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Induced-expression of osmolyte biosynthesis pathway genes improves salt and oxidative stress tolerance in *Brassica* species

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Osmolytes and other compatible solutes help to improve abiotic stress tolerance by modulating different physiological and metabolic processes in plants. In the present study, we assessed the relative role of antioxidant enzyme mediated defence system and osmotic adjustment mechanism for salinity stress tolerance in *Brassica* species. For this, a pot experiment was carried out with four *Brassica* genotypes from two species (*B. juncea* and *B. campestris*) and three different levels of salinity treatment. Salinity stress resulted in significant build-up of oxidative stress level (H_2O_2 and superoxide radical content, and lipid peroxidation) with the progressive increase in soil salinity. Relatively tolerant cultivars, CS 52 and CS 54 showed lesser oxidative stress and higher antioxidant enzymes activities (superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase) under the highest level of salt stress. Accumulation of organic osmolyte *viz*. glycine betaine and trehalose increased sharply under salinity stress especially in *B. juncea* cultivars. Gene expressions of *BADH* and *T6PS*, which regulates the biosynthesis of glycine betaine and trehalose were higher in *B. juncea* cultivars (CS 52 and CS 54) than T 9 (*B. campestris*). The results of the present study clearly showed a definite role of both osmoprotection and enzyme driven antioxidant defence mechanism as the basis of salt-tolerance in these *Brassica* genotypes and their simultaneous induction possibly brought more favourable cellular environment and salt tolerance in CS 52 and CS 54.

Keywords: Abiotic stress, Antioxidant enzymes, Gene expression, Osmoprotection, Salinity stress

Soil salinity is one of the most important abiotic stresses limiting crop production throughout the world, leading to severe reduction in productivity of many crops, especially showing detrimental effects at the time of germination, seedling establishment and flowering stages through ionic and osmotic stresses as well as oxidative damage[1]. Increased soil salinity negatively affects growth of crop plants, and the continued salinization of arable land provides an increasing threat to global crop production, especially in irrigated systems[2]. Knowledge on factors responsible for salinity tolerance will help in increasing the salinity tolerance of crop plants and consequently contribute to the maintenance of crop yields.

Salt stress usually has dual effects on plants comprising of osmotic and ionic component of the stress on whole plant and leaf physiology. Due to limited supply of water under saline condition and partial closure of stomata often plants face transient reduction of C-supply to chloroplasts leading to excess photo-reducing potential generated as a result of light reaction of photosynthesis and that tends to accumulate inside the leaf tissue and generates additional electrochemical energy[3]. The plants then try to dissipate this surplus energy via production of cytotoxic reactive oxygen species (ROS) like superoxide anion ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), which started to accumulate inside cell leading to development of oxidative stress symptoms[4]. Plants usually employ stringent regulation to maintain its ROS homeostasis to avoid the ill effects of oxidative stress under saline condition via different complex networking and balancing between cellular oxidants and antioxidants[5].

Plants usually have more than one strategy to respond to salinity stress. Among them, accumulation

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Abbreviation: APX, Ascorbate peroxidase; BADH, Betaine aldehyde dehydrogenase; CAT, Catalase; GB, Glycine Betaine; GR, Glutathione reductase; GSSG, Oxidized glutathione; POD, Peroxidase; ROS, Reactive oxygen species; SOD, Superoxide dismutase; SOR, Superoxide radical; TBARS, Thiobarbituric acid reactive substances; Treh, Trehalose; T6PS, Trehalose-6-phosphate synthase

of osmolytes or compatible solutes like, proline, sugars, sugar alcohols, glycine betaine etc. help in osmoregulation and thus protection of cellular structures and macromolecules[2]. Some of these can be essential elemental ions like K^+, while most of them are organic solutes. Glycine betaine (GB), and a non-toxic highly water soluble compatible solute found most commonly in the cell at high concentration, under stress in many cultivated plant species but also reported to be absent from some crop species likes rice and *N. tabacum*[6]. The level of GB synthesis in plants is most often regulated by the activity of the enzyme betaine aldehyde dehydrogenase (BADH), hence it is considered as the key regulatory step for GB biosynthesis[7]. With an increase in NaCl concentration from 0 to 500 mM, 2–4-fold increase in BADH activity was observed in the leaves and roots of barley. Consequently, almost 8-fold increase was observed in *BADH* mRNA levels in the leaves of salt treated plants[8]. Trehalose (Treh) is a non-reducing disaccharide composed of two glucose residues bound by α-α-(1→1) linkage. Treh is present in wide range of organisms, from bacteria to higher plants and invertebrates, and is often associated with stress tolerance[9]. In plants, the only mechanism for Treh biosynthesis is through trehalose-6-phosphate synthase (T6PS) pathway, so Treh content in plants strictly regulated by the activity of key regulatory enzyme T6PS[10].

Plants do differ at species or even genotypic level to their responses towards salinity stress. *Brassica*, one of the major oilseeds worldwide has more than one cultivated species and there exists significant variability in salinity induced responses or stress tolerance. Understanding the tolerance behavior with respect to species specific differential response and factors associated with salinity tolerance will help in developing salinity tolerant crop plants with a reduced yield loss under stressful environment. Hence, the present study focuses on establishing the hypothesis that (i) there must be more than one mechanism (oxidative stress tolerance and accumulation of compatible solute) exist for salinity tolerance in *Brassica* species to trigger stress induced response; and (ii) quantification of differential response at both protein (enzyme) or transcript level.

