INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
12–624/2013/15–4–725–730
http://www.fspublishers.org

Full Length Article

Improved Tolerance of Cu/Zn Superoxide Dismutase and Ascorbate Peroxidase Expressing Transgenic Tobacco Seeds and Seedlings against Multiple Abiotic Stresses

Young-Pyo Lee[1], Raza Ahmad[2]*, Haeng- Soon Lee[1], Sang Soo Kwak[1], Mustafa Nawaz Shafqat[3] and Suk-Yoon Kwon[1]*
[1]*Plant Systems Engineering Research Center, KRIBB, Daejeon 305-806, Korea*
[2]*Department of Environmental Sciences (Biotechnology Program), COMSATS Institute of Information Technology, Abbottabad, 22060, Pakistan*
[3]*Department of Biosciences, COMSATS Institute of Information Technology, Islamabad, Pakistan*
*For correspondence: uafraza@yahoo.com; sykwon@kribb.re.kr

Abstract

Reactive oxygen species (ROS) are produced during seed germination but remain under-control in optimum germination conditions. Seeds being germinated in single or multiple stress conditions such as drought, excessive salt and low-temperature may face burst of ROS, which leads to poor germination and weak seedling establishment. Enzymatic activities, germination rates and seedling growth of the seeds of transgenic tobacco plants simultaneously expressing Cu/Zn-superoxide dismutase (*CuZnSOD*) and ascorbate peroxidase (*APX*) in plastids (CA seeds) under various combinations of environmental stresses (drought, salt and low temperature) were investigated and compared with the non-transgenic seeds (NT seeds). CA seeds maintained higher *CuZnSOD* and *APX* activities during seed imbibitions than NT seeds. CA seeds depicted less ion leakage after salt stress than NT seeds providing the evidence of proper functioning of *CuZnSOD* and *APX* to keep ROS production under control. When germinated in stress conditions, CA seeds germinated earlier than NT seeds and this character was more profound under double or triple stress conditions. Moreover, CA seedlings accumulated more shoot and root dry weight as compared to NT seedlings under single, double and triple stress conditions. These investigations strongly suggest that overexpression of *CuZnSOD* and *APX* helped CA seeds to keep ROS under control by detoxifying superoxide radicals and H_2O_2, which paved the way for increased germination and seedling growth in various combinations of environmental stresses.

Keywords: Abiotic stresses; Ascorbate peroxidase; ROS; Superoxide dismutase; Transgenic tobacco

Introduction

Plants are invariably exposed to various environmental stresses, like drought, salt and low-temperature. The situation gets worst when plants face multiple stresses at a time in various combinations, such as drought/salt, low-temperature/drought, low-temperature/salt and low-temperature/drought/salt. These environmental stresses alone or in combination adversely affect germination, growth and productivity of plants (Munns and Tester, 2008; Farooq *et al*., 2009; Yadav, 2009; Mahmood *et al.,* 2012). Although little is known about the adverse effects of different combinations of abiotic stresses on plants, one of the major causes of damages by multiple environmental stress is oxidative injury imposed by excessive production of reactive oxygen species (ROS) (Hasegawa *et al.,* 2000). ROS are capable of damaging various cellular molecules, such as lipids, proteins and nucleic acids, which subsequently leads to membrane damage and cell death (Shah *et al.,* 2001). Plants have evolved various mechanisms to minimize injuries caused by oxidative stress via ROS-scavenging enzymes and low molecular weight antioxidants (Lu *et al.*, 2010). In higher plants, among others superoxide dismutase (SOD) and ascorbate peroxidase (APX) play central role in ROS scavenging system (Noctor and Foyer, 1998).

