Cytosolic ascorbate peroxidase and Cu, Zn-superoxide dismutase improve seed germination, plant growth, nutrient uptake and drought tolerance in tobacco

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Abstract

The effects of over-expression of two cytosolic antioxidant enzymes (Cu,Zn-SOD and/or APX) on plant nutrition, gas exchange, chlorophyll fluorescence, seed viability and germination in transgenic tobacco (*Nicotiana tabacum* cv. Xanthi) under deficit irrigation or salinity conditions were investigated. Three transgenic lines of tobacco were used in this study: line 17, harboring 2 copies of the cytosolic CuZn-SOD (*cytsod*) gene; line 51, with 2 copies of the cytosolic APX (*cytapx*) gene and line 39, harboring one copy of each gene.

Over-expression of cytosolic antioxidants enzymes in tobacco plants resulted in a better growth performance that correlated with an improved photosynthetic capacity and nutrient uptake. Moreover, *cytsod* or *cytapx* genes promoted seed germination, and enhanced tolerance to mild water stress. In addition, this enhanced antioxidant capacity protected seeds from ageing during prolonged storage, and stimulated germination under salt stress conditions. These results suggest that cytosolic antioxidant transgenes are useful tools to improve drought tolerance, nutrient uptake and seed germination under stressful conditions.

Key Words: germination; plant growth; mineral nutrition; salinity; seed aging; seed physiology.

1 Introduction

Environmental stresses exert adverse effects on plant growth and development because of the reactive oxygen species (ROS) that are overproduced under these stressful conditions. Among them, anion superoxide ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^{\cdot}OH$) are the most damaging ROS in plants (Perl-Treves and Perl 2002).

Water and salt stress are serious stress factors that limit agricultural production in many regions worldwide (Alscher et al. 1997; Hernández et al. 2001). Both, water deficit and salinity primarily affect photosynthetic CO_2 assimilation leading to a restricted plant production (Flexas et al. 2002). This lower CO_2 availability favors the ROS generation in the chloroplast inducing an oxidative stress at cellular level (Asada ~~et al.~~ 1999; Faize et al. 2011). In addition, under drought and salt stress conditions ROS production also increased in other cell compartments such as mitochondria or peroxisomes (Alscher et al. 1997; Hernández et al. 1993; Mittova et al. 2003; Bartoli et al. 2004).

Germination can also induce ROS production (Hendry 1993; McDonald 1999; Kranner et al. 2010), being traditionally considered as a negative effect. Seeds rapidly increase oxygen uptake and oxidative phosphorylation in order to support the energy for the germination process (Tommasi et al. 2001). Production of H_2O_2 has been demonstrated during the early imbibition stage of seeds of soybean (Puntarulo et al. 1991), tomato (~~Morahashi~~ 2002), radish (Schopfer et al. 2001), wheat (Caliskan and Cuming 1998), and sunflower (Baily et al 2002). If ROS over-generation is not tightly controlled, they can cause

detrimental effects during the initial phases of growth and development of embryos and seedlings (Cakmak et al. 1993).

Seeds can also be subjected to the detrimental effect of ROS production during extreme desiccation and storage, and they can lose germination ability and viability during prolonged storage periods (McDonald 1999). The excess of ROS accumulation induces a reduced germination rate, which is considered as an indication of seed ageing. Aged seeds loss plasma membrane integrity (Priestley et al. 1985) which leads to an inability of cells to maintain osmotic turgor, and therefore to seed death (Parrish et al. 1982). For these reasons, antioxidative mechanisms have been regarded as being of particular importance for the success of germination (de Gara et al. 1997; Tommasi et al. 2001). The antioxidant mechanisms are categorized into enzymatic and non-enzymatic antioxidants. Non-enzymatic antioxidants include ascorbate (ASC), glutathione (GSH), tocopherol, flavonoids, and carotenoids. The antioxidant enzymes are important to cope with abiotic stress disorders in adult plants as well as for the success of germination (Bailly et al. 2004; Barba-Espín et al. 2010). They include superoxide dismutase (SOD), the ASC-GSH cycle enzymes, catalase and peroxidases that are involved in the scavenging of ROS in plant cells (Noctor and Foyer 1998; Asada 1999). Among the antioxidative enzymes, SODs and ascorbate peroxidases (APXs) play a pivotal role in ROS detoxification in higher plants (Asada 1999). SODs are metaloenzymes that catalyzes the dismutation of $O_2^{\cdot -}$ to O_2 and H_2O_2 (Fridovich 1975). APXs, the main enzyme of the ASC-GSH cycle, are one of the most important key enzymes that scavenge potentially harmful H_2O_2 in different cell compartments

(Noctor and Foyer 1998; Asada 1999; Jiménez et al. 1997; Diaz-Vivancos et al. 2006).

Beside their detrimental effect, ROS can also play a key role in the completion of germination and should be considered as a messenger or transmitters of environmental cues during seed germination (Bailly et al. 2008, Barba-Espín et al. 2010; 2011; Diaz-Vivancos et al. 2013). In recent works, it has been reported that the imbibition of pea seeds with low H_2O_2 levels increased the germination rate as well as the growth of the seedlings in a concentration-dependent manner, linked to the induction of some proteins related to plant signaling and development, cell elongation and division and cell cycle control, as well as to a strong decrease in ABA contents (Barba-Espín et al. 2010; 2011; Diaz-Vivancos et al. 2013). In *Arabidopsis thaliana*, germination was associated with an accumulation of superoxide and hydrogen peroxide in the radicle (Leymarie et al. 2012). In radish, germination was also accompanied with increase in ROS originated from seed coat and embryo (Oracz et al. 2009).

