Transgenic Tobacco Overexpressing Cytosolic Superoxide Dismutase Exhibit Enhanced Tolerance to UV-B Stress

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Abstract

Stress leads to generation of reactive oxygen species (ROS) in living cells, which are scavenged by antioxidant enzymes. Effect of UV-B stress on transgenic tobacco (Nicotiana tabacum cv. Xanthi) overexpressing cytosolic superoxide dismutase (AhCuZnSOD) and untransformed control plants were compared. Transgenic tobacco plants exhibited higher levels of total antioxidant enzyme activities, lower level of malonyldialdehyde content and hydrogen peroxide accumulation under stress conditions in comparison to the untransformed control (UC) plants. Our analysis revealed that antioxidant enzyme leads to increase in the total activity of the antioxidant ezymes and thus improve UV stress tolerance in transgenic plants.
Keywords: superoxide dismutase, antioxidant enzymes, UV stress, tobacco

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I. INTRODUCTION

Ultraviolet (UV) radiation, reaching the earth's surface has increased dramatically as a result of numerous anthropogenic activities. It has been the reason for a multitude of physiological and biochemical perturbations in living organisms. The affects embody retardation of plant growth and alteration in metabolic processes such as photosynthesis, respiration, etc. Excessive production of reactive oxygen species (ROS) is one amongst the major consequences of exposure to such ionizing radiations. There are specific evidences suggesting that UV radiation-induced damage is related to acceleration in ROS generation (Zacchini & Agazio 2004), resulting in oxidative stress.

ROS are produced in response to various abiotic stresses, including ionizing UV radiation. Most of the time their levels are rigorously controlled and kept compartmentalized. However excessive and uncontrolled generation of ROS inactivates enzymes and damages important cellular components, apart from disturbing the electron transport (Joshi et al. 2011). Thus, measuring the extent of free radical-induced damage can assess the deleterious effects of UV radiation on plant growth and development. Free radical-induced lipid peroxidation causes leakage of cellular electrolytes, thereby damaging cellular integrity (Du & Jin 2000; Sharma et al 2012). Further, the presence of an array of photosensitive pigments in plants makes them prone to oxidative damage (Asada 1994; Blokhina et al. 2003; Gill and Tuteja 2010).

During the course of evolution, plants have developed many mechanisms to shield themselves against oxidative damage. The antioxidant molecules like ascorbate, glutathione and α-tocopherol and protective enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT) and monodehydroascorbate reductase, are essential components of a plant's antioxidative defence system (Negi et al 2015).

Superoxide dismutase (E.C.1.15.1.1.) is a metallozyme that forms the first line of antioxidant defense mechanism. This enzyme was first reported by Mann and Keilin (1938). Its biological function, that is ROS scavenging, was discovered by McCord and Fridovich (1969). It catalyses the dismutation of the superoxide radical and releases hydrogen peroxide.

Transgenic plants with ectopically enhanced activity of SOD are expected to have an improved tolerance against oxidative stress. Therefore, the present experiments were conducted to evaluate the potential of transgenic tobacco plants, overexpressing cytosolic SOD, in alleviating the UV induced oxidative damage.

II. MATERIAL AND METHODS

A. Plant Material and Growth Conditions:

Nicotiana tabacum (L.) cv. Xanthium was previously transformed by Agrobacterium tumefaciens with a binary vector containing AhCuZnSOD (Negi et al 2015). Independently transformed CuZnSOD lines of T_2 generation and wild-type (WT) plants were used in the present experiments. Total genomic DNA was isolated and subjected to PCR analysis. Presence of gene construct in the plants was confirmed by PCR using gene specific primers (FP5'- AAATGGTGAAGGCTGTGGC-3') and (RP 5'-CCAAACAACGGAAAGGGGT-3').

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1) UV-B Stress Tests:

15 d-old rooted WT and transgenic plants were grown in plastic pots on agropeat. After four weeks in a greenhouse, the plants were exposed to UV-B radiation provided by 30 W germicide fluorescent lamp (Osram Sylvania Danvers, USA). Samples were taken at regular intervals with increasing UV-B exposure time points and analyzed for various stress-marker molecules.

