

The Effects of *NPR1* Dependent Salicylic Acid Change in Increasing Salt Tolerance of Soybean Leaves by Acclimation

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Abstract

Non-expressor of Pathogen Related 1 (*NPR1*) is a regulatory gene of the salicylic acid (SA) signaling pathway, the detailed mechanism of which is still not well understood. This study investigated the effects of *NPR1*-dependent SA level change on increasing salt tolerance of soybean leaves with acclimation. Salt-sensitive ('SA88') and salt-tolerant ('Erensoy') soybean (*Glycine max* L.) plants were treated with increasing NaCl concentrations (25, 50, 75, and 100 mM; acclimation) and with 100 mM NaCl directly (non-acclimation) in two groups. The results showed that acclimation treatment alleviated salt-induced damage in the sensitive cultivar with increasing superoxide anion radical scavenging activity, and decreasing hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) content. However, the APX, CAT, and GST enzyme activities were increased by acclimation treatment, with the highest increase observed in GST enzymes. Interestingly, the *Gmnp1* gene expression was upregulated in all treatments but was more pronounced in non-acclimation. Furthermore, the highest increase in endogenous SA level was under acclimation treatment in 'SA88'. In conclusion, the results firstly showed that an acclimation process is useful for increasing salt tolerance in sensitive soybean plants with only ROS-induced *NPR1*-independent SA accumulation but not through the *NPR1*-dependent SA signaling pathway.

Keywords: acclimation; *NPR1*; salt stress; salicylic acid; soybean

Abbreviations: APX-ascorbate peroxidase; CAT-catalase; GST-glutathione-*s*-transferase; H₂O₂-hydrogen peroxide; MDA-malondialdehyde; *NPR1*-non pathogen related 1; POX-peroxidase; ROS-reactive oxygen species; SOD-superoxide dismutase.

Introduction

Salicylic acid (SA) is a signal molecule in plant defense responses (Chen *et al.*, 2009). Firstly, SA was found to be more pronounced under biotic stress, during pathogen attack, in the expression of pathogen-related (PR) genes, and in systemic-acquired resistance (Dempsey *et al.*, 1999; Durrant and Dong, 2004). However, in recent years, SA was found to also play a role in plant responses to abiotic stresses, such as high salinity, high temperatures, and chilling (Ashraf *et al.*, 2010; Hayat *et al.*, 2010).

Many studies about the effects of SA on plants under salinity were reported in literature. For example, salt-induced oxidative stress in *Hordeum vulgare* was minimized by an SA-mediated decrease in cellular malondialdehyde and reactive oxygen species (Fayez and Bazaid, 2014; Khan *et al.*, 2014). In another study, exogenously sourced SA was reported to improve salt tolerance in *Triticum aestivum* through an enhanced transcript level of antioxidant genes and increased activity of Asc-GSH pathway enzymes (Li *et*

al., 2013). Also, Ardebili *et al.* (2014) reported that SA decreased the Na⁺/K⁺ ratio and SOD enzyme activity under salinity in *Glycine max* L. plants.

Salt stress induces reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, in plants (Parida and Das, 2005). High ROS levels can result in extensive damage to proteins, DNA, and lipids, thereby affecting normal cellular functions, which can lead to permanent metabolic dysfunction and plant death (Anjum *et al.*, 2015). On the other hand, ROS also serve as stress signaling molecules and regulate the acclimation process (Miller *et al.*, 2010). Plants have a complex system for detoxifying ROS that includes nonenzymatic (ascorbate, glutathione, and tocopherols) and enzymatic (SOD, CAT, APX, POX, and GR) antioxidants. Several experiments have shown that plants with a successful antioxidant defense system have a higher tolerance to environmental stress factors (Shalata *et al.*, 2001; Azevedo Neto *et al.*, 2006; Seckin *et al.*, 2010).

Non-expressor of PR genes (*NPR*) is a master coactivator of PR1 and most SA-induced genes and was the

first redox sensor described for SA-regulated genes (Mou et al., 2003). The current knowledge indicates that SA-promoted redox modification of cysteine residues in *NPR1* determines the levels of active, reduced, and monomeric forms of *NPR1* in the nucleus (Kinkema et al., 2000; Tada et al., 2008; Lindermayr et al., 2010). The levels of nuclear *NPR1* are also regulated by other SA-mediated mechanisms, such as proteasome-mediated degradation and phosphorylation (Pajeroska-Mukhtar et al., 2013).

