Journal of Soil Salinity and Water Quality 11(1), 18-30, 2019

Predicted Model to Reveal the Mechanism of Salt Tolerance in *Brassica juncea*

Jogendra Singh, PC Sharma*, Vijayata Singh and Parveen Kumar

ICAR-Central Soil Salinity Research Institute, Karnal-132001, Haryana, India
**Corresponding author E-mail: pcsharma.knl@gmail.com*

Abstract

Research was conducted to explore and predict model of the mechanism of salt tolerance in mustard (*Brassica juncea*) using four genotypes CS 54 (salt tolerant variety), CS 52-SPS-1-2012 (salt tolerant mutant), CS 614-4-1-4-100-13 (salt sensitive mutant) and Pusa bold (salt sensitive variety) under saline irrigation water (EC_{iw} 12, and 15 dS m^{-1}). Genotype CS 52-SPS-1-2012 followed by CS 54 performed better under imposed salt stress due to differentially regulation of Na^+ accumulation in the roots and main stem, restriction of Na^+ influx from root to shoot, maintaining higher net photosynthetic traits under saline stress compared to CS 614-4-1-4-100-13 and Pusa bold. Further, expression profiling of salt responsive antiporters (*SOS1, SOS2, SOS3, ENH1* and *NHX1*) and antioxidant (*APX1, APX4, DHAR3* and *MDHAR6*) genes elucidated their involvement in different components of salt tolerance mechanism including; ion efflux from root to soil, ion accumulation in vacuoles, retrieval of ions from xylem and increased tissue tolerance to high concentrations of toxic ions and accumulation of compatible solutes and significant role for imparting salt tolerance in Indian mustard. Predicted model based on these results, suggested the tree-fold effect of salt stress on mustard plants its counteract on these toxic paths for salt tolerance.

Key words: Gene expression, Indian mustard, Photosynthetic traits, Salt stress

Abbreviations: P_n= Rate of photosynthesis (µmol CO_2 m^{-2} s^{-1}); g_s = Stomatal conductance (mmol m^{-2} s^{-1}); E = Rate of transpiration (mmol H_2O m^{-2} s^{-1}); $_i$WUE = Instantaneous water use efficiency [µmol (CO_2) $mmol^{-1}$ (H_2O)]; C_i/C_a = CO_2 assimilation (µmol CO_2 mol^{-1}); PAR = Photo synthetically active radiation; *SOS* = Salt overly sensitive; *ENH* = Enhancer of *SOS; NHX* = Vacuolar Na^+/H^+ antiporters; *APX* = Ascorbate peroxidase; *DHAR* = Dehydroascorbate reductase; *MDHAR* = Mono-dehydroascorbate reductase

Introduction

Salinity is currently one of the most severe abiotic factors, limiting agricultural production. The Indian mustard (*Brassica juncea)* is a major oilseed crop for such areas. However, salinity affects as 50-90% yield reduction across the world. The Indian mustard (*Brassica* spp.) occupies a third place for source of vegetable oil in the world due to its considerable economic and nutritional value. Soil salinity creates a bottleneck for normal growth and development of crops in two ways: primarily, by disturbing the osmotic relationship of tissues and, secondarily, by specific ion effects. The adverse effects of salinity on mustard are the reduction in seed germination percentage, early seedling growth, plant height, seed yield (Singh *et al.,* 2016) as well as changed oil quality (Singh *et al.,* 2014) affecting a variety of physiological and biochemical processes.

Photosynthesis is one of the most important physiological phenomena, severely affected by salinity and is mediated through a reduction in stomatal conductance and internal CO_2 pressure (Yan *et al.,* 2012). The increasing salinity results in decrease photosynthetic rate. It may also involve stomatal and non-stomatal restrictions and suppression of photochemical processes (Hichem *et al.,* 2009). Stomata affect plant iWUE also by controlling CO_2 flux, and regulate water flux in plants to reduce water loss (Hentschel *et al.,* 2016). A decreased iWUE under salinity have been associated with the lower CO_2 assimilation and transpiration which resulted due to plant susceptibility to salinity (Abedinpour, 2017; Cruz *et al.,* 2017). The increased iWUE under salinity indicated plant salt tolerance that can enhance plant productivity, and necessary for sustainable food production in salt stress prone environments (Sikder *et al.,* 2016). Salinity stress negatively

affects stomatal conductance (g_s) in all the crop plants but the effect was higher in salt sensitive genotypes (James *et al.,* 2002). Besides, the salinity reduced the rate of photosynthetic CO_2 assimilation rate (as a function of C_i). This reduction may be due to reduced stomatal conductance and inhibition of the CO_2 availability for carboxylation. While, direct effects of NaCl on photosynthetic apparatus caused non-stomatal inhibition of photosynthesis which is independent of stomatal closure (Stoeva and Kaymakanova, 2008; Stepien and Johnson, 2009) which further lowers the biomass production and decreased yield (Almeida *et al.,* 2017). Keeping this in view, changes in photosynthetic traits along with ionic parameters were analyzed in salt tolerant and sensitive genotypes of Indian mustard to ascertain their association with salt tolerance.