B. Superoxide Dismutase Assay (SOD) Assay:

Superoxide dismutase assay was measured according to Gupta et al (1993). The activity was assayed by SOD's ability to inhibit photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm. A 3-ml reaction mixture contained 50 mM phosphate buffer (pH 7.8), 13 µM methionine, 63 µM NBT, 1.3 µM riboflavin, and 100 µl of the enzyme extract. Riboflavin was added last and the tubes were shaken and placed 30 cm below two 15-W fluorescent tubes for 10 min. The extinction at 560 nm was read against the blank. One enzyme unit of SOD is defined as that amount of protein (in mg) causing a 50% inhibition of the photoreduction.

C. Ascorbate Peroxidase (APX) Assay:

Ascorbate peroxidase activity was assayed according to Chen and Asada (1989). The assay depends on the decrease in absorbance at 290 nm as ascorbate is oxidized. Leaves were homogenized in 100 mM sodium phosphate buffer (pH 7.0) containing 5 mM ascorbate, 10 % glycerol, and 1 mM EDTA. The activity was determined in 1 ml reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0), 0.1 mMascorbate, 0.3 mM H_2O_2, and 50 µl of the enzyme extract. The reduction in ascorbate concentration was monitored by reading the change in absorbance at 290 nm continuously for 180 seconds. One unit of APX activity was defined as the amount of enzyme required to reduce 1 µmol of H_2O_2 min−1 under the assay conditions.

D. Catalase (CAT) Assay:

The activity of CAT was measured following the procedure of Aebi (1984). The assay mixture contained 3.2 mM H_2O_2 in 50 mM phosphate buffer (pH 7.0) and 100 µl of enzyme extract in a total volume of 3 ml. The CAT activity was estimated by the decrease in absorbance of H_2O_2 at 240 nm. One unit of CAT was defined as the amount of enzyme dismuting 1 nmol of H_2O_2 per min. The activity was calculated as follows.

E. MDA Content:

The Malondialdehyde content was measured according to Heath and Packer (1968). Leaf tissue, 0.5gm of leaf tissue was ground in 2.5 ml of 0.1% TCA buffer and the homogenate was centrifuged at 10,000 X g at 4 ^{0}C for 30 minutes. In a 1.5 ml tube, 1ml of aliquot of supernatant was taken and 4ml of 20% TCA with 0.5% TBA solution was added to it. The mixture was heated at 95 ^{0}C for 30 minutes followed by quick cooling in an ice bath. After 15 minutes it was again centrifuged at 10,000 g at 4 ^{0}C for 30 minutes. The supernatant was taken in a cuvette (1 ml) and absorbance was recorded at 532nm and 450nm. The MDA content was calculated using the following equation:

$$MDA\ (\mu mol\ g^{-1}\ FW) = (6.45 \times O.D._{532}) – (0.56 \times O.D._{450})$$

F. H_2O_2 Content:

H_2O_2 assay was measured according to Velikova et al (2004). 0.5 gm of leaf tissue was ground to a fine powder in liquid nitrogen to which 5ml of 0.1% (w/v) TCA was added. The homogenate was transferred to an eppendorf tube and centrifuged at 12000g for 15 min at room temperature. The supernatant was taken in a fresh tube. To 5ml of supernatant 0.5ml of potassium phosphate buffer (pH 7.0) and 1ml of potassium iodide (1M) was added. The mixture was vortexed and its absorbance taken at 390nm using a UV-Visible spectrophotometer. The concentration of H_2O_2 was determined using an equation derived from standard graph plotted with known dilution.

$$H_2O_2\ (\mu mol\ g\text{-}1\ FW) = 1 + 227.8 \times O.D.390.$$

G. Statistical Analysis:

All experiments were independently performed at least three times. Results were analyzed by Student's *t* test. The treatments are significantly different ($p \leq 0.05$) between transgenic lines and wild type plants.