Salicylic acid is essential for *NPR1* redox modification; however, the mechanism by which it controls this process remains unclear. *NPR1* reduction is catalyzed by thioredoxin *TRXh5* (Tada et al., 2008; Kneeshaw et al., 2014), which is coded by the only member of the *TRXh* gene class transcriptionally induced by SA and oxidative stress (Laloi et al., 2004; Tada et al., 2008; Belin et al., 2015). Whether *NPR1* monomerization also occurs under oxidative stress has yet to be clarified. However, a recent study on *Arabidopsis* showed that *NPR1*-dependent SA signaling is key to controlling the entry of Na^+ into the root tissue, transporting it into the shoot, and preventing K^+ loss (Jayakannan, 2015).

Acclimation is a process by which plants can improve their physiologic ability to adapt to various environmental changes. An analysis of the acclimation process in plants can be useful in improving their salt tolerance mechanism and preventing crop yield losses (Aksoy and Dinler, 2014). Some reports have determined that acclimation has an effect on ion change and exclusion, osmolyte accumulation, and the growth process; however, the detailed mechanisms of these effects have not yet been reported (Umezawa et al., 2000; Djanaguiraman et al., 2006; Saha et al., 2010; Pandolfi et al., 2012). Thus, in the present work, the changes in physiologic variables, ROS, antioxidant enzyme activities, endogenous SA level, and *npr1* gene expression were comparatively analysed in acclimated and non-acclimated soybean leaves under salinity.

Collectively, the interaction between ROS-induced antioxidant system and *NPR1*-dependent SA level change was determined in soybean leaves under salinity. The present study examined whether the change in ROS affects antioxidant enzymes through *NPR1*-dependent or-independent SA signaling in salt-acclimated soybean leaves.

Materials and Methods

Experimental design

Soybean (*Glycine max* L.) seeds were obtained from a commercial provider ('SA88', Agrova, Adana, TR; 'Erensoy', Batem, Antalya, TR). Two kind of soybean cultivars were determined as salt sensitive ('SA88') and salt tolerant ('Erensoy') through preliminary experiments. The seeds were sterilized in 5% hypochloride solution for 10 min, rinsed three times with distilled sterile water, and then sown in plastic trays (10 cm × 14 cm) filled with soil under dark conditions. After germination, seedlings were taken into a growth chamber at 25 °C with 16 h light/8 h photoperiod and light intensity of 500 $\mu\text{mol m}^{-2}\text{s}^{-1}$ with Hoagland solution for 3 weeks. There were two groups. First one, 21 days soybean plants were exposed to 0, 25, 50,

75 and 100 mM NaCl concentrations at 24h interval in Hoagland solution and the second one; 100 mM NaCl was applied for 4 days directly. After the stress treatment, the leaves of the plants were harvested and stored at -80 °C.

Analysis

Stomatal conductance

Stomatal conductance was measured on 4th day of stress treatment using a portable steady-state porometer (model SC-1, Decagon Devices, Inc., Pullman, WA, USA). Experimental Data 3 were repeated and six specimens per copy were collected.

Malondialdehyde content

The level of lipid peroxidation in leaf samples was determined in terms of the malondialdehyde (MDA) content according to the method specified by Madhava Rao and Sresty (2000). The MDA content, an end product of lipid peroxidation, was determined by using the thiobarbituric acid reaction. The MDA concentration was calculated from the absorbance at 532 nm, and measurements were corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. An extinction coefficient of 155 $\text{mM}^{-1}\text{cm}^{-1}$ was used to determine the MDA concentration.

Hydrogen peroxide content

The H_2O_2 content was determined according to Velikova et al. (2000). Fresh leaves (0.1 g) were homogenized in 5 ml of 0.1% trichloroacetic acid (TCA) and centrifuged at 12,000 rpm for 15 minutes. The supernatant (0.5 ml) was then mixed with 0.5 ml of buffer (10 mM potassium phosphate, pH 7) and 1 ml of 1 M KI. The absorbance reading was taken at 390 nm and H_2O_2 content was expressed as $\mu\text{mol g}^{-1}$ dry weight.

Superoxide scavenging activity

Scavenging ability of superoxide anion radical was evaluated by the method of negative staining technique (De Rosa et al., 1979). This system contained 0.48 ml of 0.05 M sodium carbonate buffer (pH 10.5), 0.02 ml of 3 mM xanthine, 0.02 ml of 3 mM ethylenediaminetetraacetic acid disodium salt (EDTA), 0.02 ml of 0.15% bovine serum albumin, 0.02 ml of 0.75 mM nitroblue tetrazolium (NBT) and 0.02 ml of sample solution. After at 25 °C for 10 min, the reaction was started by adding 6 mU xanthine oxidase (XOD) and carried out at 25 °C for 20 min. After 20 min the reaction was stopped by adding 0.02 ml of 6 mM CuCl_2 . The absorbance of the reaction mixture was measured at 560 nm and the inhibition rate was calculated by measuring the amount of the formazan that was reduced from NBT by superoxide.

