantlets were severely affected (data not shown). These results indicated that 100 mM NaCl allowed a better comparison between transgenic lines and nontransformed controls, and this concentration was used for further analysis.

The effect of 100 mM of NaCl on growth, measured as shoot length and weight, was studied. Although transgenic and nontransformed control plants were originated from hypocotyl slices and therefore are genetically different (beside the transformation event) under normal control conditions of culture, they behave very similarly, and only shoot length of line C2-3 was significantly larger ($P < 0.05$) than control shoots (Figure 3a). Although line C5-5 showed a slight decrease in fresh weight compared with nontransformed plants, these differences were not statistically significant (Figure 3b). However, when 100 mM NaCl was added to the media, all transgenic lines shoots were significantly larger than control shoots (Figure 3a), and shoot weights from lines C5-5 and J8-1 were also larger than weight of control shoots (Figure 3b).

Overall, in the presence of NaCl, the growth parameters (fresh weight and shoot length) were higher in transgenic lines, mainly lines C5-5 and J8-1, than in nontransformed plantlets. Under salt stress challenge, line C5-5 showed a 50% and 72% higher values for shoot length and fresh weight, respectively, than nontransformed line. In the case of line J8-1, the growth parameters analysed were about 50% higher than in nontransformed plants

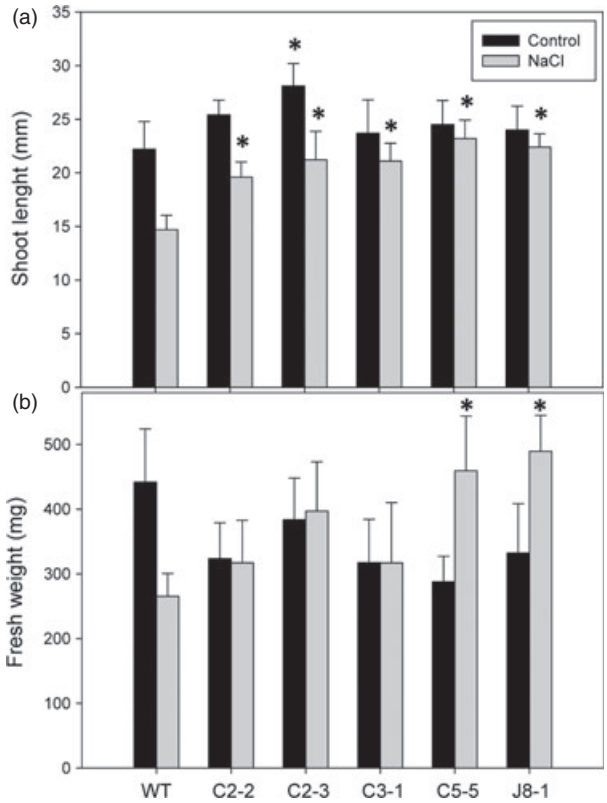


Figure 3 Effect of 100 mM NaCl treatment on (a) shoot length and (b) fresh weight. Data represent the mean ± SE of, at least, ten shoots. WT, nontransformed plants. Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild-type control for the NaCl (100 mM) and the control (0 mM) treatments according to a Dunnett's test

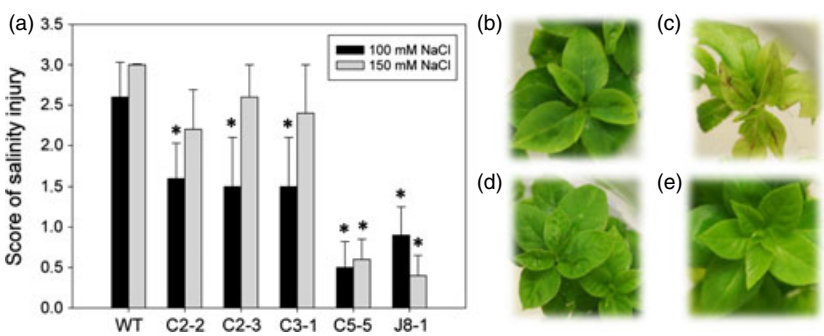


Figure 2 Salt stress tolerance of nontransformed (WT) and transgenic plum shoots. (a) Score of salinity injury measured as described in Material and Methods (data represent the mean ± SE of, at least, ten shoots). Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild-type control within each NaCl treatment according to a Dunnett's test. (b) Representative picture of nontransformed plum plantlets. (c) Representative picture showing symptoms in nontransformed plantlets induced by 100 mM NaCl treatment *in vitro*. (d) Representative picture of line J8-1 under control conditions. (e) Representative picture of line J8-1 grown in the presence of 100 mM NaCl.