Materials and Methods

Plant material and growth condition

A pot experiment was conducted at the Division of Plant Physiology, Indian Agricultural Research Institute, New Delhi with four cultivars of *Brassica viz*., CS 52, CS 54, Varuna (*B. juncea*), and T 9 (*B. campestris*). For sowing, earthen pots (30 × 30 cm) lined with 400-gauge polythene sheets were used and mixture of 10 kg of air-dried soil and farmyard manure (3:1 ratio) were filled in each pot. Bavistin @ 2 g per 100 g seeds was used as pre-sowing seed treatment. Recommended doses of N-P-K fertilizers were applied at each pot at the rate of 80:60:40 kg ha^{-1}, considering 2.24×10^6 kg of soil per hectare. At 20 DAS (days after sowing) salinity treatment was imposed by irrigating with 2.5 L of water and saline solutions, respectively for control and treated plots for every 10 kg of air-dried soil. Salinity was created by mixing Na:Ca and Cl:SO_4 salts in 4:1 ratios such that treatment S_1 contains 25, 12.5 and 12.5 mmol L^{-1} of NaCl, Na_2SO_4 and $CaCl_2$, respectively, and treatment S_2 contains 50, 25 and 25 mmol L^{-1} of NaCl, Na_2SO_4 and $CaCl_2$, respectively, while for the treatment S_0 normal irrigation water without addition of external salts were used. To see the salinity build-up, the soil samples were collected at the same time, when the plant sampling was done and the mean ECe values obtained for three distinct salinity levels were 1.65 (S_0, control), 4.50 (S_1) and 6.76 dS m^{-1} (S_2). The number of replication for each treatment was 12 in the form of pot.

Different oxidative stress parameters and antioxidant enzyme activities were estimated from fresh leaf samples while, organic osmolytes *viz*. trehalose (Trh) and glycine betaine (GB) contents were measured from dried leaf samples of third fully-matured leaf at flowering stage of the plant. Sampling was done randomly in quadruplicate from 4 pots for each treatment combinations. For gene expression studies, similar leaf samples were selected and the total RNA was extracted immediately after collection of leaf sample and kept in –80°C for further action.

Determination of oxidative stress

Superoxide radical content was estimated by its capacity to reduce nitroblue tetrazolium chloride (NBT) and the absorption of end product was measured at 540 nm[11]. Superoxide radical content was calculated according to its extinction coefficient $\varepsilon = 12.8\ mM^{-1}\ cm^{-1}$. Hydrogen peroxide was estimated by forming titanium-hydro peroxide complex by taking absorbance at 415 nm[12]. The level of lipid peroxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) content by taking the absorbance of the supernatant at 532 nm[13]. The

TBARS content was calculated according to its extinction coefficient $\varepsilon = 155\ mM^{-1}\ cm^{-1}$.

Assay antioxidant enzyme activities

Enzyme extract for superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), peroxidase (POD) and catalase (CAT) was prepared by first freezing the weighed amount of leaf samples (1 g) in liquid nitrogen to prevent proteolytic activity followed by grinding with 10 mL extraction buffer (0.1 M phosphate buffer, pH 7.5, containing 0.5 mM EDTA in case of SOD, GR, POD, CAT and 1 mM ascorbic acid in case of APX). Extract was passed through 4 layers of cheese cloth and filtrate was centrifuged for 20 min at 15000×g and the supernatant was used as enzyme.

Total SOD (EC 1.15.1.1) activity was estimated by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) by the enzyme[14]. The absorbance was recorded at 560 nm, and one unit of enzyme activity was taken as that amount of enzyme, which reduced the absorbance reading to 50% in comparison with tubes lacking enzyme. Catalase (EC 1.11.1.6) was assayed by measuring the disappearance of H_2O_2[15] in a reaction mixture (3 mL) consisted of 0.5 mL of 75 mM H_2O_2 and 1.5 mL of 0.1 M phosphate buffer (pH 7) by adding 50 µL of diluted enzyme extract. Peroxidase (EC 1.11.1.7) activity was measured in terms of increase in absorbance due to the formation of tetra-guaiacol at 470 nm, and the enzyme activity was calculated as per extinction coefficient of its oxidation product, tetra-guaiacol $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$ [16]. Ascorbate peroxidase was assayed by recording the decrease in optical density due to ascorbic acid at 290 nm[17]. Glutathione reductase (EC 1.8.1.7) was assayed as per the method of Smith *et al.*[18]. The increase in absorbance due to formation of oxidized glutathione was recorded at 412 nm spectrophotometrically and the activity was expressed as micromole of oxidised glutathione reduced per mg protein per min. Total soluble protein was determined with bovine serum albumin as a calibration standard.

Estimation of organic osmolytes

Glycine betaine (GB) content of the leaves was estimated following the method of Greive and Grattan[19] from 0.5 g of finely ground dry material. Finally, GB content from the sample was determined by measuring the absorbance of peri-iodite crystals at 365 nm after 2.0-2.5 h of incubation in 1,2-dichloro ethane. Trehalose (Treh) content was estimated spectrophotometrically at 620 nm as per the method of Ferreira *et al.*[20] from 10 mg of dried leaf sample using anthrone reagent.

Gene expression studies

For gene expression studies, the complete cDNA sequences of candidate genes in related species were obtained from National Centre for Biotechnology Information using the Basic Local Alignment Search Tool. The degenerate primer for the present study were designed manually and the quality of oligonucleotide, GC% and T_m were checked with the help of Oligoanalyzer 3.0 tool Intergrated DNA Technologies, Coralville, IA 52241, USA) (Table 1).