Proline content

The proline content of the leaves was determined according to Claussen (2005). For each treatment, 0.5 g leaf sample was ground in a mortar after addition of a small amount of glass powder and 5 mL of a 3% (w/v) aqueous sulfosalicylic acid solution. The homogenate was filtered through two layers of glass-fibers. To the filtrate (1 mL), glacial acetic acid and ninhydrin reagent (1 mL each) were

added. The closed test tubes containing the reaction mixture were kept in a boiling water bath for 1 h before the reaction was terminated at room temperature (22 °C) for 5 min. The absorbance of the reaction mixture was determined at 546 nm. The proline concentration was determined from a standard curve and calculated on fresh weight basis ($\mu\text{g proline g}^{-1}\text{ FW}$).

Enzyme and isoenzyme activities

The total soluble protein contents of the enzyme extracts were determined according to Bradford (1976) using bovine serum albumin as a standard.

SOD enzyme activity

The superoxide dismutase (EC 1.15.1.1) activity was assayed by its ability to inhibit the photochemical reduction of nitroterazolium blue chloride (NBT) at 560 nm (Beauchamp and Fridovich, 1973). Equal amounts of protein were subjected to nondenaturing polyacrylamide gel electrophoresis (PAGE) as described by Laemmli (1970), excluding the omission of sodium dodecyl sulfate. For the separation of SOD isoenzymes, 4.5% stacking and 12.5% separating gels under constant current (60 mA) at 4 °C were used.

APX enzyme activity

The ascorbate peroxidase (EC 1.11.1.11) activity was measured according to Nakano and Asada (1981). The assay depended on the decrease in absorbance at 290 nm as ascorbate was oxidized. The reaction mixture contained 50 mM Na-phosphate buffer (pH 7.0), 50 mM ascorbate, 0.1 mM EDTA Na₂, 1.2 mM H₂O₂, and 0.1 ml of enzyme extract in a final assay volume of 1 ml. The concentration of oxidized ascorbate was calculated by using an extinction coefficient of 2.8 mM⁻¹ cm⁻¹. One unit of APX was defined as 1 mmol ml⁻¹ ascorbate oxidized min⁻¹. The separation of APX isoenzymes was performed by non-denaturing PAGE at 4 °C with 4% stacking and 12.5% separating gels under a constant current (30 mA) and supported by 10% glycerol with a carrier buffer containing 2 mM ascorbate (Navari-Izzo *et al.*, 1998). The APX isoenzymes were detected in the gels as reported by Mittler and Zilinkas (1993).

POX enzyme activity

Peroxidase (POX; EC 1.11.1.7) activity was determined according to the method of Herzog and Fahimi (1973). The reaction mixture contained 3,30-diaminobenzidine-tetrahydrochloride dihydrate (DAB) solution containing 0.1 % (w/v) gelatine, 150 mM Na-phosphate-citrate buffer (pH 4.4), and 0.6% H₂O₂. A unit of POX activity was defined as 1 mol ml⁻¹ H₂O₂ decomposed min⁻¹. Electrophoretic POX separation was done according to Seevers *et al.* (1971). Leaf samples containing 50 mg protein were subjected to 10 % Native-PAGE. POX isoenzymes were detected by staining with 200 mM Na-acetate buffer (pH 5.0) containing 1.3 mM benzidine and 3% H₂O₂ for 30 min. The gels were then stored in 7% acetic acid solution.

CAT enzyme activity

Catalase activity was estimated according to the method designed by Bergmeyer. This method measures the initial rate of disappearance of H₂O₂ at 240 nm. CAT isoenzymes

were analyzed on 7.5 % non-denaturing PAGE at 4 °C, 30 g protein per lane were loaded. Gels were stained for CAT activity according to Woodbury *et al.* (1971).

GST enzyme activity

Glutathione-s-transferases (EC 2.5.1.18) activity was determined by the method of Habig *et al.* (1974) by following the increase in absorbance at 340 nm due to the formation of the conjugate 1-chloro-2,4-dinitrobenzene (CDNB) using reduced glutathione (GSH) as substrate. Equal amounts of protein were run in 10% (w/v) native PAGE according to the method of Laemmli (1970) and stained for GST activity using the method of Ricci *et al.* (1984). Briefly, after electrophoretic run, the gel was equilibrated in 0.1 M potassium phosphate buffer (pH 6.5) for 10 min and transferred to reaction mixture containing 4.5 mM GSH, 1 mM CDNB and 1 mM nitrobluetetrazolium in 0.1 M potassium-phosphate buffer (pH 6.5) at 37 °C for 10 min. Then, the gel was incubated at room temperature in 0.1 M Tris/HCl (pH 9.6) containing 3 mM phenazine methosul-phate.