(Figure 3). Taken together, these results suggest that lines C2-2, C2-3 and C3-1 exhibited a moderate tolerance to NaCl, whereas lines C5-5 and J8-1 could be considered as highly salt tolerant.

Effect of cytsod and cytapx transgenes on the antioxidative metabolism

The effect of salinity in antioxidative metabolism was studied in both transformed and nontransformed plum plantlets. When transgenic lines were compared with the nontransformed control within each treatment, under control conditions, lines C2-2 and C3-1 showed significantly higher APX activity than nontransformed plants (Table 1). Interestingly, these lines, where APX activity was increased from 51%–60%, harboured both transgenes (Figure 1). Regarding the rest of the ascorbate–glutathione cycle enzymes, when compared to nontransformed plants, no significant differences in MDHAR or GR activities were observed with the exception of lines J8-1 and C2-3, respectively, in which significant inhibitions of around 50% were recorded. However, all transgenic lines exhibited lower DHAR and CAT activities, with the exception of lines C5-5 and C2-2, respectively, which showed similar values to nontransformed plants (Table 1). POX activity was significantly increased in lines C2-2 and C5-5 and decreased in line J8-1, whereas no changes were observed in the rest of transgenic lines. Finally, under control conditions, SOD activity in transgenic lines remained similar to nontransformed plants except for line C2-3, in which a 40% increase in SOD was observed (Table 1).

The effect of 100 mM NaCl on enzymatic antioxidant defences was investigated on lines showing moderate NaCl tolerance (C2-2, C2-3 and C3-1) and the two highly salt-tolerant lines (C5-5 and J8-1). APX, CAT and SOD ($P < 0.01$) as well as GR and POX ($P < 0.001$) were significantly influenced by NaCl treatment. However, MDHAR and DHAR were not affected by the salt treatment. Salt stress induced a significant decrease in APX activity in line C5-5 but a strong increase (almost threefold) in APX activity in line J8-1 (Table 1). NaCl treatment significantly increased MDHAR activity in line C2-2 as compared with the nontransformed plants. DHAR and GR activities were similar in transgenic lines and nontransformed wild-type; in contrast, CAT activity decreased in all the transgenic lines. Finally, POX activity significantly increased in line C2-2 but decreased in lines C5-5 and J8-1, whereas SOD activity increased in lines C2-2 and C2-3 by salt stress (Table 1).

The effect of salt stress on the oxidative stress parameters, H_2O_2 contents (Figure 4a) and lipid peroxidation (Figure 4b), was also investigated. Under control conditions, transgenic lines C3-1, C5-5 and J8-1 showed lower H_2O_2 contents than nontransformed plants. Moreover, salt stress significantly increased the H_2O_2 contents. Within the NaCl treatment, all transgenic lines had lower levels of H_2O_2 than nontransformed wild-type plants. Salinity produced a significant decrease in lipid peroxidation values in lines C5-5 and J8-1, whereas there was an increase in line C2-3 as compared with nontransformed plants (Figure 4b).

Effect of cytsod and cytapx transgenes on ascorbate and glutathione contents

We analysed the effect of salt stress on the nonenzymatic antioxidants ASC (Table 2) and GSH (Figure 5) contents. It is important to highlight that under our experimental conditions, oxidized glutathione (GSSG) levels were very low, being the GSH pool mostly reduced, and surprisingly, no differences in the redox state of glutathione were observed by salt stress (data not shown). Under control conditions, all the transgenic lines showed elevated concentrations of GSH when compared to the nontransformed plants, and the GSH levels were 2.5- to 3-folds higher in lines C2-2, C3-1 and C2-3 and twofolds higher in the highly salt-tolerant lines C5-5 and J8-1 than in nontransformed plants (Figure 5). In the presence of 100 mM NaCl, the level of GSH dramatically decreased ($P < 0.001$), but still comparing with the wild-type control plantlets, all transgenic lines had significantly higher GSH values in the salt treatment (Figure 5).