Gene expression of *BADH* and *T6PS* were studied in leaf tissue. The leaf samples, collected from both control and treated plants were subjected to the total RNA extraction using RNAeasy kit (Qiagen Inc., Chatsworth CA, USA, Cat No: 749040) as per the manufacturer's instruction. DNase I (Qiagen Science, Maryland, USA) was used to remove the DNA contamination from the RNA samples. One µg of total RNA was reverse transcribed using gene specific primers and Qiagen one step RT-PCR kit. PCR conditions were standardized by gene-specific primers for tubulin. Linear amplification for semi-quantitative RT-PCR was obtained with 27 cycles. Reactions were conducted under the following conditions; initial PCR activation step: 15 min at 95ºC, reverse transcription: 30 min at 50ºC, denaturation: 1 min at 94ºC, annealing: 1 min at 57ºC (BADH) and 60ºC (T6PS and tubulin), extension: 1 min at 72ºC, final extension: 10 min at 72ºC using My Genie 32 Thermal Block PCR (Bioneer, Korea). The amplified

Table 1 — Details of the forward and reverse primers used for gene expression study along with expected amplicon sizes

Name	Sequence	Length (bases)	GC %	Tm (ºC)	Product size (bp)
BnBADH-F	CTG GAA GCA ACA TTA TGA CTT CTG	24	41.7	61.9	633
BnBADH-R	AAC ATG GCT GAG AAC AGT TGA C	22	45.5	62.7	
BnT6PS-F	TTC TGT TCT GAA ATC TCT GTG TG	23	39.1	60.4	542
BnT6PS-R	TTC AAT ATG CTC TCA AAC ATG TC	23	34.8	59.2	
Tubulin-F	CAG CAA TAC AGT GCC TTG AGT G	19	57.9	60.0	360
Tubulin-R	CCT GTG TAC CAA TGA AGG AAA GCC	24	50.0	62.2	

products were then used for electrophoresis on 1.2% agarose gel under standard electrophoretic condition (120 volts; 0.4M Tris-borate with 1 mM EDTA at pH 8.0) and with known concentration of DNA markers. Ethidium bromide stained gels were visualized under Uvi Pro Gel Documentation system (Uvitec, England) and band intensity were compared using GelQuant.NET software. After purification, the cDNA, thus obtained were cloned in pTz57R/T vector and transformed in DH5α strain of *E. coli* cells for both the genes. The transformed and recombinant plasmid showing resistance to ampicillin were selected for further analysis. The confirmed isolated plasmids were subjected to restriction analysis using Kpn I and Hind III enzymes to confirm the presence of cloned insert. The confirmed recombinant plasmids were sequenced at Xcelris Labs Limited, Bodakdev, Ahmedabad, India.

Statistical analyses

The ANOVA of experimental data was analyzed[21] and F-test was carried out to check the significant differences among the treatments by SPSS 16.0. The LSD (least significant difference) values were computed at 5% level of probability.

Results

Effect of salinity stress on oxidative damage and lipid peroxidation

Production of various reactive oxygen species (ROS) is an important phenomenon under salinity stress, especially accumulation of superoxide radical and hydrogen peroxide and increase in lipid peroxidation. Compared to control or untreated plants, the plants grown in saline conditions showed sharp increase in production of superoxide radical (SOR) and hydrogen peroxide, and lipid peroxidation at flowering stage of the plant (Fig. 1); however, in tolerant cultivars *viz*. CS 52 and CS 54, the production of these ROS was comparatively lesser than susceptible ones. The SOR content showed least increase in the cultivar CS 54 (56.6%), whereas highest increase was observed in Varuna (91.5%) under highest level of salinity treatment (Fig. 1A). Similarly, H_2O_2 content was also increased least in CS 54 (40.4%), under S_2 treatment, while the susceptible cultivars Varuna and T 9 showed as high as 51.1 and 58.6% increase in H_2O_2 content under the same treatment level (Fig. 1B). Salinity induced lipid peroxidation (measured in terms of TBARS content) was increased with progressive increase in soil salinity level (Fig. 1C). The TBARS content showed highest increase in Varuna and T 9 (93 and 82%, respectively) under the highest level of salinity treatment, while the least increase was observed in CS 54 (29%) and CS 52 (24%) under same treatment level.

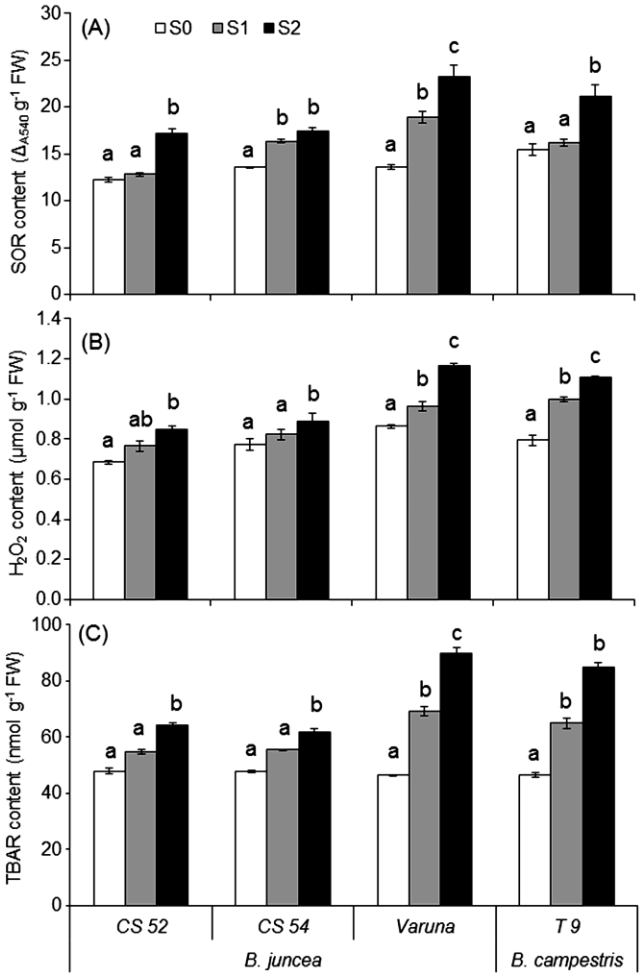

Fig. 1 — Changes in (A) superoxide radical content (ΔA_{540} g^{-1} fresh weight); (B) H_2O_2 content (µmol g^{-1} fresh weight); and (C) lipid peroxidation (nmol of TBARS content g^{-1} fresh weight) in leaves of *Brassica* cultivars in response to different levels of salinity stress. [Vertical bars show ± SE of mean (n = 4). Means sharing the same letter for an individual cultivar are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. S_0, S_1 and S_2 represent three different soil salinity levels]

