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Salicylic acid alleviates methyl viologen induced oxidative stress through transcriptional modulation of antioxidant genes in *Zea mays* L

Pranjal Yadava[1]*, Nepolean Thirunavukkarasu[2], Palminder Kaur[1], Kaliyugam Shiriga[2], Ishwar Singh[1]*

[1]ICAR - Indian Institute of Maize Research, Pusa Campus, New Delhi 110 012, India
[2]Division of Genetics, ICAR- Indian Agricultural Research Institute, Pusa Campus, New Delhi 110 012, India
*Corresponding author: E-mail: isingh.dmr@gmail.com; pranjal.yadava@icar.gov.in

Abstract

Salicylic acid (SA) is an important growth regulator that participates in both biotic as well as abiotic stress response in plants. Both-biotic and abiotic stresses are characterized by elevated cellular reactive oxygen species (ROS). We examined the biochemical and molecular effects of SA on the ROS scavenging system in maize. Pre-treatment of maize plants with SA prior to stress, reduced electrolyte leakage, enhanced protein accumulation, improved root biomass and grain yield. SA application also led to a general enhancement in the biochemical activities of three major ROS scavenging antioxidant enzymes, viz. superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX). However, corresponding transcripts of various antioxidant isozymes depicted a dynamic response for different SA doses. Under stress, pre-treatment with 0.5 mM SA led to enhanced expression of all the Sod genes analyzed while higher SA concentration repressed many Sod genes. *Cat 1* showed clear dose-dependent repression in response to graded concentrations of SA, while *Cat 3* showed inverse dose-dependent activation in response to graded concentrations of SA. *Apx 1* was found to be up-regulated at 0.5 mM SA, while higher doses of SA, i.e. 1.0 and 1.5 mM led to its repression. All the three concentrations of SA repressed *Apx 2* gene. Taken together, our results indicate that SA has the potential to alleviate oxidative stress in maize through biochemical and transcriptional modulation of ROS scavenging pathway. Also, the concentration of SA has a great bearing in its alleviating role, with lower concentrations (viz 0.5 mM) being more desirable.

Keywords: salicylic acid, reactive oxygen species, antioxidant genes, oxidative stress, maize

Introduction

Salicylic acid (SA) has long been known for its role in the responses to biotic stresses in plants, especially, its ability to induce systemic acquired resistance against viral diseases (Gaffney et al, 1993). It participates in diverse signalling pathways and has cross-talk with various plant growth regulators and hormones. Recent studies have suggested that it can also modulate pathways leading to adaptation to abiotic stresses in plants (Kang et al, 2014). One of the common consequence of pathogen attack as well as that of incidence of abiotic stress is the rapid burst of reactive oxygen species (ROS). Persistent accumulation of high levels of ROS is detrimental for plant survival. Therefore, plants have evolved mechanisms to scavenge harmful ROS, in which, antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT) and peroxidases (POX) play a major role. There is an increasing interest in the interactive role between SA and ROS (Dat et al, 2007). SA occurs naturally in plants in very low amounts and it is known to be involved in transpiration, stomatal closure, nutrient uptake, protein synthesis, inhibition of ethylene biosynthesis, plant growth, thermogenesis, flower induction, photosynthesis, enzyme activities and other important regulatory physiological processes (Yusuf et al, 2013). There have been conflicting views on the effect of SA on ROS-mediated oxidative damage. Initial evidence indicated that SA was a potent inhibitor of heme-containing enzymes, viz. CAT and POX, and an inducer of hydrogen peroxide (H_2O_2) (Harfouche et al, 2008), thus further exacerbating accumulation of ROS under stress conditions. SA was also proposed as both a potent inducer of the NADPH-oxidase and an inhibitor of alternative oxidase, thus capable of indirect regulation of the redox status of the plant. To reconcile a considerable body of contradictory views on the question, whether SA is upstream of ROS or vice versa, several researchers proposed that SA and H_2O_2 form a self-amplifying feedback loop (Vlot et al, 2009).