Transformed lines presented a significant accumulation of total ASC contents when compared to the nontransformed plants. Moreover, lines C2-2, C3-1 and C5-5 showed higher levels of reduced ASC. It is important to highlight that all transgenic lines had a more reducing ASC redox state than nontransformed plants under both control and stress conditions (Table 2). Line C2-2 displayed the most reducing redox state in the NaCl treatment (Table 2) that could be correlated with the observed increase in MDHAR activity (Table 1). Salt stress significantly decreased total and reduced ASC contents ($P < 0.001$). However, under salinity conditions, only line C5-5 showed a significantly

Table 1 Effect of 100 mM NaCl on APX, MDHAR, DHAR, GR, POX, CAT and SOD activities on *in vitro* plum plantlets

Treatment	Line	APX	MDHAR	DHAR	GR	POX	CAT	SOD
0 mM NaCl	WT	395.1	756.4	203.7	118.3	373.4	24.9	83.7
	C2-2	599.1*	781.1	113.8*	118.6	551.4*	20.0	78.9
	C2-3	402.2	748.5	153.1*	61.3*	268.8	14.7*	116.9*
	C3-1	679.4*	648.1	125.5*	92.1	310.0	15.9*	88.8
	C5-5	303.8	706.3	227.6	95.8	501.9*	15.7*	78.6
	J8-1	470.9	376.9*	115.7*	84.7	199.1*	16.3*	74.6
100 mM NaCl	WT	360.3	586.7	166.1	121.8	803.3	34.8	83.3
	C2-2	285.5	823.9*	145.7	128.6	1017.6*	19.6*	112.6*
	C2-3	352.2	711.5	154.1	128.5	801.3	20.5*	109.3*
	C3-1	304.8	675.9	130.9	106.8	873.8	15.1*	86.2
	C5-5	159.7*	560.8	157.5	110.8	606.6*	20.8*	88.6
	J8-1	971.9*	613.8	139.2	124.7	565.7*	24.5*	86.9

APX, MDHAR, DHAR and GR are expressed as nmol/min/mg protein. POX is expressed as μmol/min/mg protein. CAT is expressed as mmol/min/mg protein and SOD as U/mg protein. WT, nontransformed plants. Data represent the mean of, at least, four replications.

*Significant differences between transgenic lines and the wild-type plants within each NaCl treatment test ($P < 0.05$).

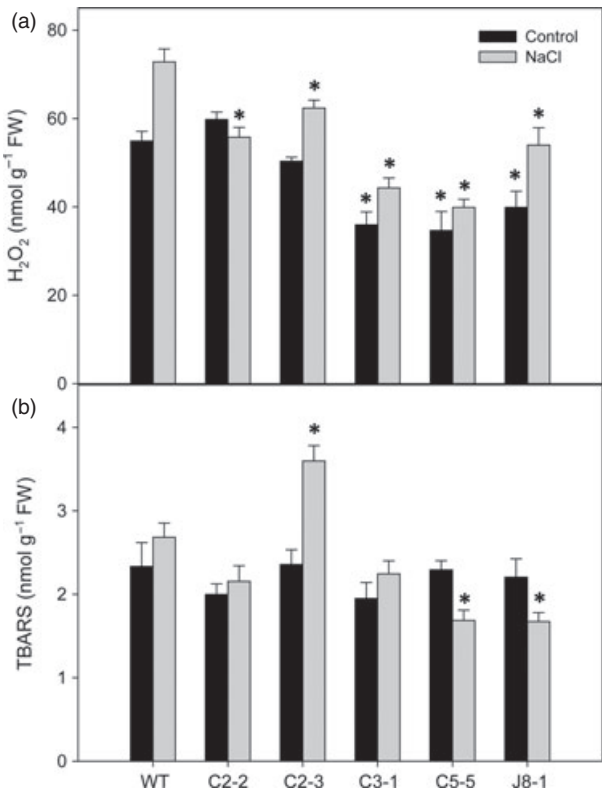


Figure 4 Effect of 100 mM of NaCl on (a) endogenous contents of H_2O_2 and (b) lipid peroxidation levels (measured as thiobarbituric acid-reactive substances; TBARS). Data represent the mean ± SE of at least four replications. Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild-type control for the NaCl (100 mM) and the control (0 mM) treatments according to a Dunnett's test. WT, nontransformed plants.