III. RESULT

A. Enzyme activity assays:

Antioxidant enzyme activity was assayed in the AhCuZnSOD overexpressing tobacco plants subjected to UV-B stress. Exposure of tobacco plants to increasing intervals of UV-B radiation induced a significant increase in all the antioxidant enzymes (SOD, APX and CAT), in untransformed control as well as transgenic plants. The increase in activity of these enzymes was greater in the transgenic lines as compared to the untransformed control plants. In the absence of UV-B stress, the SOD activity in the transgenic lines S2 and S4 plants increased by ~ 1.5 fold, whereas in the presence of 8hr UV-B stress, transgenic lines S2 and S4 showed 1.9 and 2.1 fold against untransformed control (UC). At 16hr UV-B stress, transgenic lines S2 and S4 showed 2.1 and 2.3 fold against untransformed control (UC) and 24hr UV-B stress 2.4 and 2.5-fold higher activity was observed in transgenic line (S2, S4 and S5) as compared to UC (Fig. 1).

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Under normal condition, the APX activity also increased with ~1.4 fold, whereas under 8hr of UV-B stress the transgenic lines (S2 and S4) showed 1.5 and 1.7 fold higher as compared to UC. On 16hr and 24hr of UV-B stress, the APX activity was higher in transgenic lines as compared to UC by 2.0 and 2.2 fold (S2) and by 2.1 and 2.3 fold (S4) (Fig. 2). Under similar condition of UV stress, the CAT activity also increased by 1.3, 1.4 and 1.9 fold in transgenic lines (S2) and 1.5, 1.6 and 2.0 fold in transgenic line (S4) as compared to UC (Fig. 3).

Plants under stress tend to accumulate ROS that leads to membrane lipid peroxidation and Malondialdehyde (MDA) accumulation under oxidative stress. To check whether plants overexpressing AhCuZnSOD under stress were affected leading to H_2O_2 accumulation and lipid peroxidation, the H_2O_2 and MDA content was measured in the presence and absence of UV radiations. While there was no significant difference observed in the H_2O_2 and MDA content in the absence of UV stress, under stress conditions the results showed that there was an increase in the MDA and H_2O_2 content in untransformed control as well transgenic plants. However transgenic plants showed lower MDA and H_2O_2 content as compared to untransformed control plants. MDA content increased but was lower in transgenic lines as compared to untransformed control plants. The MDA content under 8hr, 16hr and 24hr of UV-B stress, the MDA content was higher by 1.2, 1.3 and 1.5 fold (S2) and 1.2, 1.3 and 1.6-fold increase in line S4 (Fig. 4). Similarly, in the H_2O_2 content, stress treated plants increased by 1.2-1.5 fold in line S2 and ~ 1.3-1.5 fold in line S4 as compared to untransformed control. The H_2O_2 content showed linear relation with the MDA content and with the increase in H_2O_2 in all the lines under stress, (Fig. 5).

IV. Discussion and Conclusion

Reactive oxygen species are formed in all aerobic organisms including plants and therefore the method is inevitable. Plants have evolved a complex antioxidant defence mechanism that consists of enzymatic and non-enzymatic pathways to eliminate ROS accumulation whereas maintaining level for signalling. Enzymatic pathways include superoxide dismutase (SOD) that dismutates O2- into H_2O_2 and has been considered to act as the 'first line of defence' against oxidative stress in plants (Alscheret al., 2002). Increased activity of SOD in response to environmental stresses has been reported in different plant species, which suggests its possible role in eliminating ROS from cells under stress (Yasar and Fikret 2013). Therefore, in the present study the stress alleviation by overexpression of the cytosolic superoxide dismutase, isolated from salt tolerant cell lines of Arachis hypogaea has been attempted in nicotiana tabacum.