Seed germination is a crucial step which ensures continuity of plant's life cycle. During germination seeds quit quiescence and progressively activate metabolism to synthesize macromolecules required for successful germination. Germination demands high energy and oxidative phosphorylation, which can be quantified as early as a seed imbibes (Hourmant and Pradet, 1981; Cai *et al.,* 2011). Oxidative phosphorylation paves the way to yield ROS, which if remains unchecked can hamper germination (Thompson *et al.,* 1987). The over production of ROS may lead to the oxidative stress and deterioration of seeds. ROS levels are particularly increased when seeds are germinated in stressful conditions, such as sub-optimal-low temperature, salinity and drought. Seeds loose viability under oxidative stress, which damages vital cellular functions (Harman, 1986). Induction of antioxidant

To cite this paper: Lee, Y.P., R. Ahmad, H.S. Lee, S.S. Kwak and S.Y. Kwon, 2013. Improved tolerance of Cu/Zn superoxide dismutase and ascorbate peroxidase expressing transgenic tobacco seeds and seedlings against multiple abiotic stresses. *Int. J. Agric. Biol*., 15: 725–730

enzymes during germination stage ensures increased viability and germination (Gidrol *et al.,* 1994).

To ensure healthy and productive plantation under various stress conditions, it is required to increase seed germination and seedling establishment by fortifying the antioxidative mechanism via overexpression of antioxidant enzymes (Ahmad *et al.*, 2013). In our previous study, we developed and characterized transgenic tobacco (*Nicotiana tabacum cv*. Xanthi) plants, which express Cu/Zn superoxide dismutase (*Cu/ZnSOD*) and ascorbate peroxidase (*APX*) in plastids (referred to as CA plants, Kwon *et al.,* 2002). These plants exhibited enhanced total SOD and APX activities than non-transgenic tobacco plants (referred to as NT plants) and new isoenzymes of APX and SOD were also found in these transgenic plants. The CA plants depicted enhanced tolerance against methyl viologen (MV)-mediated oxidative stress than NT plants. Additionally, these CA plants showed enhanced seed longevity and germination after prolonged storage (Lee *et al.,* 2010). We hypothesized that overexpression of *CuZnSOD* and *APX* in transgenic tobacco plants may lead to improved tolerance against abiotic stresses during germination and seedling growth. In the present study, we attempted to determine enhanced seed germination and *in-vitro* plant growth of CA plants expressing both *CuZnSOD* and *APX* in plastids under single and combinations of multiple environmental stress conditions.

Materials and Methods

Plant Material and Culture Conditions

CA transgenic tobacco plants expressing both CuZnSOD and APX under the control of a CaMV35S promotor (Kwon *et al.,* 2002) and NT plants were used as plant material. The plants were maintained in a greenhouse (16 h days, 30°C day and 22°C night). Seeds were harvested 90 days after anthesis (DAA) and utilized for further experiments.

Germination Assay

For germination assays, CA and NT seeds were surface-sterilized as described by Lee *et al.* (2010). Briefly, seed were washed with 70% (v/v) ethanol for 10 sec and 2% (v/v) Clorox for 15 min and washed thoroughly several times with autoclaved distilled water. The sterilized seeds of NT and CA plants were cultured on petri dishes containing Murashig-Skoog medium (MS medium) or MS medium containing 4% PEG 8000 for drought stress (D) and/or for salt stress 100 mM NaCl (S). For low-temperature stress (L), petri-plates were incubated at 15°C in an incubator. Germination rate of NT and CA plants seed against multiple stresses were also performed in different combination as: drought and salt (DS), low-temperature and salt (LS), low-temperature and drought (LD), low-temperature, drought and salt (LDS). Seed germination was scored at radical protrusion of 2 mm in length. For growth experiments, two-day old seedlings were incubated in 500 mL bottle and challenged against various stresses described earlier and incubated for 55 days.

All germination and growth experiments were conducted in an incubator (16/8 daynight) and at 25 or 15°C. All assays were replicated at least three times; each replicate contained 25 seeds for germination assay and 10 seedlings for growth related experiments. Dry weight of plantlets was measured by drying them for 3 days at 70°C in a drying oven.

Enzyme Extraction and Assay

Seeds of CA and NT plants after a specified interval of imbibition were ground in liquid N_2. The homogenates were suspended in buffer specific to analyze SOD and APX activities as described by Lee *et al.* (2010). Analysis of SOD isoenzymes and APX via native gel assay was performed as described by Beauchamp and Fridovich (1971) and Mittler and Zilinskas (1994), respectively.