Table 2 Effect of 100 mM NaCl on ascorbate (ASC), expressed as μmol/g fresh weight, contents in *in vitro* plum plantlets

Treatment	Line	Total ascorbate	Reduced ascorbate	Redox state†
0 mM NaCl	WT	3.11	2.39	0.77
	C2-2	3.94*	3.76	0.95
	C2-3	4.25*	4.01*	0.94
	C3-1	4.22*	4.01*	0.95
	C5-5	5.68*	5.19*	0.91
	J8-1	4.52*	3.94	0.87
100 mM NaCl	WT	2.74	1.80	0.65
	C2-2	2.82	2.52	0.89
	C2-3	3.67*	3.15	0.86
	C3-1	2.94	2.15	0.73
	C5-5	4.75*	3.52*	0.74
	J8-1	3.21	2.56	0.80

WT, nontransformed plants. Data represent the mean of at least four replications.

†Redox state was calculated as the ratio reduced ascorbate/total ascorbate.

*Significant differences between transgenic lines and the wild-type plants within each NaCl treatment test ($P < 0.05$).

higher reduced ASC content than nontransformed plantlets. Finally, total ASC level was significantly higher in lines C2-3 and C5-5 (Table 2).

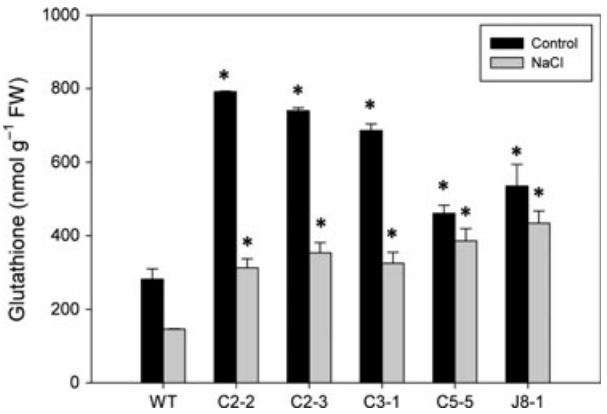


Figure 5 Effect of 100 mM of NaCl on total glutathione (GSH) contents. Data represent the mean ± SE of at least four replications. Asterisks represent significant differences ($P < 0.05$) between each transgenic line and the wild-type control for the NaCl (100 mM) and the control (0 mM) treatments according to a Dunnett's test. WT, nontransformed plants.

Effect of cytsod and cytapx transgenes on mineral content

Transgenic lines showed an increase in Ca, Zn and Mn and a decrease in Fe and Cu contents when compared to nontransformed plants (Tables S1 and S2). Salt stress also produced an alteration in the mineral content. As expected, salt treatment produced a strong increase in the Na levels. Ca, Zn and Mn levels decreased by NaCl in all the plantlets analysed, reaching in the case of Ca and Zn similar values independently of the transformation. In the presence of salt, a slight decrease or no effect on Fe content was observed except in line J8-1, which showed high levels of Fe when compared with the rest of the assayed plantlets under salt stress conditions (Tables S1 and S2). Interestingly, Cu level declined in all plantlets except in those harbouring only cyt*apx* transgene (lines C2-3 and J8-1). Finally, no important differences were observed in K, Mg, S, B and Mo contents neither by transformation nor by salt stress (Tables S1 and S2).

Discussion

Several of the transgenic plum lines exhibited *in vitro* salt stress tolerance when 100 mM of NaCl was applied. This was clearly evidenced by reduced visible symptoms such as chlorosis and necrosis. Salt stress leads to the suppression of plant growth and development as it was observed in the nontransformed plants, but these effects were less pronounced in transgenic lines. This might be due to the ability of their cells to uptake water or nutrient, because high osmotic pressure resulting from high salinity may restrict plant cells to uptake water and some minerals (Cicek and Cakirlar, 2002). Rhodes and Samaras (1994) described that growth inhibition under osmotic pressure might be mainly due to the reduction in cytoplasmic volume and the loss of cell turgor as a result of osmotic outflow of intracellular water. Reduction in plant growth and water content as a result of salinity has also been reported in several other plant species such as *Zea mays* (Ashraf and McNeilly, 1990), *O. sativa* japonica type (Lutts *et al.*, 1996), *Triticum durum* (Lutts *et al.*, 2004) and *Saccharum officinarum* (Errabii *et al.*, 2006). Surprisingly, under higher NaCl concentration (150 mM), the most tolerant lines were those expressing either cyt*sod* (line C5-5) or cyt*apx* (line J8-1) alone. In