Changes in activities of antioxidant enzymes

In the present study, salinity-induced increase in antioxidant enzyme activities was observed especially in the tolerant cultivars CS 52 and CS 54. The highest increase in SOD activity was observed in CS 54 (44%), followed by CS (25%) under the highest level

of salinity treatment (S_2), while it was reduced by 25% in T 9 under similar level of stress (Fig. 2A). Similarly, the CAT activity was also increased with increasing level of salinity stress, especially in CS 52 and CS 54 and the increase was highest in CS 54 (175%), followed by CS 52 (55%), while the change was not significant for Varuna and T 9 under the highest level of salinity treatment (S_2) (Fig. 2B). The POD activity increased 83.9 and 53.2%, respectively in both the tolerant cultivars CS 52 and CS 54 under S_2 treatment from that untreated plants, while the increase was least and non-significant in Varuna (Fig. 2C). The activities of GR showed sharp increase from 3.7 unit in control to 19.2 unit in S_2 treated plants in CS 52 (more than 4-fold), while it was increased from 3.2 unit in control to 14.6 unit in S_2 in CS 54 (~3.5-fold) (Fig. 3A). On the contrary, the increase was not significant in both Varuna and T 9. The APX activity showed more than 150% increase both the tolerant cultivar CS 52 and CS 54 under highest level of salinity treatment (S_2). Although, the increase in APX activity was significant in both Varuna and T 9 under S_2 treatment level but the degree of increase was much lower (12 and 33%, respectively) compared to CS 52 and CS 54 (Fig. 3B).

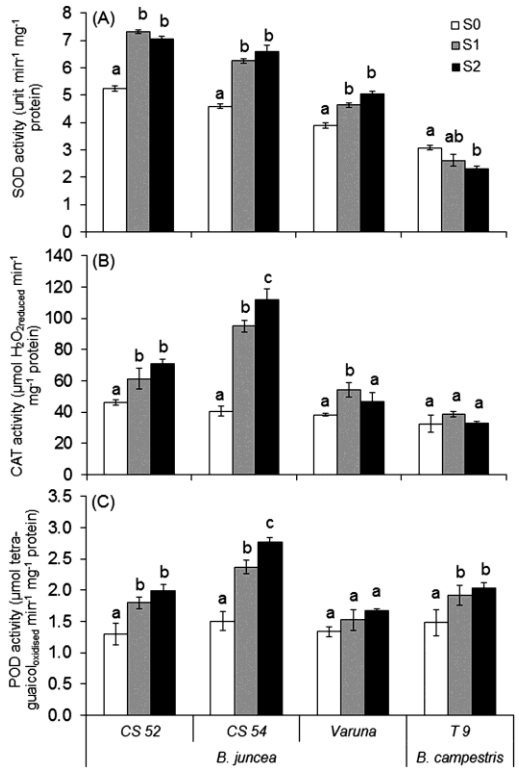

Fig. 2 — Changes in (A) SOD activity (unit mg^{-1} protein min^{-1}); (B) CAT activity (µmol H_2O_2 reduced mg^{-1} protein min^{-1}); and (C) POD activity (µmol tetra guaiacol formed mg^{-1} protein min^{-1}) in leaves of *Brassica* cultivars in response to different levels of salinity stress. [Vertical bars show ± SE of mean (n = 4). Means sharing the same letter for an individual cultivar are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. S_0, S_1 and S_2 represent three different soil salinity levels]

Glycine betaine and trehalose contents

GB content increased at both the salinity levels in the leaves of all the genotypes. The GB content was almost tripled under S_2 treatment in CS 52 and CS 54, while the rise was comparatively lower in T 9 (94%) as compared to control under S_2 treatment (Fig. 4A). Treh content increased in all the genotypes with the increase in salinity levels. The increase was highest in CS 54, followed by CS 52, where almost 100% increases were observed compared to that of control plants. The genotype T 9 showed least increase i.e. 42 and 48% under S_2 salinity level at flowering and post flowering stages, respectively (Fig. 4B).

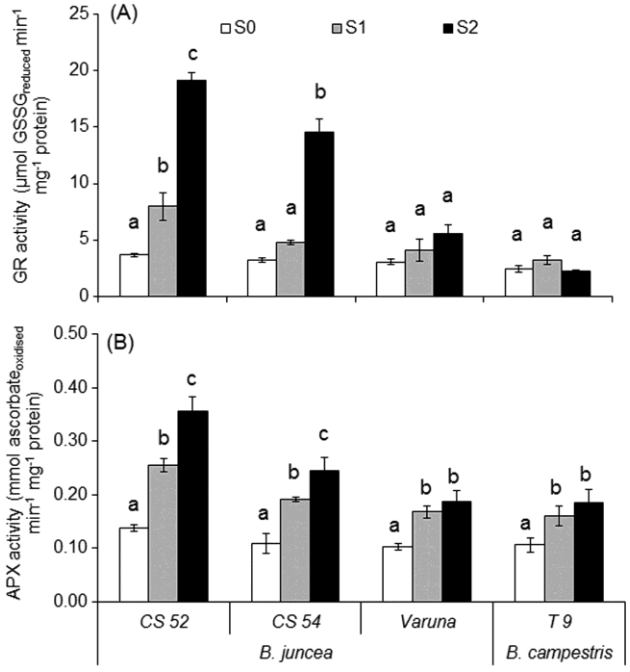

Fig. 3 — Changes in (A) GR activity (µmol GSSG reduced mg^{-1} protein min^{-1}); and (B) APX activity (µmol ascorbate oxidised mg^{-1} protein min^{-1}) in leaves of *Brassica* cultivars in response to different levels of salinity stress. [Vertical bars show ± SE of mean (n = 4). Means sharing the same letter for an individual cultivar are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. S_0, S_1 and S_2 represent three different soil salinity levels]