SA level

SA levels were determined in accordance with Flores *et al.* (2011) with the use of (UHPLC-MS/MS; AGILENT 6064, Belgium). Stock standard solutions of individual compounds (with concentrations ranging from 200 to 300 mg/L) were prepared by exact weighing of the powder and dissolution in methanol (HPLC-grade, Sigma, USA).

Non-expressor of pathogen related gene expression

Relative quantification of gene expression and statistical analysis of all qRT-PCR data (pair wise fixed reallocation randomisation test) were performed using the REST software according to Pfaffl *et al.* (2002).

RNA isolation, cDNA synthesis, and real-time RT-PCR assay

RNA extraction was performed using Tripure reagent (Roche) according to the manufacturer's instructions. The integrity of total RNA was checked spectrophotometrically using a NanoDrop Spectrophotometer ND-2000 (Labtech International), followed by gel electrophoresis. cDNA synthesis was performed from 4 g total RNA using a Transcriptor 1st strand cDNA synthesis kit (Roche) according to the manufacturer's instructions and cDNAs of independent biological replicates (n=3) from same treatments were pooled into single samples. Subsequently, transcript levels were analyzed in a LightCycler 480II real-time PCR cycler (Roche) using a FastStart Essential DNA Probes Master kit (Roche) according to the manufacturer's instructions. Reaction conditions were 95 °C for 600s, followed by 45 cycles of 95 °C for 10 s, 56 °C for 10 s, and 72 °C for 1 s. Relative quantification of gene expression and statistical analysis of all qRT-PCR data (pairwise fixed reallocation randomization test) were performed using the REST software according to Pfaffl *et al.* (2002).

GmNPR1-specific products were obtained using the following primers: forward primer, 5'-TCAGATGATGTTGAGCTTGTTAAAC-3'; reverse primer ACCAAGTACCTCAGAAACAACCTT. Actin beta gene was used as reference gene. GmAct F;

GAGCTATGAATTGCCTGATGG, *GmAct* R; CGTTTCATGAATTCAGTAGC, Probe upl 61 (roche). Primer design was designed by using Acs number: XM, Acs number NM, NCBI and ensemble gene banks by us.

Statistical analysis

The experiment were repeated three times independently, and each data point was the mean of three replicates (n=6). Statistical variance analysis of the data was performed using ANOVA and differences among treatments were compared using Tukey's post-hoc analysis with least significant differences at the 5% level. In all the figures, the spread of values is shown using error bars representing standard errors (SE) of the means.

Results

Changes in stomatal conductance

Stomatal conductance was increased by 25% in acclimation group according to control groups in 'SA88'. In 'Erensoy', acclimation treatment also increased this value by 31% according to control group (Table 1).

Changes in malondialdehyde (MDA) and (H₂O₂) content

MDA content was increased 2.11 fold in acclimated group and 3.09 fold in non-acclimation group in 'SA88'. Otherwise, acclimation treatment increased 2.08 according to control in 'Erensoy' (Fig. 1A). Hydrogen peroxide was not changed under treatments in 'Erensoy'. This value was 63% in acclimation group and 2.3 fold in non-acclimated group according to control group in 'SA88'. Non

acclimation group increased H₂O₂ content by 46% according to acclimation in 'SA88' (Fig. 1B).

Changes in superoxide scavenging activity

Superoxide radical scavenging activity increased by 24% in acclimation group as compared to control but this percentage was 92% in non-acclimation group in 'Erensoy'. However, this value was increased 2.6 fold in acclimation group and 3.2 fold in non-acclimation group in 'SA88' (Fig. 1C).

Changes in proline content

Acclimation treatment caused a significant increase in proline content (7.79 fold) of the control while it was 17.95 fold in non-acclimation group in 'SA88'. However, this value was also 6.9 fold higher in non-acclimation group than in the control in 'Erensoy' (Fig. 1D).

Changes in antioxidant enzymes and isoenzymes activities

SOD enzyme activity increased in all treatment according to control group in both cultivars. The effect was more pronounced (84%) in non-acclimation while it was 61% in acclimation group in 'SA88' while this was 24% in 'Erensoy'. Otherwise, SOD enzyme activity increased by 14% in non-acclimation as compared to acclimation group. Six different SOD isoenzymes were determined in both soybean leaves. The more pronounced was in SOD4 isoenzyme expression. SOD4 was upregulated by 49% in acclimation and 48% in non-acclimation (Fig. 2 A,B,C).