Furthermore, salinity tolerance is the result of an interdependent series of molecular events comprising of particular gene activation and/or regulation of a range of salt stress-responsive genes (Passaia *et al*., 2013). Presently, the role of nine major salt responsive genes/sequences; *SOS1*, *SOS2, SOS3, ENH1*, *NHX1*, *APX*, *APX4, DHAR3* and *MDHAR6* involved in ionic and oxidative genes modules was studied with respect to the salt tolerance in Indian mustard. SOS pathway contains *SOS1, SOS2 and SOS3,* play a major role for salt tolerance in *Brassica crops* (Kumar *et al.,* 2009; Chakraborty *et al.,* 2012; Sharma *et al.,* 2015; Singh *et al.,* 2019).

This study aimed to dissect salt tolerance mechanism into its components and add molecular, physiological and biochemical tools to our selection approach, which earlier considered only biomass production and ion accumulation. We also explore the role of major salt responsive genes/sequences and photosynthetic traits in the mechanism of salt tolerance in the Indian mustard.

Material and Methods

Plant material and experimental setup

The experimental materi-al comprised of four Indian mustard genotypes; CS 54 (national check for salt tolerance), CS 52-SPS-1-2012 (highly salt tolerant mutant), Pusa bold (salt sensitive cultivar) and CS 614-4-1-4-100-13 (highly salt sensitive mutant). The study was conducted at ICAR-Central Soil Salinity Research Institute, Karnal, India. Initially, 15 seeds of each genotype were sown at depth of 1 cm in 20 kg capacity ceramic pots filled with sand in net house. A hole at the bottom of each pot plugged with glass wool facilitated drainage of extra irrigation water. The pots were irrigated by ¼ strength Hoagland solution prepared in normal tap water (control, 2 dS m^{-1}), saline water (EC_{iw} 12.0 and 15.0 dS m^{-1}) and maintained at full strength field capacity. Earlier, Singh and Sharma (2016) have reported EC_{iw} 12 dS m^{-1} as the threshold limit for Indian mustard. The saline water for irrigation was prepared in ¼ strength Hoagland nutrient solution with addition of NaCl, Na_2SO_4 and $CaCl_2$, maintain Na:Ca and Cl:SO_4 ratios of 4:1 which reflect the major ion compositions of naturally occurring saline waters/soils.

The pots were arranged in a factorial randomized block design (RBD) experiment with four replications. To maintain the respective salinity level in the root zone, pots were irrigated daily till the maturity of the crop. Plant sampling (10 plants per genotype) in the three replications for ionic accumulation, compartmentalization and estimation of photosynthetic traits was done at the flowering stage (52 days after sowing). Further, other one replication was used for gene expression studies and sample of 10 plants per genotype from control, EC_{iw} 12 and 15 dS m^{-1} salt stress, were taken (52 days after sowing); frozen immediately in liquid nitrogen and stored at –80°C before RNA isolation.

Irrigation with saline water was continued in three replications until the harvesting of the crop to estimate yield to correlate and validate the results of photosynthesis and gene expression studies. Remaining five plants per pot (after ionic and photosynthetic traits estimation) in three replications were harvested at maturity, and air dried before to record their seed yield under different salinity regimes.

Measurement of photosynthetic traits

The P_n, E, g_s, intracellular CO_2 concentration and leaf to air vapour pressure deficit ($VPD_{leaf\text{-}air}$) was measured using portable infrared gas analyzer (*LI-*

6400XT, Li-COR, USA). All the parameters were determined during the course of the experiment between 10:00 and 11:30 h in sunlight when weather conditions were; PAR ~700 µmol m^{-2} s^{-1}, relative humidity ~70%, temperature 25 ± 1°C and air CO_2 355 µmol mol^{-1}. Further, iWUE (µmol mol^{-1}) was calculated as P_n/E, and the CO_2 assimilation (µmol CO_2 mol^{-1}) was calculated as the ratio between internal CO_2 (C_i) and ambient CO_2 (C_a) concentration.

Measurement of ion concentration

The ion concentrations in shoot (mid and top shoot), roots and branches and leaves (basal, middle and top) were estimated using di-acid method (Piper, 1942) containing HNO_3 and $HClO_4$ acid (9:4) for complete understanding of the pattern of ion partitioning under imposed salt stress. The concentrations of Na^+ and K^+ in the samples and standards were estimated using ion specific filters in a flame photometer (Corning EEL, UK). The Standard curves of Na^+ and K^+ were plotted and estimated the ion concentrations as mg g^{-1} dry weight (DW) in the samples and calculated Na^+/K^+ ratio.

Statistical analysis

Statistical analyses and analysis of variance was done using the SAS 9.3 software (SAS Institute Inc., Cary, USA).