Gene expression studies

For all the treatment combinations, the amplified cDNA of 633 bp was obtained for all the genotypes. Except CS 52, the expression level of *BADH* mRNA was found relatively low in control plants of the genotypes. Compared to control, high level of mRNA expression was observed under S_2 treatment condition in CS 54 (~ 6-fold) and CS 52 (~ 3-fold). In case of the cultivar T 9, the changes in the expression level was not significant at both S_1 and S_2 treatment level, while in case of Varuna, the increase in *BADH* transcript level was only significant under S_2 treatment, although the degree of increase was much less compared to CS 52 and CS 54 (Fig. 5). The partial cDNAs of *BADH* had been registered in NCBI Gene Bank (accession No. JN967676, JN967677, JN967678 and JN967679; for CS 52, CS 54, Varuna and T 9, respectively). The partial cDNA and deduced amino acid sequences of BADH were compared with *Brassica napus* and *Arabidopsis thaliana* using ClustalW (2.1) multiple alignment (Fig. 6). All the four genotypes, showed approximately 72% similarity with *A. thaliana*, while among the *Brassica* genus, the similarity was 98%. That indicates that the cloned portion of the *BADH* gene was highly conserved in all the *Brassica* species/genotypes. Conserved domains were identified using 'PROSITE' (release 20.61) and the partial amino acid sequence of BADH showed two conserved domains: (1) ALDEHYDE_DEHYDR_GLU, Aldehyde dehydrogenases glutamic acid active site (PS 00687) (amino acid residues 4-11). (2) ALDEHYDE_DEHYDR_CYS, Aldehyde dehydro–genases cysteine active site (PS 00070) (amino acid residues 32-43).

In case of *T6PS* gene again a clear-cut amplicon of 542 bp size obtained in all the genotypes in every treatment combinations. Lesser expression was observed in control plants; however, very prominent bands were found in tolerant group of *B. juncea* genotypes (CS 52 and CS 54) under highest level of salinity treatment. Although, the progressive increase in expression of *T6PS* mRNA with increasing level of soil salinity was observed in relatively susceptible cultivars, but as a whole the level of expression was

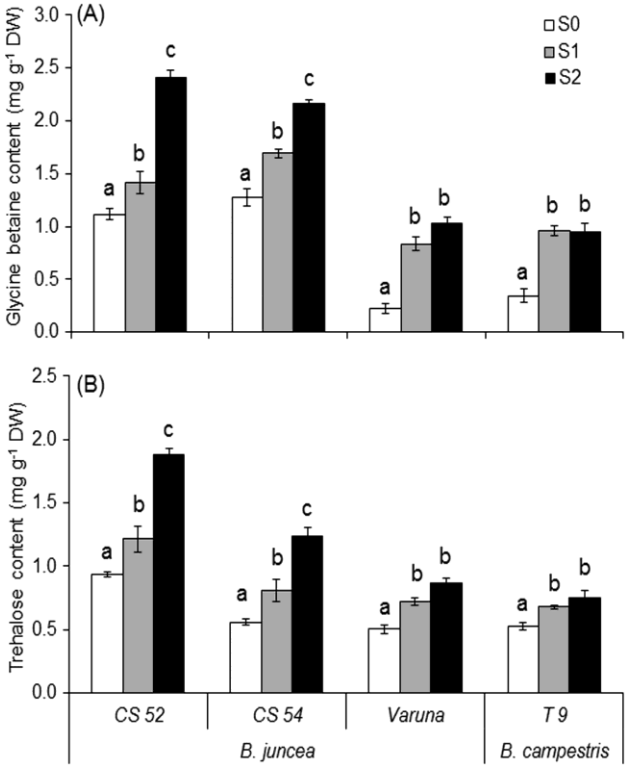

Fig. 4 — Changes in (A) glycine betaine; and (B) trehalose accumulation in the leaves of *Brassica* cultivars in response to different levels of salinity stress. [Vertical bars show ± SE of mean (n = 4). Means sharing the same letter for an individual cultivar are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. S_0, S_1 and S_2 represent three different soil salinity levels]

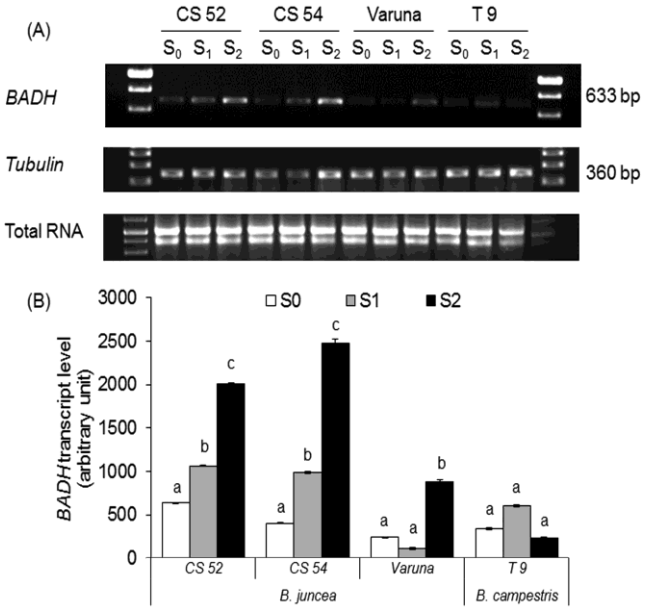

Fig. 5 — Gene expression of *BADH* in the leaves of *Brassica* genotypes under different salinity levels. (A) Semi-quantitative RT-PCR; and (B) quantification of mRNA levels normalized with tubulin as internal control. [Vertical bars show ± SE of mean (n = 4). Means sharing the same letter for an individual cultivar are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. S_0, S_1 and S_2 represent three different soil salinity levels]