Previously, we transformed tobacco (*Nicotiana tabacum* cv. *Xanthi*) plants with cytosolic *Cu-ZnSOD* and/or *APX* genes. These transgenic lines displayed greater tolerance to mild water stress, by stimulating the antioxidant capacity in both the cytosol and the chloroplast (Faize et al. 2011). We extended this work to see if this higher antioxidative capacity can also be important to increase the germination of the derived T1 seeds and vigor of the tobacco seedlings. We also attempted to study the effect of these cytosolic transgenes in plant growth, mineral content, photosynthesis (V_{cmax} and J_{max} parameters) and seeds physiology under unfavorable environmental conditions.

2 Material and methods

2.1 Plant material, seed germination, plant vigor and biomass

Three transgenic lines of tobacco (*Nicotiana tabacum* cv. *Xanthi*) (T0 plants) were used in this study: line 17 harboring 2 copies of the cytosolic *Cu-ZnSOD* (*cytsod*) gene; line 51 with 2 copies of the cytosolic *APX* (*cytapx*) gene and line 39 harboring one copy of each gene. All of these cytosolic genes are under the control of the constitutive duplicated CaMV35S promoter (Faize et al. 2011). Transgenic and non-transgenic plants were grown in pots with peat substrate in the greenhouse under a 16/8 h day/night photoperiod at 25ºC.

T1 seeds were collected from T0 plants 60 days after self-pollination at anthesis and used for further experiments. They were used immediately (new seeds) or stored for up to 4 years at 8ºC (aged seeds). New seeds were germinated on Petri dish on three layers of filter paper disks soaked with 5 ml of sterile distilled water and incubated at 25ºC up to 10 days.

In order to investigate the effect of ectopic expression of *cytsod* and *cytapx* genes on seed germination and plant growth vigor, seed germination rates were tested in non-transformed and transgenic T1 lines. Seeds were surface sterilized with diluted sodium hypochlorite solution (2%) for 5 min followed by three washes with sterilized distilled water. They were then germinated on three layers of filter paper disks soaked with 5 ml of sterile distilled water or with 5 ml of 100 mM NaCl solution. Seeds were incubated at 25ºC with a16/8 h photoperiod. The rate of germination was determined every 2 days by counting seeds with radicle emergence of at least 2 mm in length. The time course (measured in days) taken to reach 50% of germinated seeds was

also determined (T50). All assays were replicated four times using 50 seeds per replicate.

Growth parameters, such as roots and shoots length and shoots fresh weight from germinated seeds were measured 14 days after seed imbibition, and seedlings were transferred to pots with peat substrate and acclimatized in an environmental-controlled greenhouse. The height and the weight of shoots were measured again at 4 and 8 weeks after germination.

2.2 Water stress assay, gas exchange measurements and chlorophyll fluorescence analysis

Three week-old control (non-transformed) and transgenic plants (T0 plants) grown in a greenhouse were deprived from water during 3 days. Control plants were irrigated with 50 ml of water every day during the three days duration of the experiment (Faize et al. 2011).

Photosynthetic capacity of leaves was estimated from the analysis of A-Ci response curves. Measurements were made using an open gas exchange system (Li6400, Li-Cor Inc., Nebraska, USA) with an integrated leaf chamber fluorometer (LI-6400-40, Li-Cor Inc., Nebraska, USA). The curves were performed under saturating irradiance (1800 µmol m^{-2} s^{-1}) and under constant leaf temperature (25ºC) by controlling the CO_2 concentration of inlet air in 11 steps from 50 to 1400 µmol mol^{-1}. Diffusion leaks when performing the curves were taking into account by applying the manufacturer's equation to determine the diffusion coefficient. Six leaves per line and water treatment were measured. Photosynthetic parameters maximum rate of carboxylation by ribulose 1,5-biphosphate carboxylase/oxygenase (Rubisco) (V_{cmax}) and the

maximum rate of electron transport (J_{max}) were determined according to the Farquhar model of leaf photosynthesis (Farquhar *et al.,*1980). The temperature dependencies of photosynthetic parameters were calculated according to Bernacchi et al. (2002) modified taking into account the effect of mesophyll conductance (g_m). A-Ci curves were fitted with a non-rectangular hyperbola version of the biochemical model of leaf photosynthesis following Ethier and Livingston (2004). The Ci cut-off point was determined based on the method proposed by Ethier et al. (2006). From this analysis V_{cmax}, J_{max} and g_m were determined.

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 tobacco lines after 3 days. After dark-incubation of plants (15 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 PSII quantum yield (Y(II)), the non-photochemical quenching (NPQ) and the coefficients of non-photochemical quenching (qN), and the photochemical quenching (qP) were analyzed.

2.3 Mineral content

Mineral content was analyzed on the leaves of non-transformed and transgenic tobacco T0 plants grown in normal conditions or exposed to 3 days mild water stress. Leaves were washed with distilled water, dried at 65 ºC, 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, USA) with HNO_3:H_2O_2 (4:1, v/v) and then used for macro- and micro-nutrient determination with inductively coupled plasma-optical omission spectrometry (ICP-OES), in a Thermo ICP-ICAP 6500 DUO (Thermo Scientific, England).