RNA extraction, quality analysis, and cDNA preparation

Total RNA was extracted from shoot tissues of control and salt-stressed plants using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA (1 µg) was further digested with DNaseI of *Sigma-Aldrich* following the instruction manual. RNA was treated with 1U of RNase inhibitor (RNAse K; Sigma) and stored at –80°C till further use. To verify quality and integrity of the RNA samples, it was run on 1% agarose gel and the 28S and 18S bands were confirmed. Furthermore, the absorbance ratio at 260/280 and 260/230 nm were determined by Nanodrop (Spectrophotometer ND-2000, Thermo Fischer, USA). The double-stranded cDNA was synthesized from the mRNA by gene-specific primers (Sharma *et al.*, 2015) (Supplementary file 1) by using high capacity cDNA reverse transcription kit (Applied Biosystems, USA) as follows: 6 µl of nuclease free water, 2 µl of 5 X reaction mix, 1 µl of 1000 ng/µl RNA and 1 µl of enzyme. The mixture was incubated for 10 min at 25°C, 1h at 42°C, 5 min at 95°C followed by 10 min at 4°C. The cDNA was immediately stored at –20°C till further use.

Quantitative real-time PCR analysis

In the present study, expression analysis of nine salt responsive genes/sequences (Sharma *et al.*, 2015) was carried out using qRT-PCR (Table 1). The qRT-PCR was performed using Fast SYBR Green Master Mix (Sigma-Aldrich, USA) on ABI Step One Plus real-time amplification thermal cycling system (Applied Biosystems, USA). Amplification reactions were conducted as above in triplicate and normalized using *Actin* gene as an internal control. The total volume of the reaction mixture was 25 µl, which consisted of 2 µl cDNA, 1 µl of forward and reverse primers (1 nmol each), 10 µl of SYBR Green and 11 µl of nuclease-free water. The relative expression level of the transcripts was calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). For expression analysis results, Heatmapper (Babicki *et al.*, 2016) was used for generating heat map.

Results and Discussion

Variable response of morpho-physiological traits to salinity

Analysis of variance exhibited the significant mean square for the studied traits which depicted significant genetic variation for P_n, gS, E, $_i$WUE, CO_2 assimilation, yield/plant, shoot Na/K and root Na/K ratio. Mean squares of the salinity levels under pot study were significant for all the traits under study indicated significant differences of these traits for control and salinity. The significant interaction effect of salinity levels × genotypes revealed the variable response of genotypes by expression of traits over the salinity (Table 2).

Photosynthesis and associated traits under salinity

The net photosynthesis, stomatal conductance, water use efficiency and transpiration rate

Table 1. Gene-specific primers used in real-time PCR analysis (Sharma *et al.*, 2015)

	Gene Name	Primer sequences (5′-3′)	
SOS1	Salt Overly Sensitive 1	F	GTTGCTCGGTGGACTCTTGA
		R	AAAACCACAATTGCCGTCCC
SOS2	Salt Overly Sensitive 2	F	TTTAGGGCTCATACGCGCAA
		R	CAAGTGTTTCACCAGCAGCC
SOS3	Salt Overly Sensitive 3	F	GGAGGAGTTTCAGCTTGCCT
		R	CGACCGTACAAACTCCCCAA
ENH 1	Enhancer of SOS3-1	F	TCCACTCCTCGTCTCCTCTCTT
		R	ATCTTCCAACGAAACTCCCTGAA
NHX1	Sodium/Hydrogen exchanger	F	CCCCGGAGAATCGAGAATATC
		R	CGAGATTGGAAGAGCAAAGACA
DHAR3	Dehydroascorbate reductase	F	GGCTTATCAAGGGATTCGGTTT
		R	CGCCATAGCCACTGTTCCTAAC
APX1	Ascorbate peroxidase; superoxide dismutase 1	F	TTGGGTTGGTGGAGGGTTT
		R	AGATCCTTCCGAGTACATTAAGCAA
APX4	Ascorbate peroxidase; superoxide dismutase 4	F	TCCCGACAACACTTTTTCTTTTC
		R	GCAGTCCCAGCGAGTTTGA
MDHAR6	Monodehydro ascorbate reductase 6	F	TTGTGTTCTGCTACACTCTTTAAAGCT
		R	AACTGGAAATAGAAGAGACATAGAAAGACTT

Table 2. Analysis of variance for photosynthetic traits, ionic ratio, and yield under saline environments

Source of variation	D.F.	Mean sum of squares							
		P_n ($\mu molCO_2$ m^{-2} s^{-1})	gs (mmol CO_2 m^{-2} s^{-1})	E ($\mu molCO_2$ m^{-2} s^{-1})	$_iWUE$ (μmol mol^{-1})	CO_2 assimilation (μmol CO_2 mol^{-1})	Yield/ plant (g)	Shoot Na/K	Root Na/K
Replication	2	5.28	0.11	0.28	0.86	0.02	13.28	3.33	10.04
Genotypes	3	252.23**	0.53**	1.34**	41.47**	1.19**	732.25**	218.12**	3812.3**
Salinity	4	855.88**	0.37**	21.58**	13.89**	0.30**	873.96**	730.62**	3863.6**
Interaction (salinity × genotype)	12	16.62**	0.71**	3.15**	3.63**	0.97**	36.88**	78.37**	1087.3**
Error	38	1.85	0.08	0.25	0.57	0.27	4.45	1.30	3.89