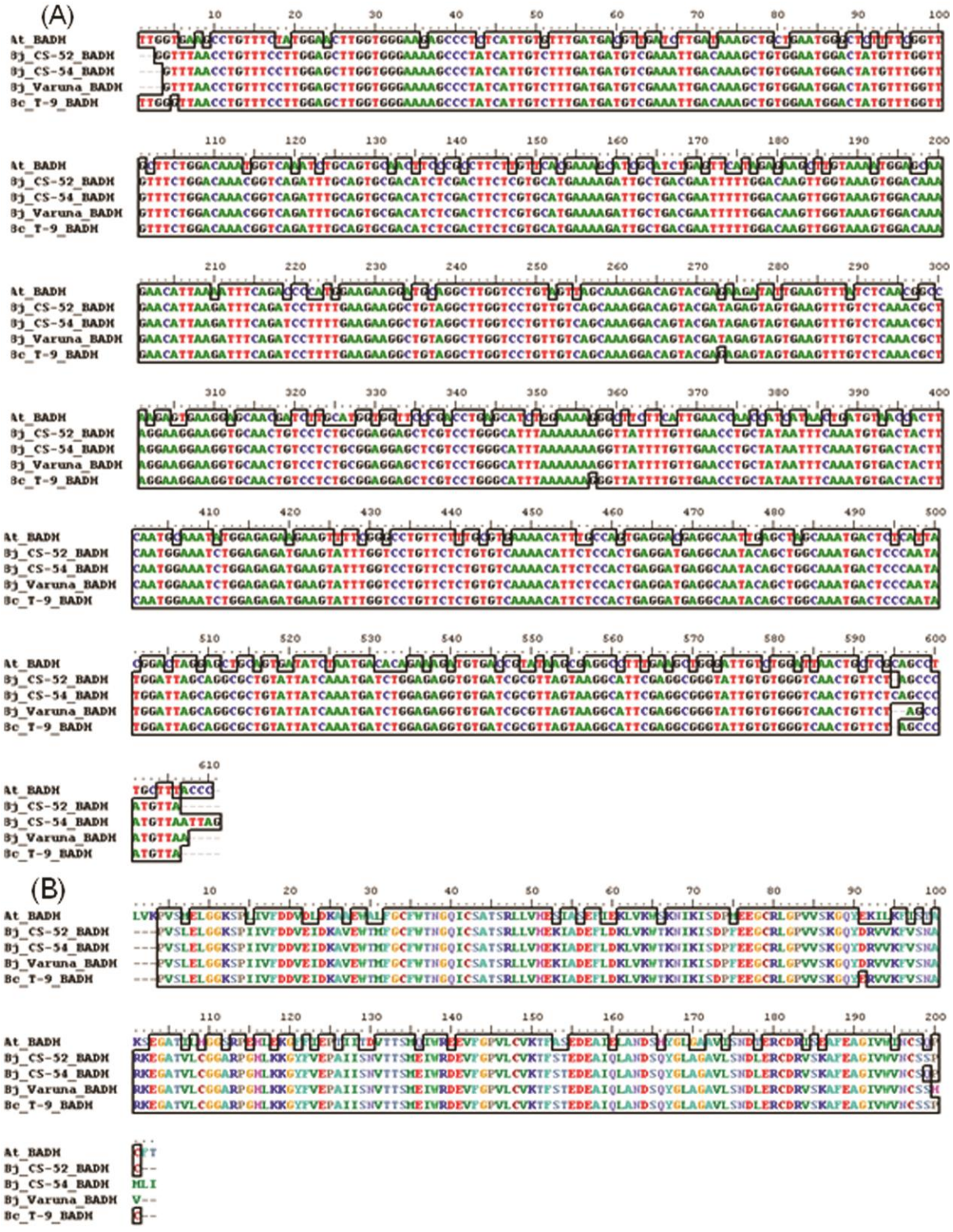

Fig. 6 — Clustal W (2.1) multiple sequence alignment and comparison of (A) partial coding; and (B) deduced protein sequences of *BADH* in leaf tissues in *B. juncea* genotypes CS 52, CS 54 and Varuna and *B. campestris* genotype T9 with *A. thaliana* (Gene Bank Acc. No. AY093071.1 and AAM13070.1) (outlining box shows conserved region).

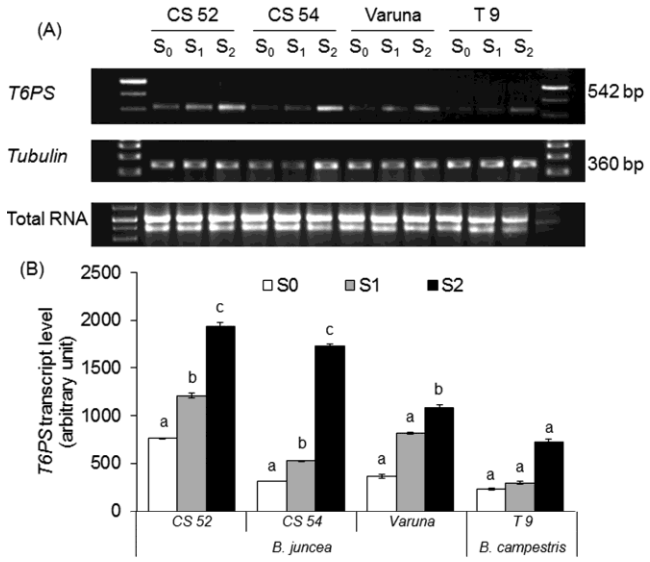

Fig. 7 — Gene expression of *T6PS* in the leaves of *Brassica* genotypes under different salinity levels. (A) Semi-quantitative RT-PCR; (B) quantification of mRNA levels normalized with tubulin as internal control. Vertical bars show ± SE of mean (n = 4). Means sharing the same letter for an individual cultivar are not significantly different ($P \leq 0.05$) according to Duncan's multiple range test. S_0, S_1 and S_2 represent three different soil salinity levels.

lower in both Varuna and T 9 (Fig. 7). The partial cDNAs of *T6PS* have been registered in NCBI Gene Bank (accession No. JN967680, JN967681, JN967682 and JN967683 for CS 52, CS 54, Varuna and T 9, respectively). All the genotypes, except CS 52 showed approximately 82 and 91% similarity with *A. thaliana* and *B. napus*, respectively, while among the genotypes similarity was 80-85%. CS 52 showed 73 and 80% similarity with *A. thaliana* and *B. napus*, respectively (Fig. 8).