There have been several studies, where pre-treatment of plants with SA has been shown to confer abiotic stress tolerance of various degrees. Cucumber and tobacco plants pre-treated with SA exhibited enhanced protection to oxidative stress induced by methyl viologen (MV) application at 3 days after the SA pre-treatment (Strobel and Kuc, 1995). Similarly, pre-treatment of barley seedlings with SA in the dark via the transpiration stream fully blocked the

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subsequent light-induced inhibitory effect of MV on photosynthesis and also decreased the MV-induced production of H_2O_2, lipid peroxidation, and electrolyte leakage (Ananieva et al, 2002), while inducing the activities of certain antioxidant enzymes (Ananieva et al, 2004). These results were also confirmed using SA-deficient NahG transgenic rice plants, which showed great sensitivity to MV. These plants also had lower glutathione content, which may explain the greater sensitivity of these plants to oxidative damage and also suggests that SA may regulate glutathione synthesis in rice (Kusumi et al, 2006).

Although exogenous SA mediated perturbation in cellular redox state under stress has been studied in many plants, its mechanism of action, especially its effect on transcriptional dynamics is unexplored. This study was undertaken to determine the physiological and biochemical changes in maize plants treated with SA during MV-induced oxidative stress, and to investigate the role of this plant growth regulator in altering the transcriptional dynamics of the antioxidant genes. Elucidating the mechanisms of plant growth regulator mediated stress adaptation in plants would pave way for developing appropriate spray based technologies or transgenic crops that are resilient to environmental stresses.

Materials and Methods

Plant growth and treatments

This experiment was conducted at the Maize Physiology Field Research Facility, ICAR- Indian Agricultural Research Institute, New Delhi, India (Latitude: 280°38'23"N, Longitude: 770°09'27"E, Altitude: 228.61 masl) during July-October 2012. The mean maximum and minimum temperatures during the period of study were 39.1 and 13.9°C, respectively, while total rainfall was 481.8 mm. HQPM 1, a single cross (HKI 193-1 x HKI 163) quality protein maize hybrid was grown in micro-plots of 3 rows with 2 m row length with 10 plants in each row. The crop was irrigated only once just after sowing and remained completely rainfed thereafter. Recommended fertilization and management practices were followed. Plants were sprayed with different doses of SA @ 0.5, 1.0 and 1.5 mM at the flowering stage. All the spray formulations contained Tween 20 (0.1%) as surfactant. The control plants were mock sprayed with only Tween 20 dissolved in distilled water. Artificial oxidative stress was induced in the plants by spraying 200 µM MV. Leaves were harvested from five different plants per treatment at two stages i.e. after 48 h from application of SA (post treatment) and after 48 h from MV application (post stress); snap freezed and stored at -70°C.

Root growth and yield

At harvest, the data on total root biomass and root length were recorded and expressed as g per plant and cm per plant, respectively. The cobs were harvested from the SA treated and untreated plants at physiological maturity (30 % grain moisture). The final grain yield at 14% grain moisture was calculated as g per plant.

Membrane stability

Five leaf discs weighing 100 mg each from five different plants per treatment were washed, dried and placed in a glass test tube containing 20 ml deionized water in replicates of three for each treatment. The samples were kept for 24 hrs at room temperature and initial conductance values (E1) were recorded with conductivity meter. The content was then boiled for 30 min in a boiling water bath (100°C), cooled to room temperature and final conductivity values (E2) were determined. Cell membrane injury was represented in terms of total electrolyte leakage (%) and calculated by following formula:

$$Electrolyte\ Leakage\ (\%) = \frac{Conductivity\ before\ boiling\ (E1)}{Conductivity\ after\ boiling\ (E2)} x100$$

Total soluble proteins and anti-oxidant enzyme assays

Leaf samples (1 g) were homogenized in 10 ml ice-cold 50 mM potassium phosphate buffer (pH 7) containing 100 mg g^{-1} of polyvinyl pyrrolidone (PVP) in mortar and pestle and centrifuged at 10,000 rpm for 30 min at 4°C. The supernatant was then collected and stored at -20°C. The supernatant collected was used for different antioxidant enzyme assays after estimating total protein content (Bradford, 1976) using bovine serum albumin as standard.