2.4 Statistical analysis

The effects of the overexpression of *cytsod* and/or *cytapx* on leaf mineral nutrition and gas-exchange parameters measured in non-transformed and transgenic lines under well-irrigated and deficit irrigation conditions were tested by a two-way ANOVA, whereas those related to seedling growth parameters in T1 plants were tested by one-way ANOVA. Within each irrigation treatment, lines were compared with non-transformed controls by a Dunnett's test.

3 Results

3.1 Effect of *cytsod* and *cytapx* on maximum rate of carboxylation by Rubisco (V_{cmax}) and maximum rate of electron transfer (J_{max})

The effect of *cytsod* and *cytapx* on V_{cmax} and J_{max} was investigated in plants well irrigated and in plants subjected to ~~deficit irrigation~~ (Table 1). V_{cmax} data were affected by the "Line" and "Irrigation Treatment" factors ~~and an interaction between both factors was observed~~. However, J_{max} and the ratio J_{max}/V_{cmax} were affected only by the "Irrigation Treatment" factor.

Under well irrigated conditions, line 39 displayed a significantly higher V_{cmax} than the non-transformed control, but the transgenic lines 17 and 51 presented a significant lower V_{cmax} value than non-transformed line. Line 39 also exhibited the highest J_{max} values (Table 1). However, under deficit irrigation conditions, V_{cmax} and J_{max} decreased in all cases and values were quite similar in all tobacco lines. Finally, the ratio J_{max}/V_{cmax} was similar in all lines both under well irrigated and under deficit irrigation conditions although an increase in non-transformed plants and in line 39 occurred (Table 1).

The images of chlorophyll fluorescence parameters showed that under well irrigated conditions lines 39 and 51 displayed the highest qP and Y(II) values, whereas non-transformed plants showed the highest values for the non-photochemical quenching parameters (qN and NPQ) (Fig.1). Under deficit irrigation conditions a decrease in the photochemical quenching parameters [qP and Y(II)] as well as the qN parameter was observed in non-transformed plants (Fig.1). A decrease in qP also occurred in line 51 that was accompanied by an increase in the NPQ parameter, related to the safe dissipation of the excess light energy. However, under deficit irrigation conditions, line 17 did not change the qP parameter and even an increase in the non-photochemical parameters (qN and NPQ) was observed. Finally, in line 39 deficit irrigation also decreased qP, but ~~unchanged Y(II) and NPQ values were observed~~ (Fig. 1).

3.2 Effect of *cytsod* and *cytapx* and water stress on mineral macro- and micro-elements

Leaf mineral content (macro and micro-elements) was determined in both non-transformed tobacco lines and transformed lines under deficit of irrigation

and under well irrigated conditions. The ANOVA analysis revealed that the ~~data for~~ all the macro-nutrients, ~~except Ca~~, ~~were affected by~~ the “Line” studied. However, the “Irrigation Treatment” factor had a significant effect only in Na and Mg contents, whereas a significant interaction was observed for the C, Na, Ca and Mg levels (Table 2). Regarding the micro-nutrients, “Line” and “Irrigation Treatments” factors, as well as the interaction between them, had a significant effect only on Mn contents. All the studied micro-nutrients were affected by the “Line” used. An interaction “Line*Irrigation Treatments” was also observed for Zn contents (Table 3). Under well irrigated conditions all the transformed lines showed slightly lower C contents than non-transformed plants (Table 2). Line 39 had significantly higher Na and B contents than non-transformed, whereas line 51 showed higher Mg, B, Mn and Zn levels than the non-transformed plants (Tables 2 and 3). Under deficit irrigation conditions the most important changes were observed in line 51 that presented the highest Na, B and Mn contents, whereas line 17 also showed higher Mn levels than non-transformed plants (Tables 2 and 3).

3.3 Effect of *cytsod* and *cytapx* on seed germination under various conditions

The effect of ectopic expression of *cytsod* and *cytapx* on seed germination was investigated in new and aged seeds as well as on seeds soaked in 100 mM NaCl (Fig. 2). For this experiment, we used seeds produced by self-pollinating the T0 lines and therefore around 6% of the seeds were non transgenic (corresponding to a segregation 1:16 of two independently inherited copies of the *nptII* gene).

When new seeds were sown in the presence of 100 mM of NaCl germination rate was severely affected in the control (only 2% of seeds germinated after 10 days, Fig. 2A). The percentage of germination was also affected in the line 39 (around 20% of seeds germinated after 10 d). This line showed significant differences in the germination rate with the non-transformed control at 5 days. However, germination rate was less affected in lines 17 and 51 (T50 at 5d and 7d, respectively), reaching a germination rate of about 67% for line 17, and about 58% for line 51 (Fig 2A).

Germination rate was also determined in aged seeds that were stored for 4 years at 8ºC (Fig. 2B). Germination was severely affected in the non-transformed seeds (less than 20% of seeds germinated after 10 days), while it was barely affected in the line 39 (T50 of 3.5 d) and not affected in the lines 17 and 51 (T50 of 2 d) (Fig 2B). According to Dunnett's test the three transgenic lines exhibited significantly higher germination ability than the non-transformed control ($P<0.05$).