APX enzyme activity increased by 9% in acclimation and decreased by 31% in non-acclimation in 'SA88' compared to control group whereas it did not change in

Table 1. Effects of acclimation and non-acclimation salt stress on stomatal conductance (g_s) ($mmol\ m^{-2}\ s^{-1}$) in sensitive 'SA88' and tolerant 'Erensoy' soybean (*Glycine max* L.) cultivars

Cultivar/ g_s	'SA88'			'Erensoy'		
	C	A	NA	C	A	NA
($mmol\ m^{-2}\ s^{-1}$)	74.35±5.90 ^a	93.36±0.90 ^b	93.36±0.90 ^b	26.22±3.22 ^a	34.35±3.52 ^b	24.85±4.87 ^a

Legend: Control group (C), acclimation (A), non-acclimation (NA). Columns with different letters represent significantly different ($P < 0.05$) values

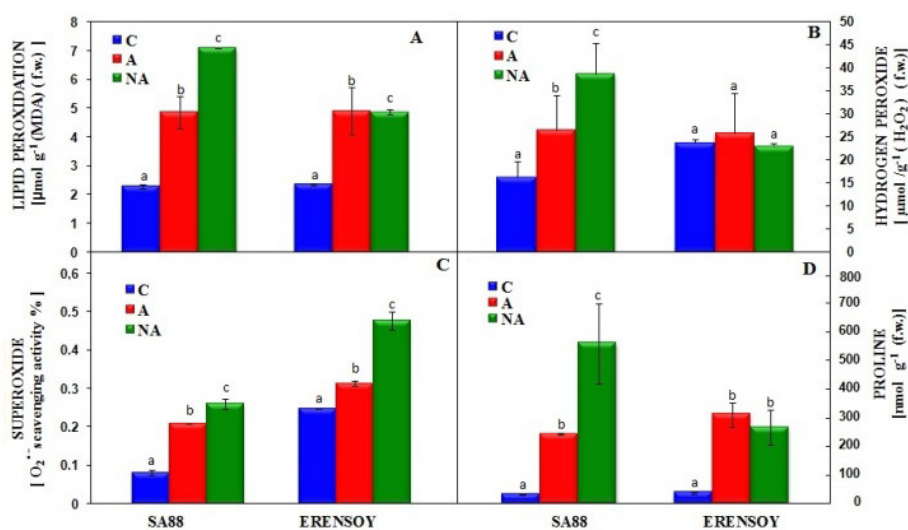


Fig. 1. Effects of acclimation and non-acclimation to salt stress on leaf malondialdehyde (MDA) content (A), hydrogen peroxide (H₂O₂) content (B), superoxide radicals (O₂[•]) scavenging activity (C), proline content (D) in sensitive 'SA88' and tolerant 'Erensoy' soybean (*Glycine max* L.) cultivars. Control (C), acclimation (A), non-acclimation (NA). Columns with different letters represent significantly different ($P < 0.05$) values