Superoxide dismutase (SOD) was assayed according to the method of Kakkar et al (1984). The assay mixture contained 1.2 ml of sodium pyrophosphate buffer (0.025 M, pH 8.3), 0.1 ml of phenazine methosulphate (PMS) (186 µM), 0.3 ml of nitro blue tetrazolium (NBT) (300µM), 0.2 ml of the enzyme extract and water in a total volume of 2.8 ml. The reaction was initiated by the addition of 0.2 ml of NADH. The mixture was incubated at 30°C for 90 s and arrested by addition of 1.0 ml of glacial acetic acid. The reaction mixture was then shaken with 4.0 ml of n-butanol, allowed to stand for 10 min and centrifuged. The intensity of the chromogen in the butanol layer was measured at 560 nm in a spectrophotometer. One unit of enzyme activity is defined as the amount of enzyme that gave 50% inhibition of NBT reduction in one minute. Catalase (CAT) activity was assayed using the method of Aebi (1983). The assay solution contained 50 mM potassium phosphate buffer (pH 7) and 30 mM H_2O_2 and 50 µl enzyme extract. The activity was estimated by monitoring the decrease in absorbance of H_2O_2 within 30 s at 240 nm. Unit of enzyme activity was taken as the change in absorbance per minute at 240 nm. The non-specific peroxidases (POX) activity was assayed using the method of Reddy et al (1995). To 3.0 ml of pyrogallol solution (0.05 M), 0.1 ml of the enzyme extract was added and the spectrophotometer was adjusted to read zero at

Table 1 - List of primer pairs used for the qRT-PCR amplification of 11 antioxidant genes in maize.

S No.	Gene name	Primer Sequence
1	Zm Sod2	F-GATCATTGGCCGAGCTGTTGTTGT R-TCTATGGTGTTTGCCTCGGAGGTT
2	Zm Sod4	F-AAACAAATGGGTCGCTGGACTTCG R-ACAGTTCAGCTCATCGGGTGCTTA
3	Zm Sod9(4A)	F-CGTGTTGCTTGTGGGATCATTGGA R-TCGGTGGCTACAGGTGCATAATGA
4	Zm Cu-Zn Sod (chl)	F-TGTTGCAAATGCTGAGGGCATAGC R-CCAACAACACCACATGCCAGTCTT
5	Zm Fe Sod	F-AGCACAGGTCTGGAACCATCACTT R-ACAGCTGTAAGGCTGAGCGGATAA
6	Zm Mn Sod	F-TTGTGTACCTGCTGGACCAAGTGT R-ACTACGAGCAGCAGAAAGTGGAGT
7	Zm Apx1	F-TGCTGCCTGTATGGAATCATGTGG R-TACCAACAACAGCCTAAGGGCTCA
8	Zm Apx2	F-TTGTTGACAAATACGCAGCGGACG R-ACACTCTTCTGGTTCGTTACGCCT
9	Zm Apx 8	F-AGTATGCAGATGACCAGGAAGCGT R-TCGGAAGGTGAAGAGCTTGTGTCA
10	Zm Cat1	F-ACCTGAAGCCGAGCATGTAAGGAT R-ATAATCGACCACCGACCATCAGCA
11	Zm Cat3	F-CCGGCTCAACATGAAGGCAAACAT R-TTCTCTTGTTCCTGGCGACGACAT

430 nm. To the test cuvette, 0.5 ml of H_2O_2 (1%) was added and mixed. The change in absorbance was recorded every 30 s up to 3 min in a spectrophotometer. One unit of peroxidase was defined as the change in absorbance per min at 430 nm.

Total RNA isolation and cDNA preparation

Total RNA was extracted from pools of three plants for each experimental sample using the RNeasy mini kit (Qiagen, Hilden, North Rhine- Westphalia, Germany) followed by treatment with DNase I to remove any genomic DNA contamination. Concentrations were determined with a NanoDrop spectrophotometer (Thermo scientific NanoDrop 1000). The cDNA was synthesized using Superscript III Kit (Invitrogen) from 1 µg of total RNA according to the manufacturer's protocol. The reverse transcription reaction was carried out at 44°C for 60 min followed by 92°C for 10 min. The reaction without template was included as control.