3.4 Effect of *cytsod* and *cytapx* on plant vigor and biomass

Transgenic lines (T1 plants) exhibited elevated vigor, showing significantly higher shoot and root length than the non-transformed plants (Fig. 3, Table 4). After 2 weeks, root growth was significantly higher in transgenic tobacco lines than in non-transformed plants. Root length was 2.7-fold and 1.8-fold higher in lines 17 and 39, respectively, whereas in line 51 the effect was even more evident, and root reached a length 4-fold higher than non-transformed plants (Table 4).

When compared to the non-transformed plants, shoot length was significantly higher in the transgenic lines, being 1.7-fold in line 17 and about 2-fold in lines 39 and 51, after 2 weeks of germination. Four weeks after germination all the transgenic lines showed higher vigor than the non-transformed plants, although values were only statistically significant for lines 17 and 51. Two month after germination, shoot length was about 2.5-fold higher in lines 17 and 39 whereas line 51 reached a shoot length 4.3-fold higher than to non-transformed plants (Table 4).

The transgenic lines showed also higher biomass when compared to the non-transformed plants at 2 weeks after germination, being 2.4-fold higher in the line 17, 1.6-fold in the line 39 and about 4-fold in the line 51 when compared with non-transformed plants. However, differences were significant only for lines 17 and 51 (Table 4).

4 Discussion

We generated transgenic tobacco transformed with *cytsod* (line 17), *cytapx* (line 51) or containing both transgenes (line 39). These transgenic lines showed enhanced tolerance to mild water stress that correlated with higher water use efficiency and better photosynthesis rates, as well as with increased antioxidant capacity in both soluble and chloroplast fractions (Faize et al. 2011). In this work we showed that these transgenic lines exhibited modified photosynthetic capacity, enhanced mineral content and seed germination as well as plant growth.

4.1 Photosynthetic capacity

A direct effect of water stress is disruption of photosynthesis. The maximum Rubisco activity (*Vcmax*) and electron transport capacity (*Jmax*) are the key parameters determining photosynthetic capacity (Dickson and Tomlinson, 1996). *Vcmax* is a measurement of the process by which Rubisco catalyzes the reaction of ribulose 1, 5-biphosphate (RuBP) with CO_2 to produce the carbon compounds that become triose phosphates, whereas *Jmax* reflects electron transport through the thylakoid membrane, which is critical to produce NADPH and ATP, and then provide the metabolic energy necessary to produce triose phosphates. In this study we showed that under well-watered conditions, lines 39 and 51 exhibited the highest *Jmax* values that correlated with high net photosynthesis (Faize et al. 2011) and the qP parameter. Under deficit irrigation conditions, decreases in *Vcmax* and *Jmax* were observed in all cases that correlated with decreases in qP and Y(II) and increases in NPQ in non-transformed plants and in line 51. However, lines 17 and 39 maintained Y(II) and also increased NPQ as a safe mechanism of excess energy dissipation. Some authors, point out that the reduction in *Vcmax* can be due to an inactivation or loss of Rubisco, reducing the carboxylation efficiency, while the reduction in *Jmax* seems to be associated with a diminution of sedoheptulose-1, 7-bisphosphatase, a key regulatory enzyme in the Calvin cycle (Allen et al. 1997; 2000; Nogués and Baker 2000; Ölcer et al 2001). In fact, the decrease in CO_2 assimilation would reduce the demand for ATP and NADPH, resulting in decreased electron transport (Zhou et al. 2004). The ratio J_{max}/V_{cmax} can be considered as a parameter indicating light absorbed/light used for CO_2 fixation. For this reason, values close to 1 could indicate a balanced photosynthetic

process, as observed in line 39, or even in non-transformed plants under well irrigated conditions. Lines 17 and 51 (harboring only one transgene) showed constitutive high J_{max}/V_{cmax} values. However, values greater than 1, as occurred under deficit irrigation conditions in non-transformed plants and line 39, could indicate an excess of light energy that can lead to the photoinhibition of photosynthesis as well as ROS over-generation. In fact, these tobacco lines suffered a higher decrease in photosynthetic rate (A_N) (near 50% decrease) than lines 17 and 51 (about 25% decrease) (Faize et al. 2011), maintaining the J_{max}/V_{cmax} values even under stress conditions. In addition, this response correlated with higher chloroplast and cytosolic APX and SOD levels (Faize et al. 2011), enzymes involved in the water-water cycle, which is involved in dissipation of the excess of excitation energy under environmental stress conditions (Asada 1999).

4.2 Leaf mineral nutrition and plant growth

Micro-elements such as Fe, Cu, Mn and Zn are cofactors of different metalloenzymes, including APX, catalase, peroxidases (POXs) and SOD isoenzymes (Fe-, Mn- and CuZn-SOD), along with their role in other important processes including protein synthesis, enzyme activation or carbohydrate metabolism (Marschner 1997). The content ~~in~~ these micro-elements was somehow higher in transgenic lines than in non-transformed plants. This could be related with the enhanced activity of those metalloenzymes described previously (Faize et al. 2011). Moreover, line 51 presented higher B content under well irrigated and deficit irrigation conditions that ~~was parallel with a better membrane protection~~ (Faize et al. 2011). Boron is an essential micronutrient for

plant growth and development, and it is involved in a number of metabolic pathways and functions such as cell wall synthesis and structure lignification, carbohydrate metabolism, phenol metabolism and plasma membrane integrity (Marschner 1997).