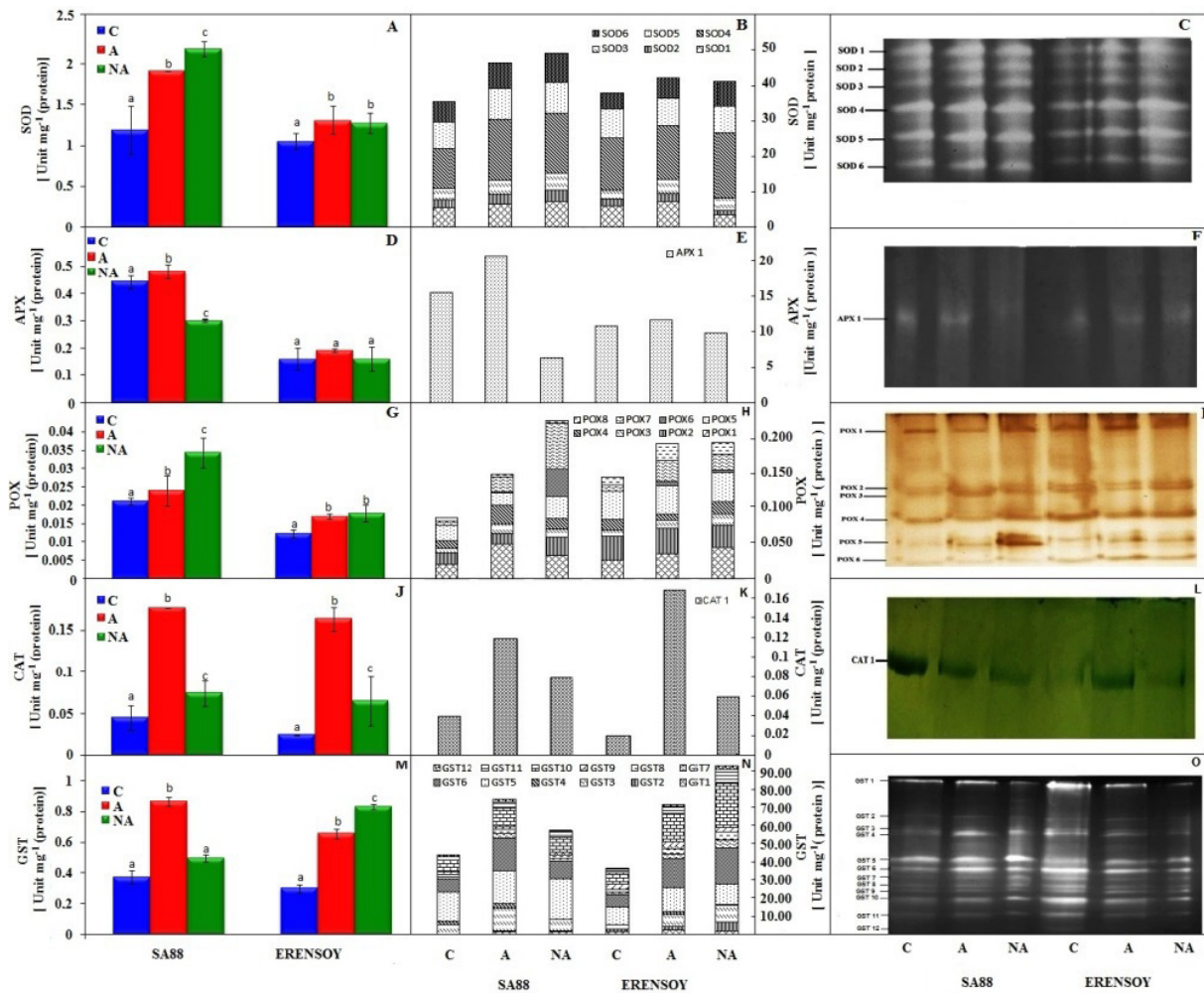


Fig. 2. Effects of acclimation and non-acclimation to salt stress on leaf activity staining and % induction of SOD (A-B-C), APX (D-E-F), POX (G-H-I), CAT (J-K-L), GST (M-N-O) isoenzymes and total activity in sensitive ‘SA88’ and tolerant ‘Erensoy’ soybean (*Glycine max* L.) cultivars. Control (C), acclimation (A), non-acclimation (NA). Columns with different letters represent significantly different ($P < 0.05$) values. in sensitive ‘SA88’ and tolerant ‘Erensoy’ soybean (*Glycine max* L.) cultivars

‘Erensoy’. However, it was decreased by 37% in non-acclimation as compared to acclimation. Only one APX isoenzyme (APX1) was determined in both cultivar. APX1 was upregulated in acclimation as compared to control group significantly in ‘SA88’ whereas there was no change in ‘Erensoy’ (Fig. 2 D,E,F).

Acclimation treatment induced POX enzyme activity by 9% while it was more pronounced (61 %) in non-acclimation in sensitive one. In contrast, POX enzyme activity decreased by 47% in acclimation as compared to non-acclimation in ‘SA88’. Nevertheless 8 different POX isoenzymes were determined in both cultivar. The most significant increase was POX7 (11 fold) gene expression in non-acclimated group in ‘SA88’ (Fig. 2 G,H,I).

Compared to control, the increase was 4.25 fold under acclimation, while this value was 8 fold in tolerant. This activity was also increased by 75% in non-acclimation as compared to control group in ‘SA88’ while this was 3 fold in tolerant. Nevertheless, acclimation group increased CAT activity by 75% as compared to non-acclimation. Similarly, CAT 1 isoenzyme was upregulated by 3 fold in acclimation while it was 2 fold in non-acclimation group as compared to control in ‘SA88’. Acclimation treatment lead to induce

CAT 1 expression by 8.5 fold according to control while this was 3 in non-acclimation treatment (Fig. 2 J,K,L).

GST enzyme activity was not affected in non-acclimated group according to control but acclimation treatment resulted in a higher activity of GST (2.32 fold) in ‘SA88’. Otherwise, acclimation treatment caused the increase GST activity by 43% as compared to non-acclimation in sensitive. For the acclimated group, when compared to control, the peak activity raised 2.16 fold for GST enzyme while it was 2.73 fold in non-acclimation. However, non-acclimation group increased by 26% in compared to acclimated group in tolerant one. 12 different GST isoenzymes were determined in both cultivar and the most significant increase was in GST5, 6 and 10. Otherwise GST5 (38%), GST6 (2.3 fold) and GST10 (2.5 fold) were increased in acclimation group according to control while GST5 (14%), GST6 (2.8 fold) and GST10 (3.8 fold) were in non-acclimation group in ‘Erensoy’.