Enhanced enzyme activity in plants is an adaptive mechanism under photo-oxidative stress. Overex- pression of SOD could easily augment this limited capacity of the plants to detoxify superoxide thereby strengthening the antioxidant defence system of the affected plant. An increase in total SOD activity under increasing levels of UV-B stress could result in improved protection against ROS by detoxifying O2- (Bornman et al 1997) Free radical generation is one of the initial cytochemical responses of plants exposed to ionizing radiations. These free radicals potentially initiate lipid peroxidation, leading to extensive membrane damage (Sreenivasulu et al. 2007). Du et al. (2003) have reported an increase in MDA content in Taxus cuspidata exposed to UV-C radiation. In our experiments, we found lower level of MDA content in transgenic lines as compared to WT. This observation suggested that the degree of damage to cell membrane of transgenic plant is significantly less than in WT, therefore, the higher activity of AhCuZnSOD in transgenic tobacco had played its roles in eliminating ROS, and thus prevented the membrane lipids from peroxidation. Hence H_2O_2 and MDA level serve as biomarkers for plants under stress. Lower H_2O_2 and MDA content in transgenic lines as compared to the untransformed control plants under stress indicates that the transgenic plants were able to get rid of ROS (H_2O_2). The lower level of H_2O_2 in transgenic lines may be due to the scavenging of ROS by the elevated antioxidant enzymes. Similar observation has also been reported by other groups in SOD overexpresing lines (Faize, 2011; Lee, 2013)

Thus a higher enzyme activity of transgenic plants seems to be the main cause for improved growth characteristics and enhanced tolerance of the transgenic plants to UV-B-induced oxidative stress. As ROS are a common denominator in plants exposed to a variety of environmental stresses, therefore strengthening of antioxidant defence pathway could be a viable strategy to develop stress-tolerant crop plants.

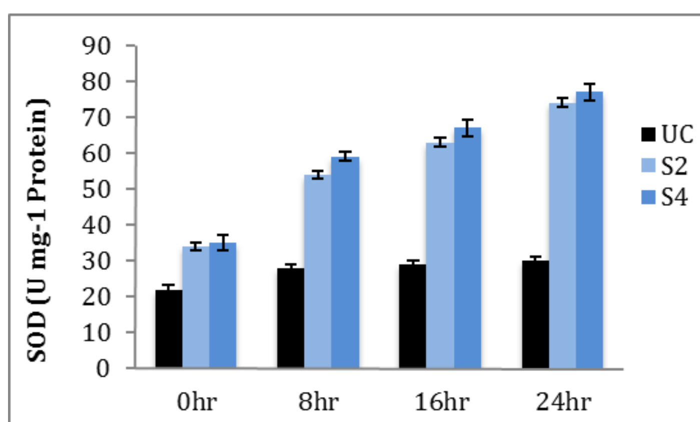

Fig. 1: Superoxide dismutase activity in the leaves of untransformed control and transgenic Nicotiana tabacum plants exposed to UV-B radiation for different intervals of time. Line above bars represents mean ± SE (n = 3). The treatments are significantly different ($p \leq 0.05$) between transgenic lines and wild type plants

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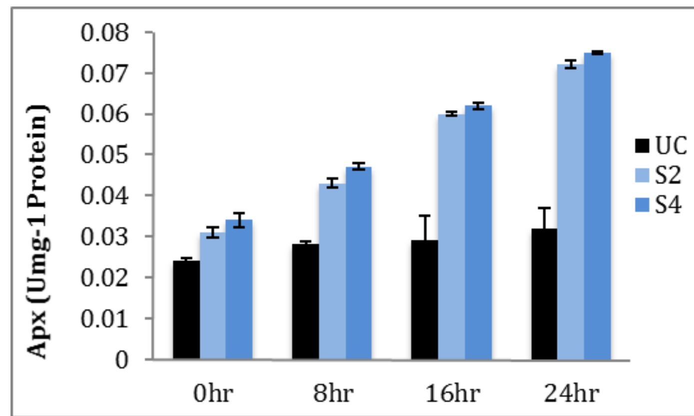

Fig. 2: Ascorbate peroxidase activity in the leaves of untransformed control and transgenic Nicotiana tabacum plants exposed to UV-B radiation for different intervals of time. Line above bars represents mean ± SE (n = 3). The treatments are significantly different ($p \leq 0.05$) between transgenic lines and wild type plants.