*, ** = Significant at P≤0.05 and P≤0.01 level of significance, respectively.

decreased substantially in the all genotypes evaluated at higher salinity (EC_{iw} 15 dS m^{-1}) as compared to control (Table 3). The highest reduction in photosynthesis rate at 15 dS m^{-1} was recorded in CS 614-4-1-4-100-13 (95.76%), while the lowest was recorded in CS 52-SPS-1-2012 (55.85%) followed by CS 54 (59.07%) compared to control. Similarly, highest reduction in stomatal conductance was noted in CS 614-4-1-4-100-13 (86.46%) while CS 52-SPS-1-2012 (33.33%) displayed lowest reduction at EC_{iw} 15 dS m^{-1} compared to control. Salt stress significantly reduced transpiration rate in all the genotypes and the rate of reduction increased with increasing salinity stress. At EC_{iw} 15 dS m^{-1}, the highest reduction in transpiration rate was recorded in CS 614-4-1-4-100-13 (67.82%), while CS 52-SPS-1-2012 displayed the lowest reduction (48.19%), compared to control. Further, the increasing salinity, also significantly ($P < 0.05$) affected the instantaneous water use efficiency in all the genotypes evaluated. The highest reduction in $_iWUE$ was noted in CS 614-4-1-4-100-13 (86.88%) while CS 52-SPS-1-2012 (14.80%) displayed the lowest at EC_{iw} 15 dS m^{-1} compared to control. Salt stress significantly reduced CO_2 assimilation rate in all the genotypes and the rate of reduction increased with increasing salinity stress. The

Table 3. Effect of different salinity levels on photosyn-thetic traits and yield of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})							
	Control	12	15	Mean	Control	12	15	Mean
	Photosynthesis Rate (P_n) (µmol CO_2 m^{-2} s^{-1})				Stomatal Conductance (g_S)(mmol CO_2 m^{-2} s^{-1})			
CS 54	23.09	18.14	9.45	16.89	0.53	0.35	0.30	0.39
CS 52-SPS-1-2012	27.57	19.68	12.17	19.81	0.57	0.48	0.38	0.48
Pusa bold	22.86	16.80	7.97	15.88	0.46	0.27	0.19	0.31
CS 614-4-1-4-100-13	22.86	4.80	0.97	9.54	0.57	0.15	0.10	0.27
Mean	24.1	14.85	7.64	15.53	0.53	0.31	0.24	0.36
	Genotypes (G)	Salinity (S)	G × S		Genotypes (G)	Salinity (S)	G × S	
C.D. (p=0.05)	0.48	0.53	1.06		0.02	0.02	0.04	
SE d±	0.23	0.26	0.51		0.01	0.01	0.02	
	Transpiration rate (E) (mmol H_2O m^{-2} s^{-1})				Instantaneous Water Use Efficiency ($_iWUE$) [µmol (CO_2) $mmol^{-1}$ (H_2O)]			
CS 54	4.23	3.00	2.03	3.09	5.46	6.05	4.66	5.39
CS 52-SPS-1-2012	4.98	3.13	2.58	3.56	5.54	6.29	4.72	5.51
Pusa bold	4.05	2.99	1.90	2.98	5.64	5.62	4.19	5.15
CS 614-4-1-4-100-13	4.35	2.29	1.40	2.68	5.26	2.10	0.69	2.68
Mean	4.4	2.85	1.97	3.08	5.47	5.02	3.57	4.68
	Genotypes (G)	Salinity (S)	G × S		Genotypes (G)	Salinity (S)	G × S	
C.D. (p=0.05)	0.13	0.14	0.28		0.3	0.33	0.67	
SE d±	0.06	0.07	0.13		0.14	0.16	0.32	
	CO_2 assimilation (µmol CO_2 mol^{-1})				Yield per plant (g)			
CS 54	0.82	0.70	0.56	0.69	28.95	16.51	10.37	18.61
CS 52-SPS-1-2012	0.86	0.70	0.63	0.73	35.99	25.56	14.27	25.27
Pusa bold	0.85	0.67	0.53	0.68	23.14	14.52	6.61	14.76
CS 614-4-1-4-100-13	0.81	0.56	0.47	0.61	21.49	3.64	2.97	9.37
Mean	0.84	0.66	0.55	0.68	27.39	15.06	8.56	17.00
	Genotypes (G)	Salinity (S)	G × S		Genotypes (G)	Salinity (S)	G × S	
C.D. (p=0.05)	0.01	0.01	0.02		0.68	0.76	1.53	
SE d±	0.004	0.005	0.01		0.33	0.37	0.73	

highest reduction in CO_2 assimilation rate at higher salinity was recorded in CS 614-4-1-4-100-13 (41.98%), while CS 52-SPS-1-2012 (26.74%) displayed the lowest, compared to control.