GST5 (14%), GST6 (2.48 fold) and GST10 (56%) were increased in acclimation group as compared to control group whereas GST5 (37%), GST6 (40%) and GST10 (24%) were in non-acclimation group in ‘SA88’ (Fig. 2 M,N,O).

Changes in salicylic acid (SA) level

SA level was increased 4.7 fold in acclimated group and 3.4 fold in non-acclimation according to control in 'Erensoy'. However, acclimated treatment increased SA level by 27 % according to non-acclimated group. Acclimation treatment caused the accumulation of SA whereas it remained unchanged in non-acclimation group according to control group in sensitive one (Fig. 3 A).

Changes in non-pathogen related gene expression (*Gmnp1*)

NPR1 gene expression was increased in both cultivar but this value was higher in tolerant than sensitive soybean cultivar. *Gmnp1* gene expression was upregulated by 13% in non-acclimation group according to acclimated group in tolerant soybean. *Gmnp1* gene expression was upregulated by 22% in non-acclimation according to acclimation in sensitive one (Fig. 3 B).

Discussion

In the present study, acclimation application was used to explain the relation between ROS-induced antioxidant enzyme system and *NPR1*-dependent SA level change in soybean leaves under salinity. This process can be useful to

improve salt tolerance mechanism in soybean plants as it has been reported in our previous study (Aksoy and Dinler, 2014).

In this study, the stomatal conductance was increased in the acclimated groups of both cultivars but was more pronounced in 'SA88' (Table 1). Parallel to our results, a higher leaf stomatal conductivity in salt-acclimated potato plants was determined by Etehadnia *et al.* (2010). The stomatal conductance was also found to be higher in mungbean plants with pretreatment NaCl concentrations (Pandolfi *et al.*, 2016). The higher stomatal conductance in acclimated soybean leaves could be explained by Na⁺ accumulation and successful cytosol sequestration or K⁺ uptake in leaves.

Salt stress causes an increase in reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radicals, in plants. Plants have certain responses and a defense system to protect them against salt-induced oxidative damage (Lin *et al.*, 2012). This system includes several antioxidant enzymes and nonenzymatic antioxidants. In the current work, salt treatment led to a significant change in antioxidant enzymes and isoenzymes (SOD, CAT, APX, POX, and GST) in soybean leaves. Nevertheless, the acclimation process (treatment with increasing concentrations of NaCl) had a prompt effect on the antioxidant enzyme system in the sensitive soybean (Fig. 2). These findings are in agreement with those of Saha *et al.* (2010). It could be suggested that the low NaCl concentration may have induced reactive oxygen species as a signal to increase the antioxidant defense system.

Salicylic acid is a signal hormone that increases tolerance to biotic and abiotic stresses in plants (Khan *et al.*, 2012; Asgher *et al.*, 2015). Remarkably, our results showed that acclimation treatment led to induction of SA accumulation in sensitive soybean leaves (Fig. 3 (A)). Behind this, this group had the highest activities of enzymes, except for SOD and POX. This finding indicates that there may be a relation between salicylic acid level and antioxidant enzyme activity changes. Nevertheless, it is well documented that the ROS content can increase the SA accumulation in plants under stress conditions (Mivra and Tado, 2014). Specifically, the APX, CAT, and GST enzyme activities were increased by acclimation treatment, with higher increases observed in GST and CAT enzymes (Fig. 2 (M, J)). On the other hand, salt treatment increased the hydrogen peroxide and malondialdehyde contents, whereas acclimation treatment caused a decrease in both cultivars (Fig. 1 A,B). These results are in agreement with the other variables, including the change in antioxidant enzyme activity, proline accumulation, and superoxide radical scavenging activity. In the current work, non-acclimation treatment increased proline content more than acclimation group in sensitive 'SA88'. This result indicates that there is a negative correlation between proline and salinity tolerance in soybean, as previously reported by Parida Das and Mohanty (2004). In contrast, Mai and Hang (2016) found that the proline accumulation was higher in acclimated soybean leaves than in non-acclimated ones. The varying results may be explained by the differences in cultivars and treatments used.