Discussion

Differential salt sensitivity had already been reported in *Brassica* species, which might well be as a result of one or more physiological strategies associated with salt-tolerance[22-25]. In the present study also *Brassica* genotypes CS 52, CS 54, Varuna (*B. juncea*) and T 9 (*B. campestris*) responded differentially towards salinity stress. As a whole the response of CS 52 and CS 54 was found comparatively tolerant to different level of soil salinity as against Varuna and T 9. We found significant build-up of oxidative stress in the plants due to imposition of salinity stress. The level of stress measured in terms of increase in SOR and H_2O_2 production as well as lipid peroxidation under salinity stress condition, was found higher especially in the susceptible genotypes. Salinity-induced oxidative stress was also reported in *Jatropha curcas* seedling showing interaction of ROS with many cellular components, leading to significant damage to membranes and other cellular structures[26]. The ROS, including superoxide radical, hydrogen peroxide and hydroxyl radical are cytotoxic in nature, which often at higher cellular concentration promotes loss of important cytosolic K^+ level *via* activation of different voltage gated channels[27], and thus external K^+-application often found to improve salt tolerance[28]. Besides, these ROS attack membrane lipids causing lipid peroxidation, increase in membrane injury (greater ion/solute leakage), chlorophyll damage, protein denaturation and DNA mutation[29]. Under normal condition the production and scavenging of these ROS is well regulated in cell metabolism. However, under stressful conditions the formation of ROS might be more than antioxidant scavenging capacity, thus creating oxidative stress[4]. Several workers reported association of oxidative damage with various environmental stresses in different plants[27,30-32].

Usually under oxidative stress, the ROS detoxification occurs through series of enzyme driven reactions which are induced as a defence response for protection of cellular environment. The whole network of antioxidant enzymes like SOD, CAT, POD, GR and APX show induced activities to detoxify ROS under unfavourable cellular conditions[33]. Earlier studies clearly proved that salinity tolerance is often associated with the capacity of group of plants to induce and over express antioxidant enzyme activities when external stress is imposed[34]. In the present study, salinity-induced increase in antioxidant enzyme activities was observed especially in the relatively tolerant cultivars of *B. juncea* (CS 52 and CS 54) than that of *B. campestris* cultivar. Turkan *et al*.[35] reported that under varying degree of abiotic stress plants can detoxify the cellular ROS via upregulating the activities of antioxidant enzymes such as SOD, APX and CAT; while Abogadallah[33] reported that simultaneous induction of all the antioxidant enzymes might not require by the plants under salinity stress. Increased production of ROS, especially H_2O_2, reported to induce activities of major antioxidant enzymes like SOD, CAT, POD and GR in