Reduction in its photosynthetic ability under salinity stress is often associated with decline in productivity in many plant species (Chaves *et al.*, 2009). All the four genotypes evaluated exhibited a significant reduction in net photosynthesis, stomatal conductance, water use efficiency and transpiration under increasing salinity (EC_{iw} 12 and 15 dS m^{-1}) stress, as compared to control. The comparatively higher reduction of net photosynthetic rate in the salt susceptible genotypes CS 614-4-1-4-100-13 and Pusa bold than the salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 under salt stress. Instantaneous water use efficiency (iWUE) is one of the very important determinants of crop yield under salinity stress. Increasing salinity significantly ($P < 0.05$) reduced water use efficiency more in the salt sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold compared to salt tolerant genotypes CS 54 and CS 52-SPS-1-2012. Such decline of $_iWUE$ in the salt susceptible genotypes may be due to higher reduction of carboxylation rate from non-stomatal factors *e.g.* decreasing *Rubisco* and chlorophyll contents at high salinity (Qi *et al.*, 2012). Consequently, all the genotypes exhibited significant decrease in the CO_2 assimilation rate ($P< 0.05$) with increasing levels of salinity over the control. However, the higher reduction was

observed in the salt sensitive genotype CS 614-4-1-4-100-13 then the salt tolerant CS 52-SPS-1-2012. This reduction in salt susceptible mustard genotypes can be attributed to salt induced damage of photosynthetic tissue and suppression in mesophyll conductance and thereby consequent restriction of CO_2 availability for carboxylation leading to acceleration of senescence at moderate and severe stress (Chaves *et al.,* 2009). The reduction in CO_2 assimilation rate may be due to reduced stomatal conductance and limited availability of CO_2 for carboxylation process in the salt susceptible mustard genotypes and direct effects of NaCl on photosynthetic apparatus that caused non-stomatal inhibition of photosynthesis which is independent of stomatal closure (Stoeva and Kaymakanova, 2008). In fact, the greater reduction level of photosynthetic traits under salt stress in the sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold compared to tolerant genotypes CS 54 and CS 52-SPS-1-2012 might be due to the stomatal closure, which automatically limit the CO_2 assimilation under salinity (Saleem *et al.,* 2011). The lower P_n values, under salt stress, were positively related to decrease in gs and C_i (Lu *et al.,* 2009). Modifications in cytoplasmic structures and negative feedback of diminished sink activity associated with slow transport of photosynthates may speed up the senescence of plant organs and shift in the activity of enzymes are other possible reasons for the salinity induced decrease in photosynthetic traits (Chaves *et al.,* 2009).

Reduction in seed yield was recorded in the four genotypes of mustard under increasing salinity. Highest reduction in seed yield was noticed in CS 614-4-1-4-100-13 (86.18%) while CS 52-SPS-1-2012 showed the lowest reduction (60.35%) at higher salinity of EC_{iw} 15 dS m^{-1}, compared to control. The decline in seed yield was higher in salt sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold than the salt tolerant genotypes CS 52-SPS-1-2012 and CS 54. Higher reduction in seed yield of salt susceptible genotypes may be attributed to a reduction in photosynthetic rate, lesser accumulation of assimilates, inhibition of their movement towards the developing reproductive organs and transformation of leaf tissue as a sink rather than source while higher seed yield in salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 was the result of enhanced translocation of photosynthetic product towards the developing reproductive organs under salt stress (Asha and Dhingra, 2007; Singh *et al.,* 2019).

Ion accumulation and their partitioning in different tissues of plant under salinity

Increasing levels of salinity lead to elevated concentration of Na^+ in shoot over control. Maximum Na^+ accumulation was recorded in roots followed by shoot tissues of all the genotypes (Fig. 1). At the higher salinity (EC_{iw} 15 dS m^{-1}), shoot and root tissues of CS 614-4-1-4-100-13 accumulated the highest amount of Na^+ (5.4 and 8.2 times, respectively,) compared to control. On the contrary, lowest Na^+ concentration in shoot and root was recorded in Pusa bold (2.5 times) and CS 52-SPS-1-2012 (3.3 times). Further, the increasing levels of salinity lead to drastic reduction in the accumulation of K^+ in both shoot and root tissues, compared to control (Fig. 1). The highest reduction of K^+ at EC_{iw} 15 dS m^{-1} was found in the shoot (91.09%) and root (92.12%) tissues of CS 614-4-1-4-100-13 followed by Pusa bold (90.53% in the shoot and 89.34% in root). However, the lowest reduction in K^+ concentration was recorded in the shoot and root tissues of CS 52-SPS-1-2012 (84.20% and 73.66%, respectively) followed by CS 54 (84.58% in the shoot and 88.02% in root). Further, Na^+/K^+ ratio of the shoot and root was significantly lowest in the salt tolerant mutant CS 52-SPS-1-2012 (1.64 and 2.33, respectively), whereas, the ratio was highest in the salt susceptible mutant CS 614-4-1-4-100-13 (2.58 and 6.87) followed by Pusa bold (2.37 and 6.04) for shoot and root, respectively, across the salinity levels.