Several factors may contribute to the changes in element contents of the leaves. A large size of the root system will help to access nutrient with limited diffusion such as phosphates (Marschner 1997). Increased leaf mineral nutrient may help to maintain efficient photosynthesis and thus biomass. A correlation between vigor and tolerance to water stress has been reported in several plants such as *Arabidopsis* (Narang et al. 2000) and rice (Price et al. 2002). Under our experimental conditions, overexpression of *cytsod* and/or *cytapx* genes stimulated the growth of T1 tobacco plants. This was especially noticeable in line 51, which exhibited better growth parameters than the non-transformed control. Under normal conditions, as well as under drought stress, line 51 exhibited the highest maximum net photosynthesis (An) that showed a good relationship with leaf mass area (Faize et al. 2011). In addition, the elevated qP and Y(II) values observed for this line are indicative of its better photosynthetic capacity. Moreover, line 51 showed a better membrane protection as observed by its lower TBARS content and electrolyte leakage values (Faize et al. 2011).

4.3 Seed Physiology

Our transgenic tobacco plants exhibited a tight control of ROS production via an enhanced antioxidant capacity (Faize et al. 2011). ROS can act as signaling molecules regulating plant growth and development (Gechev et al. 2006), being also involved in seed germination and seedling growth (Kranner et

al. 2010; Barba-Espín et al. 2010). Increased growth by over-expression of antioxidants enzymes has also been reported in transgenic tobacco expressing pepper ascorbate peroxidase-like gene (Sarowar et al. 2005).

The reactivation of metabolism following seed imbibition may provide an important source of ROS (Bailly 2004; Kraner et al. 2010). For this reason, anti-oxidative mechanisms have been regarded as being of particular importance for the success of germination, and most of them increase during germination (De Gara et al. 1997; Tommasi et al. 2001; Bailly 2004; Barba-Espín et al. 2010). Uncontrolled ROS accumulation during germination may lead to seed deterioration. For this reason ROS accumulation must be tightly regulated by the scavenging mechanisms during seed germination (Bailly 2004). These deleterious effects of ROS, with the concomitant decrease in the antioxidative defenses, are responsible for decreased structural integrity and increased seed mortality (Priestley et al. 1985; Simrnoff 1993). Therefore, reinforcement of antioxidative system can lead to improved seed germination and plant vigor. In this sense, our results showed that overexpression of *cytapx* or *cytsod* increased seed germination, vigor and plant biomass under non-stress conditions in T1 plants.

Salt stress can affect different physiological processes, including seed germination (Darra et al. 1973; Heikal et al. 1982). Salinity is detrimental to both seed viability and vigor during germination (Unger 1978). In salt-sensitive species, germination under saline conditions produced osmotic potential increases which can inhibit the imbibition process by restricting water uptake (Bradford 1990). In the present work, we showed that salt stress drastically reduced the rate of germination in non-transformed tobacco being transgenic

lines less affected. Surprisingly, line 39, overexpressing both transgenes, exhibited an intermediate phenotype. This differential behavior could rely on the modulation of the activity of ROS scavenging enzymes (Faize et al. 2011). It has been extensively reported that salt stress induced an oxidative stress in plants (Hernández et al. 2001). In this way, the overexpression of antioxidants enzymes (APX and/or Cu,Zn-SOD) can lead to an optimal control of ROS generated during the germination process under salinity conditions. The addition of antioxidants such as reduced ascorbate or glutathione improved the germination rate of *Arabidopsis* seeds under NaCl stress (Borsani et al. 2001). Probably, the overexpression of *cytsod* or *cytapx* can minimize the salt-induced oxidative stress during the germination of tobacco seeds, improving their germination rates. Moreover, different works reported that ROS, when tightly regulated, can be beneficial for the germination process (Bailly et al. 2008; Kranner et al. 2010; Barba-Espín et al. 2010; 2011; Diaz-Vivancos et al. 2013). In previous work, we reported that the imbibition of pea seeds with low H_2O_2 levels increased the germination rate and the early seedling growth, and this response was correlated with the induction of cytosolic and stromatic *apx* transcripts and their corresponding activity as well as with the increase of other antioxidant enzymes such as monodehydroascorbate reductase (MDHAR) and POX (Barba-Espín et al. 2010). Improvement of seed germination using tylakoidal *apx* or plastidial *sod* and *apx* has been already reported in tobacco (Sun et al. 2010; Lee et al. 2010). However, this is the first report of the involvement of cytosolic ROS-scavenging enzymes during germination.

Seed germination is reported to be either suppressed or slowed down during aging (McDonald 1999). In our conditions, seed storage for 4 years

severely affected germination in non-transformed tobacco, while it had no detrimental effect on germination of transgenic seeds, probably due to the lack of detrimental ROS accumulation during storage. Viability losses during storage is a result of ROS accumulation as well as a decrease in antioxidant mechanisms, which ultimately lead to oxidative damage to essential macromolecules during seed imbibition (Bailly et al. 2008; Rajjou et al. 2008). Therefore, the regulation of ROS generation and antioxidant defenses is of vital importance to maintain seed viability. In our work, aged seeds from transformed lines showed no loss of viability after 10 days post-imbibition, contrary to the control seeds, which showed a very low germination rate, indicating serious seed deterioration during storage. Our results are in agreement with those of Lee et al. (2010) who showed that overexpression of SOD and APX in tobacco plastids resulted in enhanced seed viability of aged seeds. Their transgenic lines exhibited low electrolyte leakage and maintained their membrane integrity during imbibition, due to higher antioxidant ability, which results in a lower accumulation of ROS. Our results demonstrate that overexpression of *cytsod* and *cytapx* in the cytosol can also increase seed longevity and germination rates under unfavorable environments.