contrast, in a previous work carried out with tobacco plants transformed with the same transgenes, the overexpression of both cyt*apx* and cyt*sod* or at least cyt*apx* alleviated the damage produced by mild water stress conditions, monitored by a higher water use efficiency and better photosynthetic rates and chlorophyll fluorescence (Faize *et al.*, 2011), reinforcing the importance of the cytosolic antioxidant system in the response to oxidative stress situations (Mittler and Zilinskas, 1994; Alscher *et al.*, 1997; Hernández *et al.*, 2000, 2004a;). Salt treatments produced an oxidative stress in nontransformed plants as indicated by increases in H_2O_2 and lipid peroxidation levels. Cell membranes are the primary site of injury caused by oxidative stress situations, and ROS are the main mediators of peroxidation damage to various cellular components (Zhang and Kirkham, 1996). However, transgenic lines are capable of moderating H_2O_2 production. In addition lines C5-5 and J8-1 decreased lipid peroxidation in response to NaCl stress, suggesting a tight control of the ROS production and a more efficient protection mechanism of membrane damage as a consequence of the transgenes expression. Our results suggest that both overexpression of the introduced transgenes and salt stress can modulate the activity of enzymatic ROS scavengers as well as the ASC and GSH contents. Contradictory results were obtained by different authors regarding the effect of salt stress on the activity of antioxidant enzymes. Some works correlated salt tolerance with a higher constitutive levels of some antioxidant enzymes (Gueta-Dahan *et al.*, 1997; Tsugane *et al.*, 1999; Hernández *et al.*, 2003; López-Gómez *et al.*, 2007), whereas others authors have found that the coordinated up-regulation of the activities of antioxidative enzymes seems to be one of the mechanisms involved in the salt tolerance response (Gómez *et al.*, 1999; Hernández *et al.*, 2000, 2001; Mittova *et al.*, 2003). However, increases in antioxidant enzymes not always guarantee tolerance to a given stress, as an example, loquat plants, sensitive to NaCl, showed increased APX, MDHAR and DHAR activities when exposed to salt stress (Hernández *et al.*, 2003). The two highly salt-tolerant lines C5-5 and J8-1 displayed a different behaviour under salt stress in terms of antioxidant defences. Line J8-1 showed the highest APX activity, whereas it seems that the mechanisms of line C5-5 to deal with oxidative stress could be related to nonenzymatic antioxidant reactions rather than via enzymatic antioxidants. This assumption is supported by the fact that line C5-5 exhibited the highest levels of ASC and a fine control of GSH levels under salt stress.

Although our data indicated that the cyt*sod* transgene is functional in the transformed lines, the enzymatic analyses revealed that only line C2-3, harbouring only cyt*apx*, showed enhanced SOD activity under control and salt stress conditions. Interestingly, salinity produced an increase in SOD activity also in line C2-2, carrying both transgenes, indicating a possible crosstalk in the mechanism of SOD activation exerted by APX in plum plantlets. Moreover, transgenic lines C2-2 and C3-1 harbouring both transgenes (two cyt*sod* and one cyt*apx* copies) displayed the highest APX activity under control conditions. These results could be explained by the fact that 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; Yoshimura *et al.*, 2000; Hernández *et al.*, 2004b; Barba-Espin *et al.*, 2010; Faize *et al.*, 2011).