Quantification of ROS Release

ROS released from seeds during imbibitions was determined as described by Schoper *et al.* (2001) with some modifications as described by Lee *et al.* (2010). Briefly, potassium phosphate buffer (20 mM, pH 6.0) containing dichlorofluorescien diacetate (50 μM) (Serva, Boehringer Mannheim, Germany) was loaded with 0.1 g L^{-1} pig liver esterase (Boehringer Mannheim, Germany) and incubated at 25°C for 15 min to yield dichloroflurescein (DCFH) after deacetylation. Seeds (0.1 g) imbibed under normal conditions or in 100 mM salt solution were incubated in 1.5 mL freshly prepared ROS detection solution in a shaking incubator set at 150 rpm a shaken at 150 rpm. One mL of the solution was used for measuring fluorescence (488 nm excitation and 525 nm emission) using a fluorescence spectrophotometer (LS-50B, Perkin-Elmer, Bucks, England). ROS-detection solution devoid of seeds was used as blank and the difference between the blank and assay's values was calculated.

Statistical Analysis

Assays to record ROS release, germination percentages and *in-vitro* growth in normal and in stress conditions were repeated three times. Data were analyzed by Student's t-test by using Microsoft Excel 2007. The significance was determined at the $P \leq 0.05$ level.

Results

CuZnSOD and APX Enzymatic Activity in Germinating Seeds

Antioxidant enzymatic activities of *CuZnSO*D and *APX* during tobacco seed germination was analyzed using native polyacrylamide gel method (Fig. 1). Notably, introduced *CuZnSOD* and *APX* isoenzymes were highly detected during seed germination in CA seeds (Fig. 1a, b).

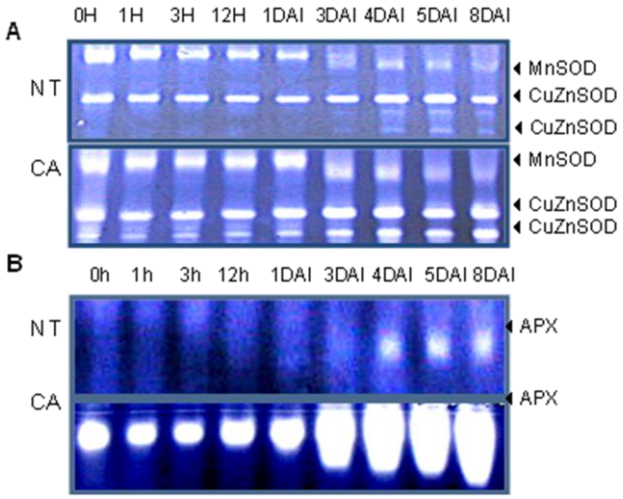

Fig. 1: Time course 0 hour (H) to 8 days after imbibition (DAI) native gel assay of superoxide dismutase (SOD) and ascorbate peroxidase (APX) during seed germination of NT and CA plants. A, *SOD* activities in the seeds of CA and NT plants during germination; B, *APX* activities in the seeds of CA and NT plants during germination