Quantitative Real Time -PCR Analysis

The sequences of the corresponding maize genes of the ROS scavenging system were obtained from the GenBank. Using these sequences, 11 sets of primers were designed using IDT PrimerQuest for further gene expression analysis (Table 1). For the quantitative real-time PCR (qRT-PCR), 1 µL cDNA (10 ng) was used in 25 µL reactions using Hot start-IT SYBR green qPCR master mix (Affymetrix) according to the manufacturer's instructions. Amplification was performed on the Mx3005P multiplex quantitative PCR system (Stratagene). For each sample, at least three replications were performed in one experiment. The threshold cycles (CT) of each test target were averaged for triplicate reactions. 18S rRNA was used as internal control (Schmittgen and Livak, 2008). The gene expression values were normalized by subtracting the mean reference gene (18S rRNA) CT value from individual CT values of corresponding target genes (ΔCT). The fold change value was calculated using the expression, where ΔΔCT represents difference between the ΔCT condition of interest (i.e. gene expression in SA treated plants after MV stress) and ΔCT control (i.e. gene expression in mock treated plants after MV stress).

Results and Discussion

Root growth and yield

The SA treated plants were monitored till the physiological maturity stage. Significantly higher root biomass and grain yield were observed in SA treated plants over the control. Although marginal increase in root length was also observed in SA treated plants over control but this increase was non-significant. The most effective concentration of SA was 1 mM, which improved root biomass and grain yield by 45.7 and 32.9% over untreated plants (Table 2), indicating that SA is capable of alleviating harmful effects of oxidative stress in maize.

Previous studies corroborate that lower concentrations of SA (viz. 0.1 mM to 0.5 mM) are most optimal in imparting stress tolerance in plants with low levels of resident SA, maize being one of them (Janda et al, 1999; Ding et al, 2002; Kang and Saltveit, 2002; Kang et al, 2003; Nemeth et al, 2002; Tasgin et al, 2003; He et al, 2005; Shi et al, 2006). Many previous studies demonstrate that SA could be a potential compound for the reduction of abiotic stress sensitivity of the crops against a range of abiotic stresses, like ozone (Rao and Davis 1999; Koch et al, 2000), salt and osmotic (Borsani et al, 2001; Molina et al, 2002), UV-B (Surplus et al, 1998; Nawrath et al, 2002), drought (Nemeth et al, 2002; Munne-Bosch and Penuelas, 2003), paraquat (Yang et al, 2004), heat (Clarke et al, 2004; He et al, 2005; Shi et al, 2006), cold (Janda et al, 1999; Kang and Saltveit, 2002; Kang et al, 2003; Tasgin et al, 2003; Scott et al, 2004) and metal stress (Metwally et al, 2003; Yang et al, 2003; Pal et al, 2005). Application of ascorbic acid has also been reported to have similar effects on root biomass and grain yield in maize under MV induced oxidative stress (Yadava et al, 2013).

Membrane stability

In order to understand the effect of SA in imparting stability to the cell membrane, we compared total electrolyte leakage in SA treated plants under stress with that of untreated plants under stress. SA treatment led to significant reduction in electrolyte leakage under stress. Under stressed condition, each of the SA treatment, i.e. 0.5, 1.0, and 1.5 mM reduced the electrolyte leakage by 2.7, 4.4 and 3.3%, respectively over the mock treated control. Interestingly, SA was capable of reducing ion leakage under unstressed conditions also, indicating its general role in imparting stability to the membrane. A reduction of 10.4, 15.4, and 11.77% in electrolyte leakage was

Table 2 - Root biomass, root length and grain yield per plant at maturity for different treatments of SA under stressed conditions. Each value represents mean of three independent replications ±SE.

SA Conc. (mM)	Root biomass (g plant^{-1})	Root length (cm plant^{-1})	Grain weight (g plant^{-1})
0.0	12.39 ± 1.45	27.36 ± 1.21	38.42 ± 2.87
0.5	15.32 ± 2.31	28.12 ± 2.07	43.42 ± 1.94
1.0	18.05 ± 1.79	29.37 ± 1.94	51.07 ± 3.17
1.5	16.21 ± 1.03	28.61 ± 1.78	46.37 ± 2.86

observed in response to 0.5, 1.0, and 1.5 mM SA under unstressed conditions (Figure 1A).

In another study also, SA application was found to reduce electrolyte leakage under different abiotic stresses. In tomato plants, application of 0.1 mM SA led to 44% and 32% reduction in electrolyte leakage in plants subjected to 150 mM NaCl and 200 mM NaCl, respectively, compared to the untreated plants (Stevens et al, 2006). Supporting evidences for SA mediated reduction in electrolyte leakage have also been reported in maize, rice and cucumber hypocotyls under chilling stress (Kang and Saltviet, 2001). Maintaining integrity of cellular membranes under stress conditions is considered an integral part of the tolerance mechanism.