The relationship of cyt*sod* and cyt*apx* overexpression with the nonenzymatic antioxidants ASC and GSH was also analysed. The oxidation of ASC and GSH under salt stress conditions has been reported in different plant species, both in leaves and in different cell compartments (Hernández *et al.*, 2000, 2001; Barba-Espín *et al.*, 2011a), leading to a more oxidizing ASC and GSH state. The complex antioxidant network of plant cells has the thiol tripeptide GSH at its centre to buffer ROS and facilitate cellular redox signalling, which controls growth, development and defence (Diaz-Vivancos *et al.*, 2010b). It is interesting to mention that in this study, no accumulation of GSSG took place in plum plantlets after NaCl treatment. The absence of GSSG detection was not correlated with GR activity, whose levels did not displayed important changes. It is possible that GSSG could be reduced by other mechanisms. In this sense, a role for thioredoxin and glutaredoxin systems in GSSG reduction has been described (Porras *et al.*, 2002; Marty *et al.*, 2009). In addition, Morgan *et al.* (2013) reported that, in yeast, the GSSG that is not immediately reduced in the cytosol is rapidly transported into the vacuole by the ABC-C transporter Ycf1. Although increases in GSSG relative to GSH are a useful indicator of oxidative stress, an important property of cellular GSH homoeostasis is that increased oxidation is generally accompanied, or rapidly followed, by increases in the total pool size (Diaz-Vivancos *et al.*, 2010b). The maintenance of a high ratio GSH/GSSG plays an important role in salt tolerance. In this sense, salt-tolerant cotton cultivars had a higher GSH/GSSG ratio than salt-sensitive lines under saline conditions (Gosset *et al.*, 1996). The transformation of plum plantlets largely increases the total GSH contents that could alleviate the oxidative stress produced when plantlets were exposed to NaCl. This response was also reported in transgenic tobacco plants overexpressing GST and GPX (Roxas *et al.*, 1997). These authors described a correlation between GSH contents and salt tolerance. In fact, GSH is necessary for an effective scavenging of H_2O_2 and hydroperoxides by GPX as well as for the maintenance of other antioxidants such as ascorbate and tocopherols (Alscher, 1989). Moreover, under salt stress, the GSH contents were strongly reduced in nontransformed plants and in lines exhibiting moderate tolerance to NaCl, whereas lines C5-5 and J8-1 showed a better control of total GSH pool that correlates with no significant changes in lipid peroxidation levels, contributing to the enhanced salt tolerance displayed by these transgenic lines.

A role for ASC in salt tolerance has also been suggested. Under control conditions, transformed plum plantlets produced a significantly higher accumulation of total ASC that was accompanied by a more reducing ASC redox state than nontransformed plants. Salt stress induced a more oxidizing ASC redox state in both nontransformed and transgenic plum plantlets. The decrease in the ASC redox state was due to a loss of total ASC pool and accumulation of oxidized ASC. A similar response had been reported in a salt-tolerant pea cultivar (cv. Puget) under salt stress. In this case, moderate (90–110 mM) and severe (160 mM) NaCl stress also decreased the total ASC pools by 20% and 50%, respectively (Hernández *et al.*, 1999). A different effect of NaCl on ASC contents was observed in two different pea cultivars differing in their sensitivity to salinity. In that case, NaCl treatment (70 mM) decreased ASC contents up to 30% in the salt-tolerant cultivar and up to 50% in the salt-sensitive cultivar (Hernández *et al.*, 2000). In spite of their presumed role as damaging agents, ROS acts as signalling molecules in regulation of stress responses as well as in developmental regulation (Barba-Espin *et al.*, 2010, 2011b; Faize *et al.*, 2013). It is well known that crosstalk between Ca^{2+} and ROS modulates the activity of specific proteins involved, directly or indirectly, in plant defence responses. Moreover, ROS

scavengers can be considered as ROS sensors because they interact directly with ROS. Thus, the increased levels of Ca^{2+} together with an enhanced antioxidant capacity could modulate the defence responses in transgenic plants. ROS signalling is made possible by homoeostatic regulation that is dependent on cellular antioxidant status. Antioxidants continuously process ROS, and they are crucial components of the cellular redox signalling network (Diaz-Vivancos *et al.*, 2010a,b). Transition metals are also active for ROS generation. Free Fe and Cu can generate hydroxyl radicals by Fenton reaction that can produce an imbalance in the cellular redox metabolism (Price and Hendry, 1991). Only in the highly salt-tolerant line J8-1, an increase in Fe and Cu was observed; however, we do not know whether these transition metals are bound to proteins or free as catalytic metals for ROS generation.

In summary, the increase in stress tolerance in plants is crucial in agriculture Because both abiotic and biotic stresses limit crop productivity worldwide. Plant tolerance to salinity is a very complex trait, being generally regarded as multigenic (Flowers, 2004). However, the alleviation of the salt-induced oxidative stress can confer a partial tolerance obtaining an improvement in stress tolerance. In this way, the use of transgenic plants may be useful for obtaining partial resistance to environmental stresses and deepen the understanding of physiological, biochemical and molecular responses of plants to abiotic stresses. This is even more important in the case of woody species, because the use of resistant rootstocks to salinity and/or drought could improve productivity in arid and semi-arid regions. In conclusion, our data suggest that transformation of plum plants with cyt*sod* and/or cyt*apx* has an influence on the antioxidant metabolism under both control and stress conditions, underlying the complexity of the regulation network of plant antioxidant defences. As a future perspective, acclimatized transgenic plum plants could constitute a useful tool to improve crop yield of *Prunus* sp. (Figure S1) and other woody plants under the frequently adverse and changing environment.