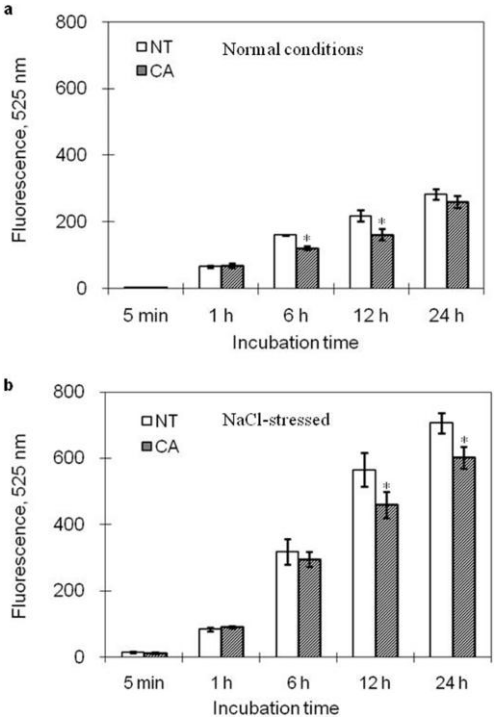

Fig. 2: Enhanced ROS scavenging in CA seeds. Time course analysis of DCFH oxidation in seeds of NT and CA plants. A 0.1 g seeds were incubated in 1.5 ml DCFH assay medium in darkness (**a**) and 100 mM NaCl treatment (**b**) and emitted fluorescence was recorded. Data are presented as mean ±SD of three independent measurements**.** *Bars* labeled with *asterisk* show significant differences between NT and CA plants by t-test at ($P \leq 0.05$)

A markedly higher expression of *CuZnSOD and APX* was noticed at 3 days after imbibition (DAI), whereas antioxidant enzyme activities of NT seeds were not detected till that time (Fig. 1a, b). Also shoot and root of CA plants showed enhanced activity of *CuZnSO*D and *APX* during seedling growth under single and combined stress conditions (data not shown). These results suggest that the CA plants have enhanced *CuZnSO*D and *APX* activity in seeds and seedlings.

ROS-scavenging in CA-seeds during Imbibition

The ROS release was quantified by determining the increment in DCF fluorescence during imbibition in DCFH solution (Fig. 2a) or in DCFH solution containing salt (Fig. 2b). The CA and NT seeds exhibited increased fluorescence in a medium containing DCFH (Fig. 2a) and this increase was more higher in salt containing DCFH medium (Fig. 2b). However, the amount of florescence in CA seeds was 20 to 25% less than in NT seeds after 12 h of imbibition (Fig. 2). CA seeds showed significantly less ROS release after 12 and 24 h of salt stress treatment as compared to NT plants, whereas in normal conditions significant difference ($P \leq 0.05$) was recorded after 6 and 12 h of imbibition. These results showed that the release of ROS in CA seeds was suppressed more strongly by overexpression of *CuZnSO*D and *APX* than that of NT plants.

Germination of CA Plants under some Abiotic Stresses

We performed germination assays under various stress conditions (Fig. 3). The CA seeds took comparatively less time to germinate than NT seeds under single stress conditions. However, CA seeds depicted significantly swift germination than that of NT seeds under multiple environmental stresses (Fig. 3b), especially, under combined stress of LDS CA seeds completed 90% germination in 24 days, while NT seeds took 30 days for 90% seed germination (Fig. 3b). Statistically, CA plants exhibited significantly ($P \leq 0.05$) better germination than NT plants in salt, low temperature, drought and salt, low temperature and drought, low temperature and salt and low temperature, drought and salt stresses (Fig. 3b).

***In-vitro* Growth of CA Plants under Multiple Abiotic Stresses**

The CA and NT plants depicted less growth retardation under single stress treatments (Fig. 4), whereas NT plants showed severe growth inhibition than that of CA plants under combined stress conditions such as DS, LD, LS and LDS. NT plants accumulated less shoot and root dry weights than that of CA plants under multiple stress conditions (Fig. 4b, c). Statistically, CA plants showed significantly ($P \leq 0.05$) higher shoot and root growth under LT, LD, LS, and LD and LS stresses, respectively. Multiple stresses also affected shoot and root lengths, and fresh weights of shoot and root (data not shown).