The significant differences in ion accumulation (both Na^+ and K^+) in shoot and roots under different salinity treatments were recorded in all the genotypes. Increasing levels of salinity lead to elevated concentration of Na^+ in shoot of salt sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold than the salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 compared to control. This higher Na^+ accumulation in the shoot of salt sensitive genotypes can be attributed to the differential cellular entry of ions under high

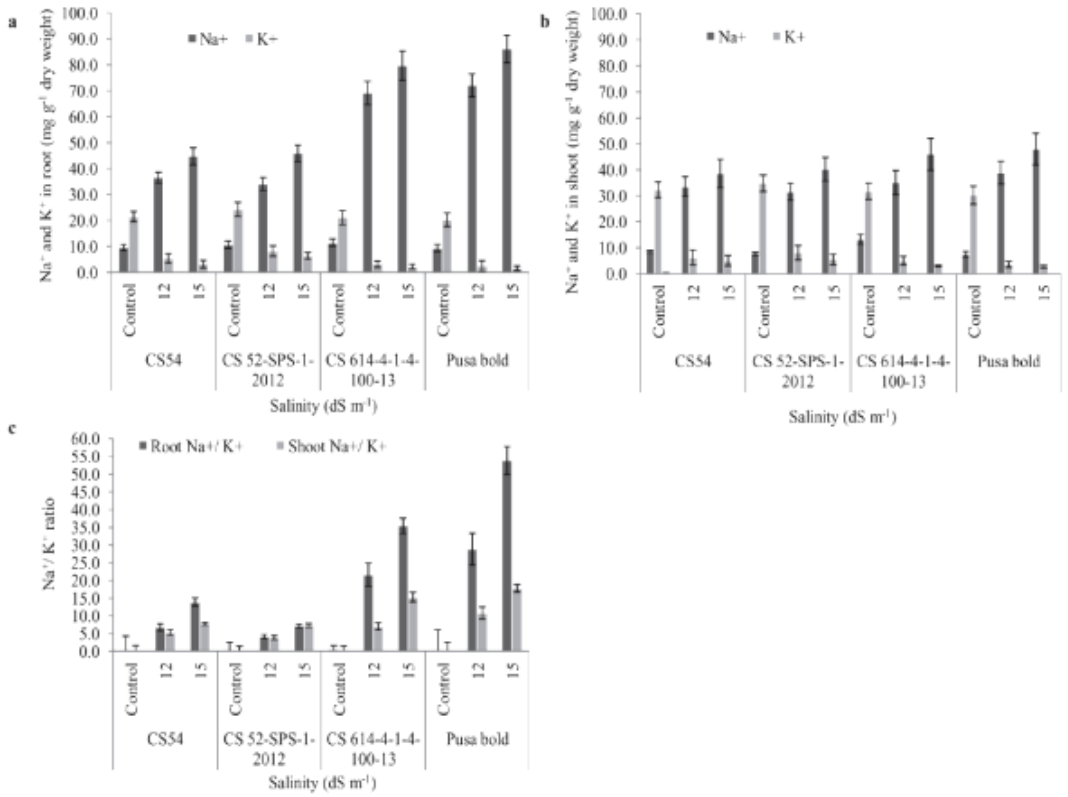

Fig. 1 Partitioning of Na^+, K^+ concentration (mg g^{-1} dry weight) in (a) root; (b) shoot; and (c) Na^+/K^+ ratio in root and shoot of salt tolerant and sensitive Indian mustard genotypes. Plotted values are the average of 10 independent biological samples per genotype from each salinity level with standard error

salinity, as the similarity in the hydrated ionic radii between Na^+ and K^+ makes it difficult for the transporter to discriminate between the two ions (Blumwald, 2000). Increasing levels of salinity lead to drastic reduction in the concentration of K^+ in both shoot and root tissues of salt sensitive genotype CS 614-4-1-4-100-13 than the salt tolerant genotype CS 52-SPS-1-2012 compared to control treatment. The enhanced internal K^+ content of the salt tolerant genotype CS 52-SPS-1-2012 might have contributed to the cellular level salt tolerance (Gupta *et al.,* 2002). One can assume that most of the harmful ions are compartmentalized into the vacuoles, thereby preserving intracellular ion homeostasis necessary for cytoplasmic metabolic activity and increase cellular osmolality to counter osmotic stress (Wang *et al.,* 2007; Singh *et al.,* 2019).