Non-expressor of PR1 (*NPR1*) is a regulatory gene of the SA signaling pathway and a master regulator of the SA-

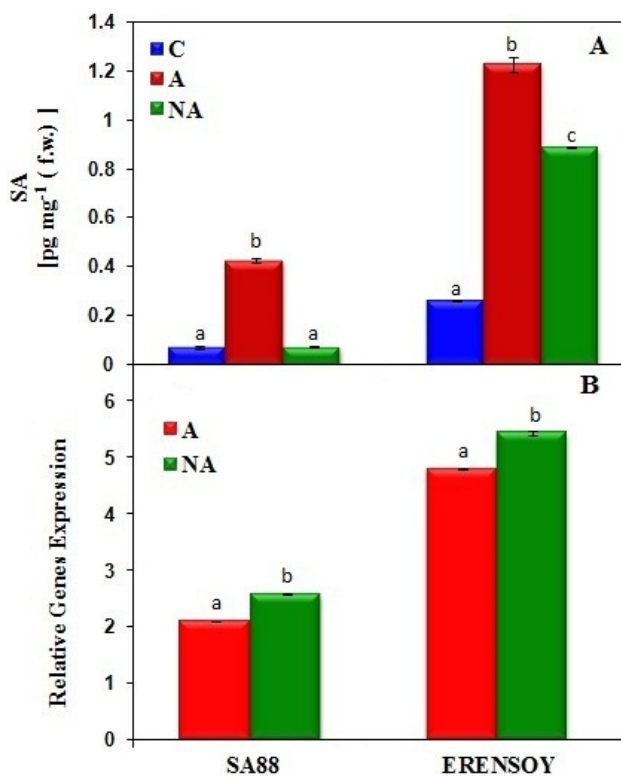


Fig. 3. Effects of acclimation and non-acclimation to salt stress on leaf salicylic acid level (A) and relative *Gmnp1* gene expression determined by qRT-PCR (B) in sensitive 'SA88' and tolerant 'Erensoy' soybean (*Glycine max* L.) cultivars. Control (C), acclimation (A), non-acclimation (NA). Columns with different letters represent significantly different ($P < 0.05$) values

mediated induction of defense genes (Wu *et al.*, 2012). Under pathogen infection or SA treatment, *NPR1* is reduced to monomers as a result of SA-induced redox changes in the cell and is translocated into the nucleus to regulate the defense gene expression (Mou *et al.*, 2003). *NPR1* was first identified and is best characterized in the model system *Arabidopsis*. However, it is found in all monocot and dicot plant species, including all economically important crops (Kuai and Despres, 2016).

Apart from biotic stresses, in recent years, some studies have reported that *NPR1* could improve salt tolerance by inducing ion channels and increasing K^+ uptake into cells and Na^+ exclusion from roots in *Arabidopsis* (Jayakannan *et al.*, 2015). This has led to the question of whether the *Gmnpr1* protein could control the acclimation process to salinity in soybean. However, the analysis carried out in the present study showed that the *Gmnpr1* gene could be upregulated by acclimation and non-acclimation treatments in both cultivars, with a higher upregulation in non-acclimated plants (Fig. 3(B)). Thus, a second question is asked: Can soybean leaves use the SA-induced independent *NPR1* pathway to increase salt tolerance?

In conclusion, *Gmnpr1* gene expression was induced in both treatments but was found to be higher under non-acclimation; the endogenous salicylic acid content was increased with acclimation treatment in 'SA88'. Based on these results, it could be suggested that the increased tolerance to salinity shown by the sensitive cultivar ('SA88') is related to SA induction due to the acclimation process in leaves (Fig. 3 A). This is in agreement with the results for all variables, such as the enzymes, MDA content, changes in H_2O_2 content, stomatal conductance. The induction indicated that the acclimation process was useful for soybean plants with SA accumulation and choosing *NPR1*-independent pathway for salt tolerance. Treatment with increasing concentrations of salt (25, 50, 75, and 100 mM NaCl) stimulated ROS accumulation and led to an increase in SA level. Early SA-inducible genes have been previously reported to code for enzymes with glutathione-dependent antioxidant and detoxifying activities, such as glutaredoxins and GST (Blanco *et al.*, 2009). Considering this information, it can be said that the *NPR1*-independent SA induction may activate the protein (GRXC9) that induces GSH and ascorbate-glutathione cycles in soybean, as has been shown in *Arabidopsis* (Herrera-Vasquez *et al.*, 2015). This suggestion may be supported by our finding of a significant increase in GST enzyme activities with acclimation treatment in 'SA88'. This is the first study to show how ROS signaling can induce *Gmnpr1* upregulation and what role the change in SA level plays under the acclimation process in soybean. Further research and molecular analyses are needed to clarify the relation between acclimation, SA, NPR, and salt tolerance in soybean.

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