Total soluble proteins

In plants, oxidative stress generally leads to enhanced synthesis and accumulation of certain proteins, especially those related to ROS scavenging and chaperone functions (Horvath et al, 2007). We also found enhanced total soluble proteins in all the plants sprayed with MV in comparison to unstressed plants. Next, we were interested to know whether application of SA had any effect on accumulation of total soluble proteins. In unstressed conditions, 48 h after SA treatment, total soluble protein content of the samples showed an inverse dose dependent response. The protein content increased by 17.8, 14.4, and 12.5% in response to 0.5, 1.0, and 1.5 mM SA, with 0.5 mM concentration being most effective in increasing the protein content. Further, we studied the effect of SA treatment on protein content under stress conditions. Under stress, different doses of SA were found to be capable of enhancing the protein content by 8.3, 3.6, and 1.2% in comparison to the mock control. Here also, inverse dose dependence as seen for unstressed conditions was observed, with 0.5 mM concentration being statistically most significant (Figure 1B).

Since, SA acts as a signalling molecule; it has potential to up-regulate several genes and their products. We also observed enhanced accumulation of total soluble proteins in response to SA application, especially so with lower concentration of 0.5 mM. Evidence for SA mediated accumulation of several classes of proteins, viz. topoisomerases, elongation factors, transcriptionally active long terminal repeat retrotransposon families, heat-shock proteins (Hsp70, Hsp17.6, Hsc70), cyclophilins, dehydrins, pathogenesis related proteins, glycine-betaine etc. have been provided in various studies (Horvath et al, 2007). Also, physiologically lower concentrations, viz 0.1 to 0.5 mM have been reported to be most effective in various studies.

Antioxidant enzyme activities

Plants exhibit a fine homeostasis between production and scavenging of ROS. The ability of the plant to rapidly quench extra ROS production during stress determines its tolerance capacity (Gill and Tuteja, 2010). SOD, CAT and POX are the important ROS scavenging enzymes in plants. Therefore, we assayed the biochemical activities of these enzymes in response to different doses of SA before and after application of oxidative stress (Figure 2). We observed that MV can drastically reduce the specific activity of SOD. There was 54.4% reduction in SOD activity in mock sprayed plants after application of MV. During unstressed conditions, lower doses of SA application

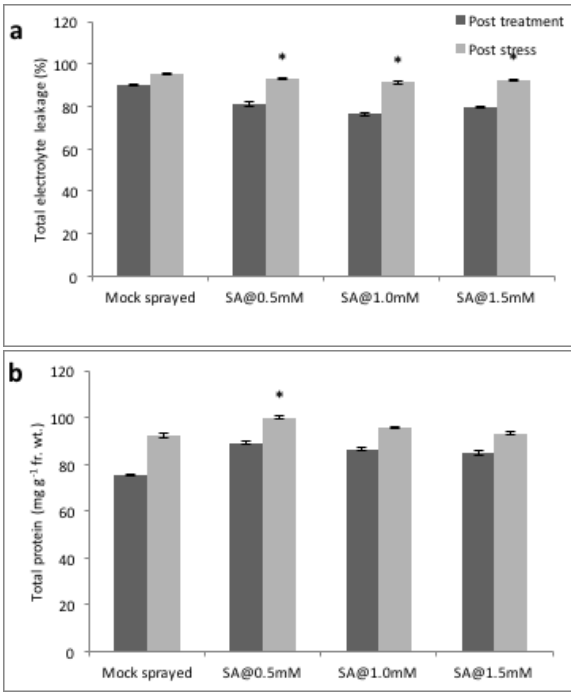

Figure 1 - Total electrolyte leakage (a) and total soluble protein (b) in response to different concentrations of SA under control and stressed conditions in maize. Each value represents mean of three independent replications ± S.E, asterisk indicates significant difference compared to the control post stress at $P<0.05$.