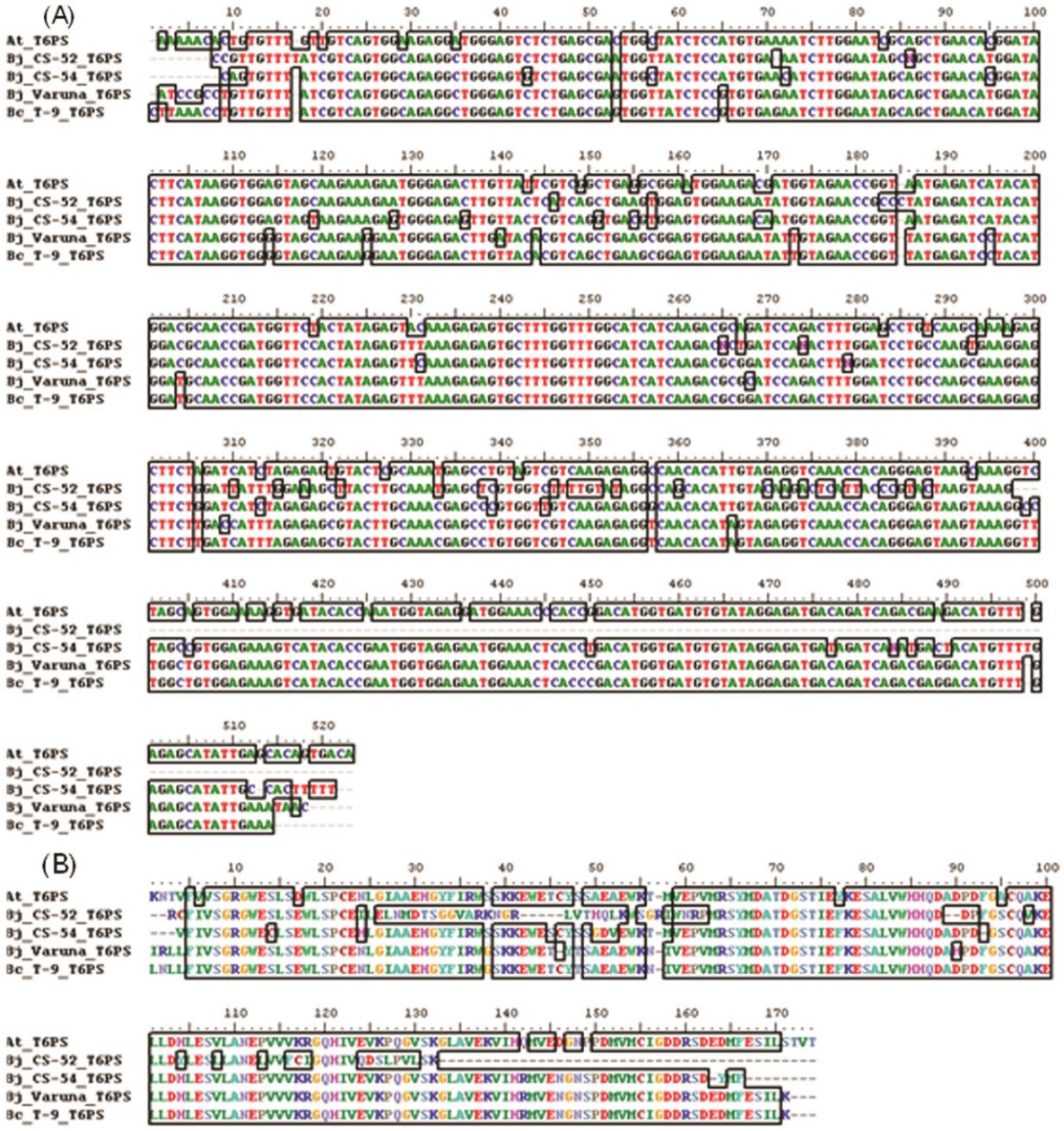

Fig. 8 — Clustal W (2.1) multiple sequence alignment and comparison of (A) partial coding; and (B) deduced protein sequences of *T6PS* in leaf tissues in *B. juncea* genotypes CS 52, CS 54 and Varuna and *B. campestris* genotype T9 with *A. thaliana* (Gene Bank Acc. No. AY096366.1 and AAM20007.1) [outlining box shows conserved region]

NaCl-stressed plants of strawberry[5]. Recent studies to confirm the role of these antioxidant enzymes towards alleviating salinity stress, showed the transgenic rice plants with higher activity of Cu/Zn-SOD with more salinity tolerance compared to wild type plants[36]. Results observed on activities of various antioxidant enzymes like SOD, APX, GR, CAT and POX under different levels of salinity treatments in both the *Brassica* species suggest relatively tolerant nature of *B. juncea* species, which is by and large associated with a continuous increase in all the five antioxidant enzyme activities under salinity stress. This might well be the reason for relatively lesser build-up of oxidative stress compared to the genotypes of *B. campestris* species.

Sharp increase in GB content observed in tolerant group of *B. juncea* genotypes (CS 52 and CS 54) under increasing salt stress, signifies their ability to

cope with salinity stress than the susceptible ones. The role of GB as osmoprotectant has long been known. GB is known to protect thylakoid and plasma membrane integrity in higher plants when exposed to salinity or high temperature stresses[6]. Wani *et al*.[37] reported alleviation of salt stress and better osmo-protection and leaf water status in genetically engineered crop plant that were capable of synthesizing more GB under salt stress condition. Greater accumulation of Trehalose (Treh) was observed in both CS 52 and CS 54, which probably explain its role in salt stress tolerance in mustard genotypes. In contrast, susceptible genotypes Varuna and T 9 showed very little increase in Treh content. Overproduction of Treh in genetically engineered rice showed increased tolerance to abiotic stress and enhanced productivity[38]. Treh accumulation in response to salinity stress has been reported earlier in crops such as *Medicago truncalata* and *Phaseolus vulgaris*[39]. During extreme drought or osmotic stress higher accumulation of Treh may be the reason for better protection of cell membranes and less enzymes/proteins denaturation[40].

In the present study, *BADH* gene expression pattern correlated with the GB content observed under salt stress. Less expression of *BADH* and GB content under salt stress in susceptible genotypes could be the reason for their higher salt-sensitivity. Similar increase in BADH activity and expression has been reported in barley under salt stress[8]. Genetically modified tobacco plants transformed with the *BADH* gene for increased activity of BADH accumulated more GB in the chloroplasts that resulted in lesser photo-inhibition of PSII under moderate heat stress[41]. A sharp increase in the *T6PS* expression under salinity stress in CS 52 and CS 54 correlates well with the greater accumulation of Treh in these genotypes. Relatively less expression of *T6PS* under salt stress in Varuna and T 9 was reflected in relatively less accumulation of Treh in these genotypes. Kosmas *et al*.[42] reported that coordinated transcriptional activation of *TPS1* gene with organ specific accumulation of Treh in the leaves and roots of cotton under drought stress. Occurrence of *T6PS* and *TPP* genes in the form of multigenic families in large group of plants and other lower organisms signifies important metabolic role of Treh in the living system[43].

In conclusion, it can be said that enough variability exists between the *Brassica* genotypes and even at the species level in terms of salinity induced responses and mechanism of tolerance. Differential response in induction of key antioxidant enzymes and variability in stress induced expression of key genes regulating osmotic adjustment in these plants seems to play major role in their salt-tolerance behaviour. This may well be the cause of differential responses in oxidative stress build-up and accumulation of compatible solutes for protection of sub-cellular structures and key macromolecules under salinity stress in *Brassica* spp. The levels of GB and Treh increased sharply under salinity stress in the *B. juncea* cultivars CS 52 and CS 54, while the increases were lesser in *B. campestris*. Gene expression pattern of *BADH* and *T6PS*, which regulate the synthesis of GB and Treh, correlated well with the higher levels of these metabolites in CS 52 and CS 54 under salt stress. This phenomenon might well be the reason for maintenance of better cell water relation, lesser membrane injury and thus created more favourable cellular environment in comparatively tolerant group of plants than the susceptible ones under salinity stress.

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