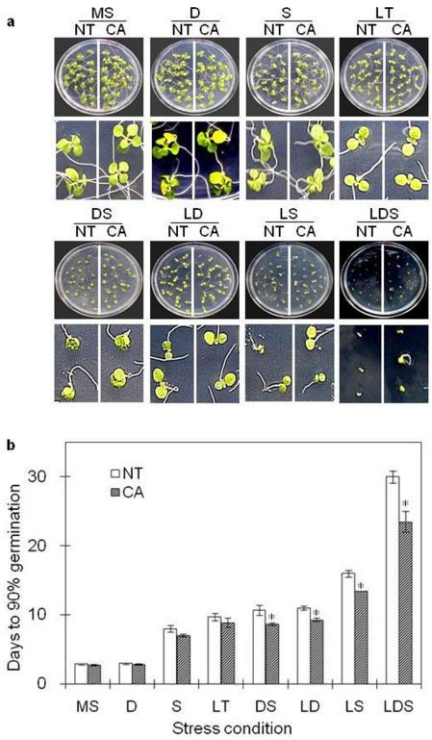

Fig. 3: Enhanced germination of CA seed under single or combined abiotic stress conditions. Twenty-five NT and CA seeds were germinated and the germination rates were recorded in Murashige-Skoog (MS) media with various environmental stress conditions for 30 days at 25ºC except low temperature (L) treatment at 15ºC. (**a**) A representative photograph of 1-week-old NT and CA seeds grown in MS medium (MS) or in MS medium treated with osmotic stress (D, MS media added with 4% PEG), salt (S, MS media added with 100 mM NaCl), low temperature (L, grown at 15ºC), drought stress and salt (DS), low temperature and osmotic (LD), low temperature and salt (LS), and triple combined stress (LDS). (**b**) Germination rates of NT and CA seeds in MS media treated with environmental stressors. Data are presented as mean ±SD of three independent treatments. *Bars* labeled with *asterisk* show significant differences between NT and CA plants by t-test at ($P \leq 0.05$)

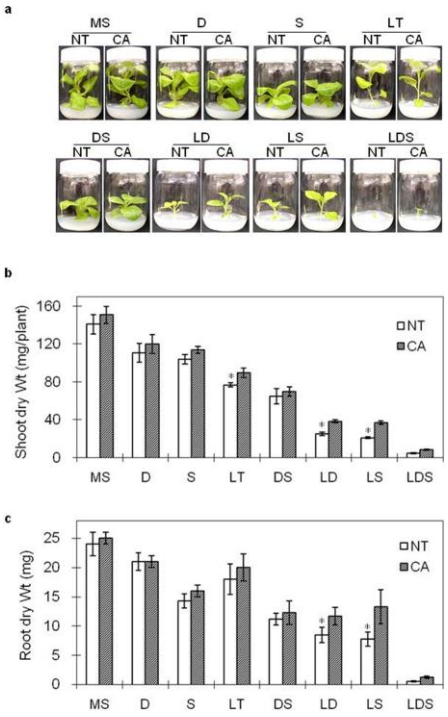

Fig. 4: Enhanced seedling growth of CA plants under various stress conditions. (**a**) A representative photograph of NT and CA seedlings grown in MS medium (MS) or in MS medium treated with osmotic stress (D, MS media added with 4% PEG), salt (S, MS media added with 100 mM NaCl), low temperature (L, grown at 15ºC), drought stress and salt (DS), low temperature and osmotic (LD), low temperature and salt (LS), and triple combined stress (LDS). (**b, c**) Shoot and root dry weight of CA and NT plants after 55 days of growth under various stress conditions. Data are presented as mean ±SD of three independent measurements. *Bars* labeled with *asterisk* show significant differences between NT and CA plants by t-test at ($P \leq 0.05$)

Discussion

ROS has gained more interest in seed science, particularly in seed aging, germination and initial plant growth. However, majority of the previous findings address the deleterious effects of ROS on seeds and seedling (Hendry, 1993; Leprince *et al.,* 1995; Reuzeau and Cavalie, 1995). In barley alurone layer, expression of SOD, APX and CAT APX, diminishes after GA treatment, which resulted in over accumulation of ROS leading to cell death in aleurone layer (Bethke *et al.,* 1999; Fath *et al.,* 2001; Fath *et al.,* 2002). The automated ROS scavenging capacity of seeds allow them to germinate efficiently but this situation may be reversed during environmental stresses, in which ROS production overwhelms its scavenging system, leading to poor germination and seedling growth.