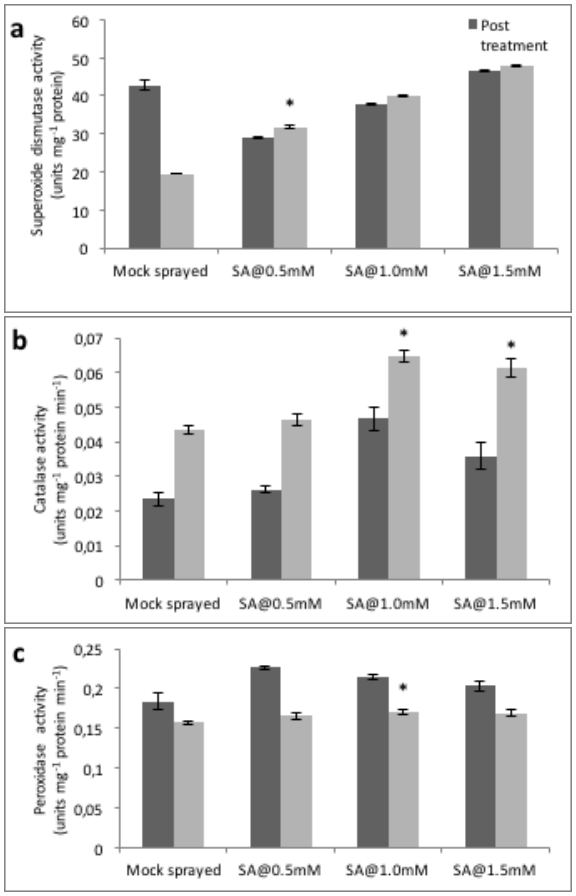

Figure 2 - Specific activities of superoxide dismustase (a), catalase (b) and peroxidases (c) in response to different concentrations of SA under control and stressed conditions in maize. Each value represents mean of three independent replications ± S.E, asterisk indicates significant difference compared to the control post stress at $P < 0.05$.

(0.5 mM and 1.0 mM) led to reduced SOD activities while the higher dose of SA (1.5 mM) increased SOD activity marginally by 9.0% . However, under stressed conditions, SA was found to have considerable effect in enhancing SOD activity. An increase of 62.6, 104.8, and 144.8% in SOD activity was observed in stressed plants pre-treated with 0.5, 1.0, and 1.5 mM SA respectively, as compared to the mock treated plants. In contrast to SOD, a general enhancement in CAT activity was observed upon induction of oxidative stress. SA was found to further increase CAT activity under stress. Under stress, CAT activity increased by 7.3, 49.3, and 41.3% in response to 0.5, 1.0, and 1.5 mM SA, with later two concentrations having most significant effect. Like SOD, non-specific POX activity also showed a general reduction under MV induced oxidative stress. Once again, confirming its role in alleviating oxidative stress, SA was found to enhance POX activity. Application of all the concentrations of SA increased POX activity in both stressed as well as unstressed conditions. Under stressed conditions, pre-treatment of 0.5, 1.0, and 1.5 mM SA increased POX activity by 5.3, 8.9, and 7.9% respectively as compared to mock sprayed plants. Thus, SA application led to enhanced activities of all the three ROS scavenging enzymes (SOD, CAT, and POX) under stress, highlighting its positive role in alleviating oxidative stress.

In our experiment, SA application led to enhanced activities of all the three scavengers, i.e. SOD, CAT, and POX. In many previous studies, SA mediated alteration in various antioxidant enzyme activities under stress has been reported. However, there exist considerable inconsistencies and variations in changes in enzyme activities. The response has been largely dependent on genotypes in question, the dose of SA, time of application and nature of abiotic stress. For example in a recent study in wheat, treatment with SA alone increased all the antioxidant activities in Gerek-79 genotype (Multu and Atici, 2013). However, the same treatments with SA decreased the activities of CAT and SOD in Bezostaya genotype, while increased that of POX. On the other hand, salt applications alone increased the activities of POX and SOD, while decreased that of CAT in Bezostaya genotype. The same salt applications increased the POX activity, while decreased the activities of CAT and SOD in Gerek-79 genotype. In plants under saline conditions, except 0.75 M NaCl, treatments with SA increased the activities of CAT, POX, and SOD in both the varieties compared with plants applied with salt alone. In plants applied with 0.75 M NaCl, however, treatments with SA decreased CAT activity. In another study, incubation of Arabidopsis leaves with SA enhanced SOD activity in a dose-dependent manner, while treatment of leaves with 5 mM SA decreased CAT by 33% compared with control leaves. In the same study, concentrations of 0 to 5 mM SA had no major effect on POX activity (Rao et al, 1997). We observed that MV application can drastically reduce SOD activity. Loss of ROS scavenging capacity due to reduction in SOD activity might be a major factor contributing to lethal effects of MV induced oxidative burst. In contrast to SOD, a general enhancement in CAT activity was observed upon induction of oxidative stress. This may be due to the crucial need of the plant to expeditiously remove harmful H2O2 produced due to MV induced severe oxidative stress.