Experimental procedures

Plant material, plasmid constructions and transformation

The *Agrobacterium tumefaciens* strain EHA105, carrying the binary vector pBinARS-SOD or pCGN1578-APX, was used for inoculation of mature seed hypocotyls slices. Constructions described in Faize *et al.* (2011) harboured, in their T-DNA, neomycin phosphotransferase (*npt*II) for aminoglycoside selection and cytosolic Cu/Zn *sod* (cyt*sod*) from *Spinacia oleracea*, or cytosolic *apx1* (cyt*apx*) cDNA from *Pisum sativum*. Cyt*sod* and cyt*apx* are under the control of the duplicated *CaMV35S* promoter plus TEV enhancer and *Nos* terminator.

Hypocotyls discs of plum (*Prunus domestica* cv. Claudia Verde) were infected with *Agrobacterium* according to Petri *et al.* (2008), and transgenic plum lines were recovered transferred to the shoot growing medium (SGM; three-fourth MS-based medium with 1 μM N^6-benzylaminopurine and 0.25 μM indole butyric acid). Plum shoots were maintained by subculturing at 4-week intervals on a shoot multiplication medium (Petri *et al.*, 2008) at 23 °C under cool white fluorescent tubes (55 μmol/m^2/s) and a 16-h photoperiod.

Southern blot analysis of transgenic shoots

Genomic DNA was isolated from 50 mg of plum leaves according to the procedure of Doyle and Doyle (1990). Southern blot analysis was performed as described previously in Faize *et al.* (2013). The PCR *npt*II, cyt*sod* and cyt*apx* fragments were labelled with digoxigenin (DIG) by means of specific primers encoding *npt*II (forward 5′-gattgaacaagatggattgc-3′ and reverse 5′-ccaagctcttcagcaatatc-3′), cyt*sod* (forward 5′-aaaggctgtggttgtt-ctaa-3′ and reverse 5′-gtcttgctgagttcatgtcc) and cyt*apx* (forward 5′-ctgctggtacttttgattcc-3′ and reverse 5′-gagagcttaagatgtcttca-3′), using the PCR DIG Probe Synthesis Kit (Roche GmbH, Mannheim, Germany). Prehybridization 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) and 0.1% (w/v) sodium dodecyl sulphate (SDS) for 15 min and twice at 65 °C in 0.5 × SSC and 0.1% SDS for 15 min. Hybridizing bands were visualized with anti-DIG antibody–alkaline phosphatase and CDP-Star (Roche) on X-ray films (Faize *et al.*, 2013).

Gene expression analysis by qRT-PCR of transgenic shoots

Leaves isolated from *in vitro* transgenic lines as well as from nontransformed plants were snap-frozen in liquid nitrogen and stored at −80 °C until use. RNA was extracted from each set using RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The expression level of cyt*sod* and cyt*apx* transgenes and a *β-actin* gene, used as the endogenous control, was determined as described previously in Faize *et al.* (2011) by real-time RT-PCR using the GeneAmp 7500 sequence detection system (Applied Biosystems, Foster City, CA).

In vitro evaluation of salinity resistance of transgenic lines

To determine the effect of saline stress on *in vitro* plum plantlets, different concentrations of NaCl (50, 100, 150 and 200 mM) were added to the shoot multiplication medium. The cultures were maintained in the growth chamber at 23 °C under a 16-h photoperiod. After 4 weeks, the shoots were collected and washed with distilled water and dried on filter paper, and the growth response was determined by measuring the fresh weight and the length of the shoots. The *in vitro* shoots were also scored for visible symptoms of salinity injury on a 0–3 scale as follows: 0—no injury, 1—yellowing on leaf edges, 2—necrosis on the leaves and 3—necrosis and yellowing on the whole leaves. Score of salinity injury was calculated according to the following formula $SI = \Sigma\ (ni \times i)/N$, where ni is the number of shoots receiving the mark i (from 0–3), and N is the total number of shoots.

Determination of antioxidative enzymes

All operations were performed at 4 °C. Plantlets shoots (500 mg) were homogenized with a mortar and pestle in 1.5 mL of ice-cold 50 mM Tris–acetate buffer pH 6.0, containing 0.1 mM EDTA, 2% (w/v) PVP, 2% (w/v) PVPP, 2 mM Cys and 0.2% (v/v) Triton X-100. For APX activity, 20 mM ascorbate was added. The homogenate was centrifuged at 14 000 ***g*** for 10 min, and the supernatant fraction was filtered through Sephadex G-25 NAP columns equilibrated with 50 mM Tris–acetate buffer pH 6.0, containing 2 mM ascorbate for APX.