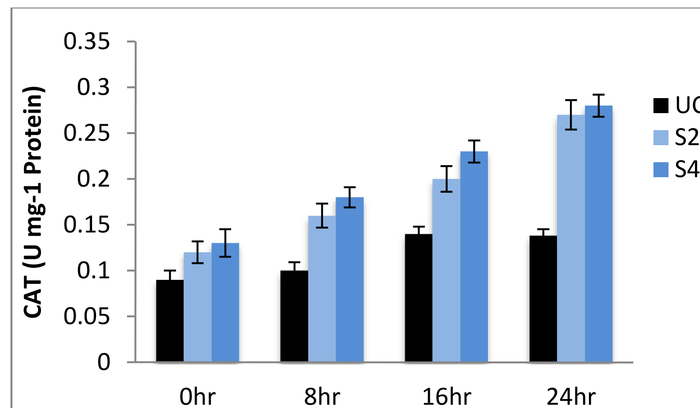

Fig. 3: Catalase activity in the leaves of untransformed control and transgenic Nicotiana tabacum plants exposed to UV-B radiation for different intervals of time. Line above bars represents mean ± SE (n = 3). The treatments are significantly different ($p \leq 0.05$) between transgenic lines and wild type plants.

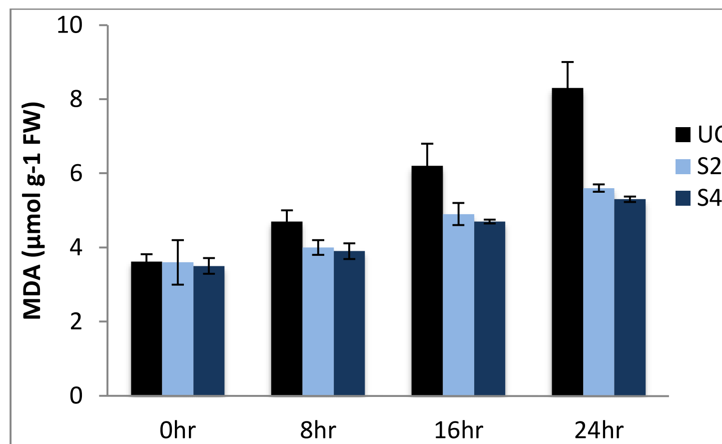

Fig. 4: Malondialdehyde content in the leaves of untransformed control and transgenic Nicotiana tabacum plants exposed to UV-B radiation for different intervals of time. Line above bars represents mean ± SE (n = 3). The treatments are significantly different ($p \leq 0.05$) between between transgenic lines and wild type plants.

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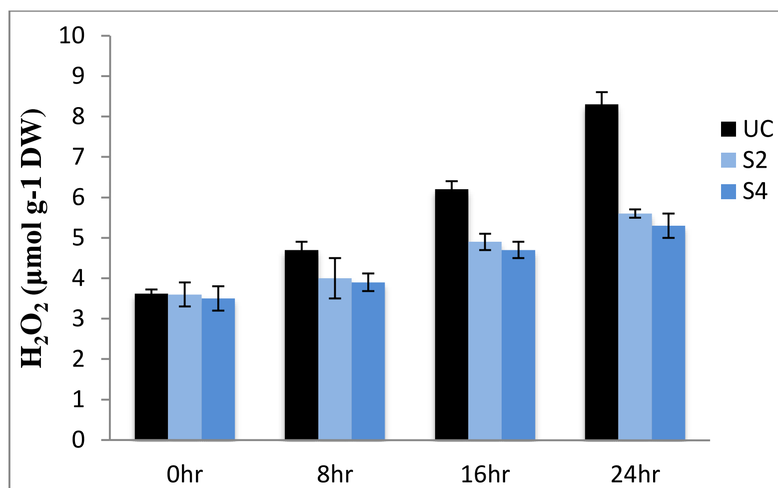

Fig. 5. Hydrogen peroxide content in the leaves of untransformed control and transgenic *Nicotiana tabacum* plants exposed to UV-B radiation for different intervals of time. Line above bars represents mean ± SE (n = 3). The treatments are significantly different ($p \leq 0.05$) between two genotypes.

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