Antioxidant gene expression analysis

In order to understand more about the molecular mechanisms that may contribute to the variation of the ROS scavenging enzymes, we studied the transcriptional dynamics of the corresponding genes. Though biochemically indistinguishable, some of these scavenging enzymes have a number of isozymes. For example, SODs are classified into three types: Mn-, Fe-, and Cu/Zn-SOD, depending on the metal found in the active site. In the higher plants, SODs are found in the cytosol, plastids, and mitochondria. In maize, different SOD isozymes are reported: four Cu/Zn cytosolic isozymes (SOD-2, SOD-4, SOD-4A,

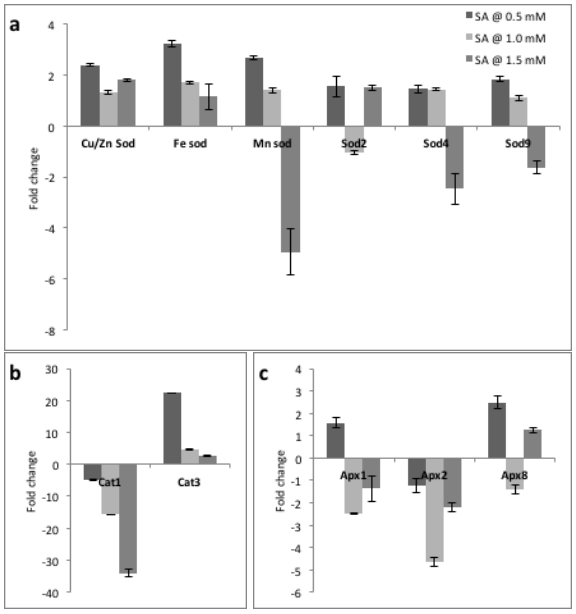

Figure 3 - Fold change in transcript abundance of various Sod (a), Cat (b), and Apx (c) genes in SA pre-treated plants under stress as compared to control plants under stress. Each value represents mean of three independent replications ± S.D. Negative values show gene repression.

and SOD-5), four mitochondrial-associated Mn SODs (SOD-3.1, SOD-3.2, SOD-3.3, and SOD-3.4), and one Cu/Zn chloroplast-associated isozyme (Baum and Scandalios, 1979; Scandalios, 1997; Guan and Scandalios, 1998). Also, in maize genome, there exist multiple gene homologs coding for one group of scavenging enzymes. We designed DNA primers that were capable of distinguishing various homologs of these genes (Table 1).

Figure 3 depicts fold change of transcript accumulation in SA pre-treated plants after 48 hrs of MV treatment as compared to mock treated controls. Under stress, pre-treatment with 0.5 mM SA led to enhanced accumulation of transcripts of all the *Sod* genes analyzed. The fold increase ranged from 1.4 times for *Sod 4* to 3.2 times for *Fe Sod* transcript. The positive fold change for other Sod transcripts was 2.3, 2.6, 1.5, and 1.8 times for *Cu/Zn Sod*, *Mn Sod*, *Sod 2* and *Sod 9* respectively. When the concentration of SA was doubled to 1.0 mM, fold increase in transcript accumulation for various Sod genes was decreased as compared to that of 0.5 mM dose, with *Sod 2* showing repression. At 1.0 mM SA concentration *Cu/Zn Sod*, *Fe Sod*, *Mn Sod*, *Sod 4* and *Sod 9* genes were up-regulated by 1.3, 1.7, 1.4, and 1.1 times respectively as compared to the mock control. On the other hand, *Sod 2* was repressed by 1.0 fold. When, the concentration of SA spray was further enhanced to 1.5 mM, even more number of *Sod* genes showed repression. In this condition, Mn Sod exhibited a repression of 4.9 times, while *Sod 4* and *Sod 9* showed 2.5 and 1.6 fold repression respectively. Thus, a dynamic response of transcript accumulation of various *Sod* genes was observed, which was also affected by the concentration of SA, with 0.5 mM SA being most effective in uniformly up-regulating various isozymes of *Sod*. At the protein level also, SA mediated enhancement of SOD enzyme activity under stress was most significant for 0.5 mM concentration. This also correlates with significantly higher total protein accumulation observed in samples pre-treated with 0.5 mM SA concentration.