In the present study, we found that CA seeds were highly tolerant against various stress conditions such as salt (100 mM NaCl), drought (4% PEG-8000), low temperature (15^{o}C) as well as combined stress DS, LD, LS and LDS during germination and growth as compared to NT seeds. *APX* is an important H_2O_2–scavengier that is found in the chloroplasts, mitochondria, peroxisomes and cytosol of of plants (Allen *et al.,* 1997). We found individual *APX* isoforms and the amount of *APX* was increased in CA seeds during germination and growth (Fig. 1). *APX* of CA seeds has a high affinity towards H_2O_2, this increased *APX* activity is most likely a reason of improved tolerance

against various environmental stress conditions. *SOD* is also considered a vital enzyme that detoxifies ROS in plants. Our data revealed that enhanced SOD activity rendered CA seeds more tolerant to various stress conditions (Fig. 1). We observed a correlation between high *CuZnSOD* and *APX* activity levels of CA seeds and scavenging of ROS under normal and 100 mM NaCl condition (Fig. 2). In plants three different isoforms of SODs exist in different organelles, which also use different functional metals (Bowler *et al.,* 1991). We demonstrated that NT and CA seeds contain three different SOD isoforms and it was noted that cytosolic CuZnSOD showed higher activity, MnSOD depicted lower activity, moreover, MnSOD activity was decreased during imbibition (Fig. 1A), but CuZnSOD activity was increased during imbibition (Fig. 1A). The ROS release can be successfully evaluated with the ROS probe, 2',7'-dichlorofluorescin (DCFH) that is oxidized by H_2O_2 to highly fluorescent 2',7'dichloro fluorescein (DCF) in a peroxidase-dependent reaction (Keston and Brandt, 1965). ROS scavengers can inhibit this reaction, which indicates that H_2O_2 and ^{-}OH participate in the oxidation of DCFH (Bass *et al.,* 1983; Cathcart *et al.,* 1983; Scott *et al.,* 1988; Simontacchi *et al.,* 1993). These results suggest that overexpressed foreign *CuZnSOD* and *APX* isoenzymes in CA seeds can contribute to scavenge oxidative stress and can be correlated with their ability to tolerate multiple environmental stresses (Fig. 2).

ROS release is implicated in all kinds of abiotic and biotic stresses (Ahmad *et al.,* 2010). ROS include superoxide (O^{-}_2) and hydroxyl radicals (.OH-) Plants have been equipped with mechanism to detoxify ROS produced during normal or stressful conditions, Among others SOD and APX play central role by detoxifying superoxide radicals and hydrogen peroxide (Noctor and Foyer, 1998). ROS accumulation in seeds and during germination may lead to seed deterioration (Bailly, 2004). These deleterious effects of ROS 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 stress tolerance and can improve seed germination and seedling establishment as well under different stress conditions.

To evaluate the enhanced protection acquired by overexpression of APX and SOD at germination (Fig. 3) and growth (Fig. 4) under various stress condition, we investigated the germination and the growth of NT and CA seeds under various single, double and triple stress conditions as described earlier. CA seeds showed improved protection against various stresses and germinated more quickly as compared to NT seeds (Fig. 3). Similarly, CA seedlings responded with higher growth rate than NT seedlings under double and triple stress conditions (Fig. 4). The extent of both delayed germination and retardation of growth under stress of NT and improved stress tolerance of CA seeds and plants may be due to the difference of SOD and APX activities which lies between transgenic and non-transgenic plants. CA seeds depicted higher activities of APX and SOD enzymes during exposure to drought, salt, LT and combined stress conditions (data not shown).

In conclusion, CA plants overexpressing *APX* and *CuZnSOD* in plastids depicted enhanced tolerance against various environmental stresses. These results prove to some extent that the elevation of ROS scavenging capacity of plants may be an effective and crucial step to increase stress tolerance at germination and seedling stage.

Acknowledgements

This study was supported by a grant from the Next-Generation Biogreen 21 program (PJ008103), the Rural Development Administration (RDA), Republic of Korea.

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(Received 08 August 2012; Accepted 17 January 2013)