5 Conclusions

Taken together, these results suggest that cytosolic antioxidants mechanisms can be useful tools to improve several physiological processes such as seed germination, nutrient uptake, plant growth and a better photosynthetic response under abiotic stress conditions.

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Figure Captions

Figure 1. Effect of deficit irrigation on chlorophyll fluorescence parameters in leaves of non-transformed (NT) and transformed tobacco lines (17, 39 and 51). Images of the coefficient of photochemical quenching (qP), the effective PSII quantum yield [Y(II)], the coefficient of non-photochemical quenching (qN) and the non-photochemical quenching (NPQ) parameter are shown. I, irrigated; DI, deficit irrigation.

Figure 2. A) Germination percentage from new tobacco seeds imbibed in 100 mM NaCl. B) Germination percentage from aged tobacco seeds. New seeds derived from non-transformed control (NT) and transformed tobacco lines (line 17, line 39 and line 51) were harvested 2 months after anthesis and stocked for 4 years at 8ºC. Data are mean and standard errors from 4 independent replicates. 50 seeds were used for each replicate.

Figure 3. Picture showing seedling growth of non-transformed (NT) and transformed tobacco lines (line 17, line39, and line 51) 3 weeks after seed germination. Bar, 1 cm.

Table
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Table 1. Maximum rate of carboxylation by ribulose 1·5-bisphosphate carboxylase/oxygenase (Rubisco) ($V_{c.max}$; µmol CO_2 m^{-2} s^{-1}). maximum rate of electron transport (J_{max}. µmol e^{-1} m^{-2} s^{-1}) and J_{max}:$V_{c.max}$ ratio in the different irrigated (I) and deficit irrigation (DI) genotypes lines. Data are the mean ± SE from 4 replicates.

Treatment	***Line***	***Vcmax***	***Jmax***	***Jmax/Vcmax***
Irrigated	Control	91.8± 18.2	101.5±21.1	1.11±0.23
	17	71.9±1.6*	100.4±5.1	1.40±0.07
	39	148.4±8.6*	140.7±12.6	0.95±0.12
	51	79.2±11.6*	113.06±7.5	1.43±0.09
Deficit irrigation				
	Control	62.1±2.2	92.5±6.8	1.49±0.08
	17	55.4±9.1	78.1±2.1	1.41±0.23
	39	50.2±2.8	82.3±5.5	1.64±0.15
	51	56.3±4.4	78.1±7.2	1.39±0.07
		[a]F values		
Source of variation		***Vcmax***	***Jmax***	***Jmax/Vcmax***
Line (A)		5.06**	1.06	1.05
Irrigation Treatment (B)		50.04***	17.49***	9.08**
AxB		9.00***	1.49	1.71

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

Table
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Table 2. Macronutrient contents in leaves from non-transformed and transformed tobacco lines (control, line 17, line39, and line 51) under irrigated (I) and deficit irrigation (DI) conditions (3 days of withholding water). Data are the mean ± SE from 3 replicates. Asterisks represent significant differences (P<0.05) between each transgenic line and the wild type control, within each irrigation treatment, according to Dunnett's test.

Treatment	Line	C (%)	N (%)	P (%)	K (%)	Na (%)	Ca (%)	Mg (%)	S (%)
I	**Control**	41.8±0.19	1.74±0.14	0.308±0.02	3.22±0.14	0.045±0.01	1.65±0.10	0.52±0.015	0.26±0.014
	Line 17	**41.1±0.23***	2.06±0.28	0.355±0.01	3.50±0.48	0.047±0.01	1.92±0.28	0.55±0.011	0.28±0.025
	Line 39	**41.1±0.20***	2,48±0.13	0.399±0.04	4.20±0.32	**0.094±0.03***	1.93±0.17	0.54±0.01	0.32±0.01
	Line 51	**41.1±0.10***	2.78±0.27	0.416±0.04	4.17±0.4	0.057±0.01	2.09±0.21	**0.62±0.03***	0.35±0.03
DI	**Control**	41.3±0.19	2±0.41	0.338±0.01	3.60±0.43	0.090±0.01	1.85±0.15	0.54±0.024	0.28±0.032
	Line 17	41.8±0.28	1.86±0.24	0.324±0.04	2.96±0.39	0.089±0.01	1.72±0.14	0.48±0.037	0.25±0.032
	Line 39	41.6±0.21	2.84±0.28	0.385±0.01	4.41±0.23	0.078±0.01	1.88±0.09	0.53±0.007	0.32±0.01
	Line 51	40.6±0.57	2.51±0.07	0.434±0.02	4.66±0.35	**0.175±0.01***	1.75±0.24	0.58±0.03	0.36±0.01

Source of variation	***[a]F values***							
Line (A)	6.42**	18.07***	13.67***	17.26***	13.95***	0.76	13.78***	19.96***
Irrigation Treatment (B)	0.11	0.11	0.02	0.89	56.0***	1.15	5.96*	0.12
AxB	5.57**	2.41	1.44	2.53	20.33***	3.43*	4.42*	1.10

[a]F values from two-way ANOVA for the different macronutrient analysed. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

Table

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Table 3. Micronutrient contents in leaves from non-transformed and transformed tobacco lines (control, line 17, line39, and line 51) under irrigated (I) and deficit irrigation (DI) conditions (3 days of withholding water). Data are the mean ± SE from 3 replicates. Asterisks represent significant differences (P<0.05) between each transgenic line and the wild type control, within each irrigation treatment, according to Dunnett's test.