We analyzed two *Cat* genes, *Cat 1* and *Cat 3* and both these genes showed contrasting transcriptional profile. *Cat 1* showed clear dose dependent repression in response to graded concentrations of SA, while *Cat 3* showed inverse dose dependent activation in response to graded concentrations of SA. Also, sensitivity of *Cat* genes to SA was seen to be very high for both activation and repression. *Cat 1* expression reduced by 5.0, 15.7, and 34.1 fold respectively in response to 0.5, 1.0 and 1.5 mM SA. On the other hand, *Cat 3* expression increased by 22.3, 4.6, and 2.6 folds respectively in response to 0.5, 1.0, and 1.5 mM SA, with 0.5 mM SA once again being most effective. Transcriptional profile of the three *Apx* genes analyzed exhibited dynamic response. *Apx 1* was found to be up-regulated by 1.6 fold at 0.5 mM SA, while higher doses of SA, i.e. 1.0 and 1.5 mM led to repression of *Apx 1* by 2.5 and 1.4 times respectively. All the three concentrations of SA repressed *Apx 2* gene. *Apx 2* transcript accumulation was reduced by 1.2, 4.6, and 2.2 times respectively in stressed plants pre-treated with 0.5, 1.0, and 1.5 mM SA. On the other hand, *Apx 8* transcript abundance increased 2.5 and 1.2 folds in response to 0.5 mM and 1.5 mM SA, while it decreased 1.4 fold in response to 1.0 mM SA.

The dynamic transcriptional profile of various genes of the ROS scavenging pathway in response to various doses of SA suggest that different ROS genes are differentially regulated by SA and may also likely to form a feedback system. For example, originally, H_2O_2 was proposed to function downstream of SA on the basis of evidence that high levels of SA can bind and inhibit H_2O_2-removing enzymes, such as CAT and APX (Durner and Klessig, 1996; Chamnongpol et al, 1998). Furthermore, APX is post-transcriptionally suppressed by SA (Mittler et al, 1998; Yuan and Lin, 2004), and CAT is downregulated at the level of steady-state mRNA (Dorey et al, 1998). Later, it was found that increased H_2O_2 levels also act upstream of SA to induce endogenous SA (Leon et al, 1995). Thus, SA and H_2O_2 compose a positive feedback loop. Moreover, endogenous SA concentration of the plant can directly affect accumulation of ROS. This has been shown in SA deficient transgenics and mutants in Arabidopsis. Transgenic Arabidopsis expressing a bacterial salicylate hydroxylase NahG, and Arabidopsis mutant sid2 (SA induction deficient) and eds5 (enhances disease susceptibility), which have a very low level of SA (below 1 μg g^{-1} fresh weight), accumulate a high level ROS and therefore prone to

more severe damages from abiotic stress (Lederer and Böger, 2003; Nawrath and Metraux, 1999; Rao and Davis, 1999; Nawrath et al, 2002; Clarke et al, 2004). Recent studies have also shown that SA is capable of inducing promoters and transcription factors (Van der Does et al, 2013).

In conclusion, our results suggest that SA have potential to alleviate oxidative stress and biochemical and transcriptional modulation of ROS scavenging pathway might be a key mechanism behind its protective role. Also, the concentration of SA is important in its alleviating role, with lower concentrations (viz. 0.5 - 1.00 mM) being more favourable. The increase in SOD, CAT, and POX activities and associated transcriptional modulation with the application of SA, strengthen the conjecture that SA might have neutralized the harmful ROS by not only acting as antioxidant molecule itself but also as a signalling molecule causing up-regulation of expression of genes and specific factors related to synthesis of antioxidant enzymes. The results of the investigation confirm the importance of exogenously applied SA in conferring protection against oxidative stress in maize through modulation of antioxidant pathway.

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