The activities of the ASC-GSH cycle enzymes and POX, CAT and SOD activities were assayed as described in Diaz-Vivancos *et al.* (2006, 2008). Proteins were determined according to the method of Bradford (1976). All measurements were carried out in, at least, three biological replicates.

Ascorbate and glutathione analysis

Shoot samples were snap-frozen in liquid nitrogen and stored at −80 °C until use. The frozen samples were homogenized in 1 mL 1 M $HClO_4$. Homogenates were centrifuged at 12 000 ***g*** for 10 min, and the supernatant was neutralized with 5 M K_2CO_3 to pH 5.5–6. The homogenate was centrifuged at 12 000 ***g*** for 1 min to remove $KClO_4$. The obtained supernatant was used for ascorbate and glutathione contents (Diaz-Vivancos *et al.*, 2010a). The glutathione and ascorbate determination was performed according to Pellny *et al.* (2009).

Determination of hydrogen peroxide and lipid peroxidation

For H_2O_2 analyses, plum plantlets (0.5 g) were homogenized with 2 mL of Tris–acetate buffer (50 mM), pH 5.0, in the presence of 5 mM KCN (Cheeseman, 2006). The measurement of H_2O_2 was based on the peroxide-mediated oxidation of Fe^{2+}, followed by the reaction of Fe^{3+} with xylenol orange (Bellicampi *et al.*, 2000).

The extent of lipid peroxidation was estimated by determining the concentration of thiobarbituric acid-reactive substances (TBARS). Leaf material (0.2 g) was homogenized in 2 mL of 0.1% trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 14 000 ***g*** for 10 min, and 0.5 mL of the supernatant obtained was added to 1.5 mL 0.5% TBA in 20% TCA. The mixture was incubated at 90 °C in a water bath for 20 min, and the reaction was stopped by placing the reaction tubes in ice. Then, the samples were centrifuged at 10 000 ***g*** for 5 min, and the absorbance of the supernatant was read at 532 nm. The value for nonspecific absorption at 600 nm was subtracted (Cakmak and Horst, 1991). The amount of TBARS was calculated using the extinction coefficient of 155/mM/cm according to Cakmak and Horst (1991).

Mineral content

Mineral content was analysed on the leaves of nontransformed and transgenic plum plantlets exposed to salt stress conditions. Leaves were washed with distilled water, dried at 65 °C, ground and stored at room temperature. Inorganic solute analysis was performed by the Ionomic Services at CEBAS-CSIC (Murcia, Spain). Briefly, samples were digested using a high-performance microwave reaction (Ultraclave; Milestone, Shelton, CT) with 11M HNO_3:9M H_2O_2 (4:1, v/v) and then used for macro- and micronutrient determination with inductively coupled plasma-optical emission spectrometry (ICP-OES), in a Thermo ICAP 6000SERIES model (Thermo Scientific, Madrid, Spain).

Statistical analysis

Data on RNA transcription were widely spread and had highly heterogeneous variances; therefore, they were transformed in their natural logarithms, and additionally, the requested probability level of the Dunnett's test was increased to 99.9%.

The effects of salt stress on the different parameters measured were tested by an ANOVA. Lines were compared with the wild-type nontransformed control plants by a Dunnett's test within each NaCl treatment. Statistical procedures were carried out with the software package SAS.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Pictures of acclimatized plum plants exposed to water-stress conditions (deprived of irrigation during 15 days). *In vitro* rooted plants were transferred to pots and acclimatized to *ex vitro* conditions in a growth chamber by progressively decreasing the relative humidity during 4 weeks. The growth conditions were: 24/18 °C, 70% relative humidity and 500 μmoL/m^2/s PAR, with a 16-h photoperiod.

Table S1 Effect of 100 mM NaCl on mineral content (macro-nutrients) in *in vitro* plum plantlets. WT, non-transformed plants. Data represent the mean of at least two replications.

Table S2 Effect of 100 mM NaCl on mineral content (micro-nutrients) in *in vitro* plum plantlets. WT, non-transformed plants. Data represent the mean of at least two replications.