Treatment	Line	Fe (ppm)	B (ppm)	Cu (ppm)	Mn (ppm)	Zn (ppm)
I	**Control**	45.08±5.61	28.94±0.64	3.99±0.71	0.52±0.015	21.54±1.42
	Line 17	48.98±4.72	31.52±2.12	4.51±0.97	0.55±0.011	24.88±2.55
	Line 39	57.39±7.03	**33.54±2.51***	5.85±0.2*	0.54±0.01	23.51±0.12
	Line 51	71.24±9.02	**41.9±1.03***	6.6±0.9*	**0.62±0.03***	**30.78±2.78***
DI	**Control**	38.26±12.45	34.44±2.15	4.4±1.56	0.54±0.024	24.87±1.74
	Line 17	45.19±5.94	31.08±1.18	4.78±1.36	**0.48±0.00***	23.11±3.32
	Line 39	61.79±5.94	34.11±1.17	5.35±0.79	0.53±0.007	28.35±3.68
	Line 51	58.08±6.88	**40.87±1.98***	6.04±0.16	**0.58±0.03***	28.23±0.32
Source of variation		**[a]F values**				
Line (A)		15.21***	44.78***	7.84**	6.05**	8.62**
Irrigation Treatment (B)		3.12	2.96	0.24	112.06***	1.02
AxB		2.54	4.49*	0.72	7.54**	3.68*

[a]F values from two-way ANOVA for the micronutrient analysed. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

Table
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Table 4.- Seedling growth parameters at various days after seed imbibition (dai) in non-transformed and in transgenic tobacco lines. Data are means and standard error from 5 to 15 replicates. Asterisks represent significant differences (P<0.05) between each transgenic line and the wild type control, within each irrigation treatment, according to a Dunnett's test.

Line	**Root length** (mm)	**Shoot length** (mm)			**Shoot fresh weight** (mg)
	14 dai	**14 dai**	**28 dai**	**60 dai**	**14 dai**
Control	2.9±0.4	7.6±1.8	71±14	78±6	308±88
17	7.8±0.9*	12.7±2.1*	129±33*	179±13	733±186*
39	5.1±0.8*	15.7±1.8*	94±10	196±42	508±51
51	11.6±1.1*	17.2±2*	129±29*	340±123*	1192±191*
	[a]F values				
	96.77***	18.09***	5.23**	10.99***	27.22***

[a]F values from one-way ANOVA for the growth parameters analysed. F-values significant at 99.9% (***), 99% (**) or 95% (*) levels of probability.

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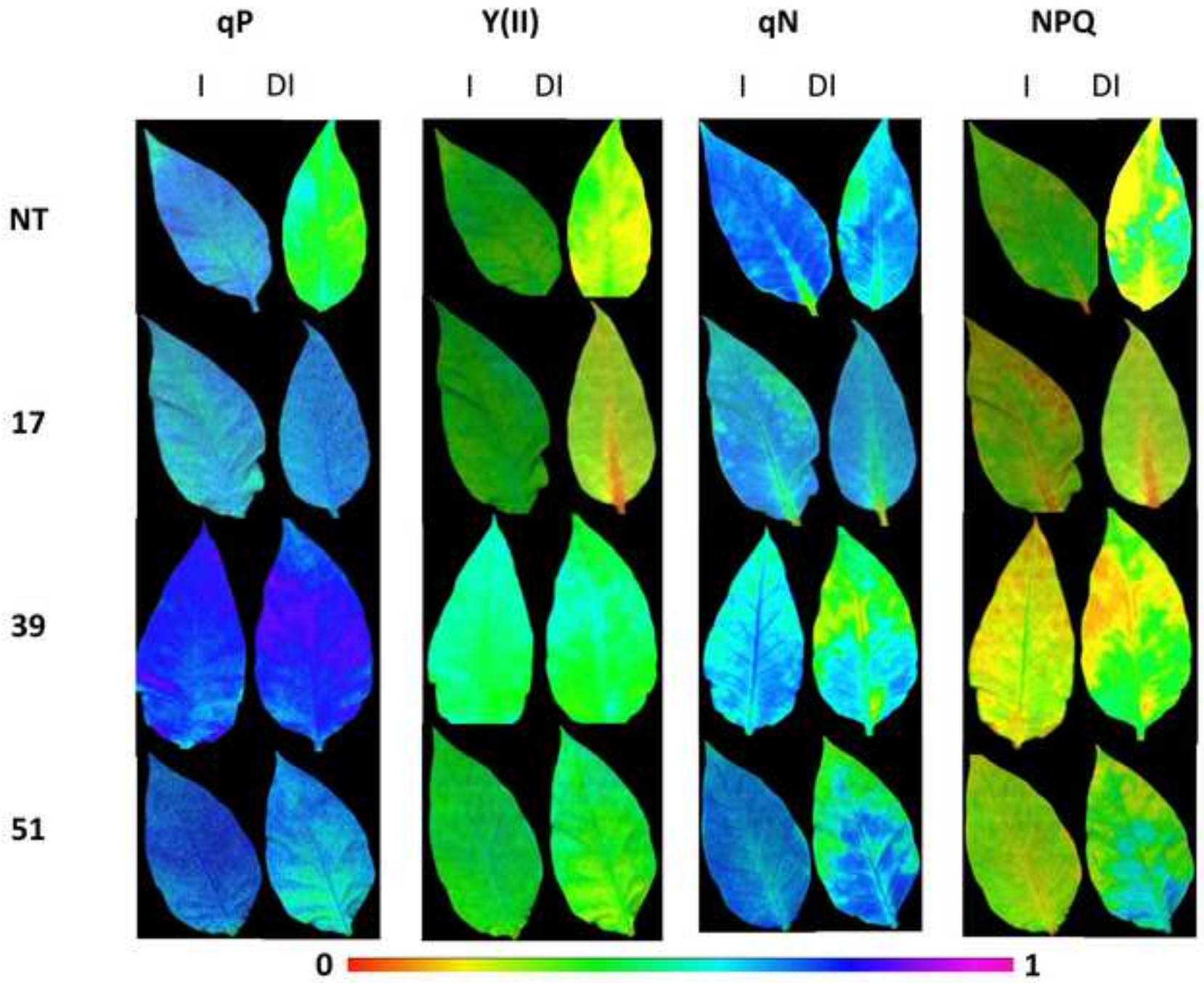