Salinity tolerance is related to the ability of the plant to maintain a lower Na^+/K^+ ratio (Ashraf and McNeilly, 2004; Almeida *et al.,* 2017) rather than simply maintaining low Na^+ concentrations. Further, genotypes were analysed for Na^+ content and Na^+/K^+ ratio in leaves (basal, middle and top), branches and shoot (middle and top) to study the partitioning behaviour of Na^+ and K^+ in different plant parts (Fig. 2). Increasing salinity levels lead to differential accumulation of Na^+ in leaves with higher Na^+ concentrations in basal leaves and lower Na^+ concentrations in leaves towards the middle and top of all the genotypes. The sensitive genotype CS 614-4-1-4-100-13 accumulated 9 times more Na^+ in the basal leaves at higher salinity level whereas the tolerant genotypes CS 54 and CS 52-SPS-1-2012 accumulated Na^+ to the tune of only 4 times to that of the control (Fig. 2a). The Na^+/K^+ ratio increase in all the genotypes with respect to salinity levels and leaf positions from top to basal. As compared to control conditions, CS 614-4-1-4-100-13 showed about 52 times higher Na^+/K^+ ratio in basal leaves, whereas CS 52-SPS-1-2012 showed on twice under increased salinity levels as compared to control (Fig. 2b). The trends of Na^+

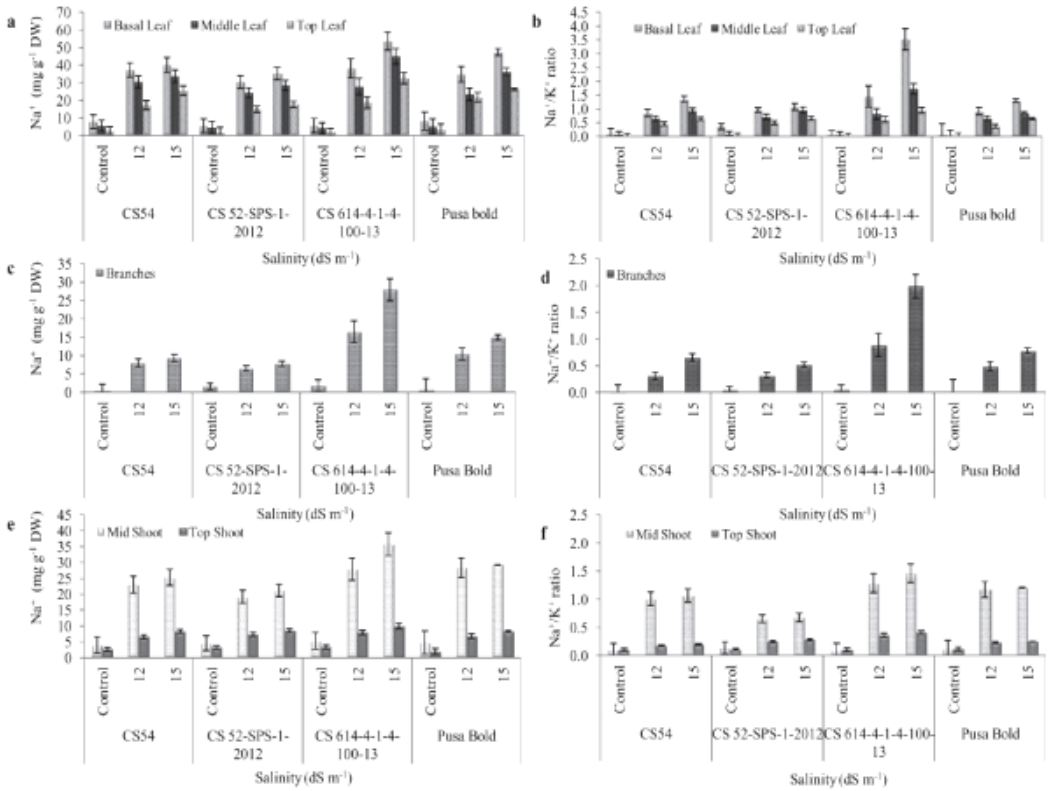

Fig. 2 Partitioning of Na^+ concentration (mg g^{-1} dry weight) and Na^+/K^+ ratio in; (a,b) leaves (basal, middle and top); (c,d) branches; (e,f) shoot (mid and top) of salt tolerant and sensitive Indian mustard genotypes