Figure 1.

Figure
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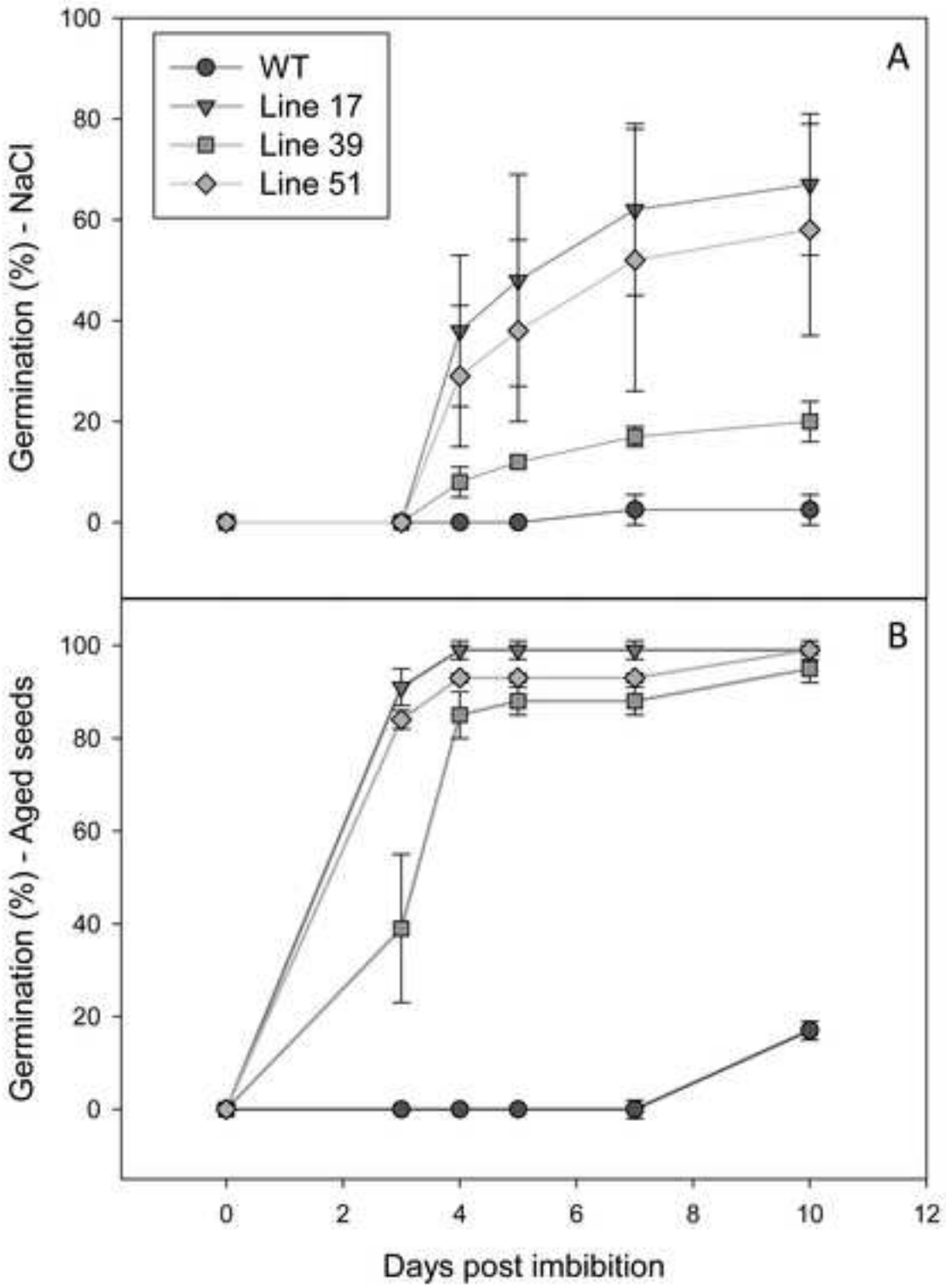

Figure 2.