concentrations and Na^+/K^+ ratio in middle and top leaves are similar to basal leaves with a lower extent. Imposed salinity stress also clearly depicted the differential pattern of Na^+ accumulation in the branches of all the four different genotypes. Both CS 614-4-1-4-100-13 and Pusa bold accumulated very high levels of Na^+ than CS 54 and CS 52-SPS-1-2012 with increasing salinity stress. Notably, CS 614-4-1-4-100-13 and Pusa bold accumulated 15 and 24 times, respectively, more Na^+ at the highest salinity EC_{iw} 15 dS m^{-1}, compared with the control. Whereas, CS 52-SPS-1-2012 was able to restrict its internal Na^+ levels to only 4 times higher level compared with the control (Fig. 2c). The Na^+/K^+ ratio increased under various imposed salinity levels in branches of all the genotypes. However, the sensitive genotype, CS 614-4-1-4-100-13 and Pusa bold displayed 42 and 51 times, respectively, higher Na^+/K^+ ratio as compared to control, at the highest salinity level. Whereas, CS 52-SPS-1-2012 was able to maintain a comparatively lower Na^+/K^+ to only 12 times higher as compared with the control conditions (Fig. 2d). Differential accumulation of Na^+ in the shoot was observed in all the genotypes with higher Na^+ concentrations in the middle portion and lower Na^+ concentrations in the top part of the shoot. CS 614-4-1-4-100-13 accumulated higher Na^+ levels in both middle and top shoot than the other three genotypes with increasing intensities of salinity stress while CS 52-SPS-1-2012 showed reduced Na^+ levels under salinity. With respect to control conditions, the sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold accumulated about 6 times more Na^+ in mid shoot at higher salinity level whereas the tolerant genotypes CS 54 and CS 52-SPS-1-2012 accumulated Na^+ to the tune of only 4 times to that of the control (Fig. 2e). The Na^+/K^+ ratio was extremely low in the top shoot as compared to the middle one in all the genotypes. The Na^+/K^+ ratio increased in mid shoot of CS 614-4-1-4-100-13 under salinity was to the tune of 16 times higher compared with the control (Fig. 2f).

The partitioning behaviour of Na^+ and K^+ in different plant parts; leaves (basal, middle and top), branches and shoot (middle and top) showed that increasing salinity levels lead to differential accumulation of Na^+ in leaves with higher Na^+ concentrations in basal leaves and lower Na^+ concentrations in leaves towards the middle and top of all the genotypes. Salinity tolerance is related to the ability of the plant to maintain a lower Na^+/K^+ ratio (Ashraf and McNeilly, 2004) rather than simply maintaining low Na^+ concentrations. The comparatively lower Na^+ content in roots of salt tolerant mustard genotypes CS 54 and CS 52-SPS-1-2012 indicated the involvement of Na^+ exclusion, while salt susceptible mutant 614-4-1-4-100-13 behaved as its accumulator by maintaining the higher concentration. Nevertheless, an increase in root Na^+ concentration, due to high salinity treatments, indicated that Na is compartmentalized in the roots (Blumwald, 2000; Baalbaki *et al.,* 2000; Munns and Tester, 2008). Controlled Na^+ uptake and lower Na^+/K^+ reduced the toxic effect of Na^+ in the cytosol and increasing cells water uptake (deVos *et al.,* 2013; Almeida *et al.,* 2017; Singh *et al.,* 2018a). Nonetheless, the roots of salt-exposed plants had the highest Na^+ concentrations of all plant parts studied. The alteration of ion ratios in the plant and differential distribution of Na^+ ions between basal, middle and top plant parts grown under high salt content is caused by the influx of Na through pathways that function in the acquisition of K^+ (Blumwald *et al.,* 2000; Kumar *et al.,* 2003; Almeida *et al.,* 2017).

Association between different physiological traits

Among the variables studied, the highest correlation coefficient recorded was between CO_2 assimilation and transpiration with r = 0.95** (Table 4). However estimated higher value of significant and positive correlation of seed yield with photosynthesis (r = 0.90**), stomatal conductance (r = 0.85**), transpiration (r = 0.82**), instantaneous water use efficiency (r = 0.64**) and CO_2 assimilation (r = 0.81**) clearly depicts that grain yield is under direct influence of these highly important physiological processes.

Table 4. Association among yield and different physiological traits of mustard genotypes

Traits	P_n	g_s	E	$_iWUE$	C_i/C_a	Yield (g/plant)	Shoot Na^+ (mg g^{-1} dry weight)	Shoot K^+ (mg g^{-1} dry weight)	Root Na^+ (mg g^{-1} dry weight)	Root K^+ (mg g^{-1} dry weight)
P_n	1.00									
g_s	0.93**	1.00								
E	0.92**	0.92**	1.00							
$_iWUE$	0.74**	0.63**	0.47**	1.00						
C_i/C_a	0.94**	0.90**	0.95**	0.62**	1.00					
Yield (g/plant)	0.90**	0.85**	0.82**	0.64**	0.81**	1.00				
Shoot Na^+ (mg g^{-1} dry weight)	-0.89**	-0.86**	-0.95**	-0.50**	-0.92**	-0.80**	1.00			
Shoot K^+ (mg g^{-1} dry weight)	0.73**	0.76**	0.83**	0.29	0.74**	0.65**	-0.88**	1.00		
Root Na^+ (mg g^{-1} dry weight)	-0.86**	-0.79**	-0.83**	-0.63**	-0.85**	-0.78**	0.93**	-0.85**	1.00	
Root K^+ (mg g^{-1} dry weight)	0.76**	0.82**	0.84**	0.35*	0.75**	0.61**	-0.83**	0.92**	-0.78**	1.00

P_n=Photosynthetic rate (μmolCO_2.m^{-2} s^{-1}); g_s=Stomatal conductance (mmolm^{-2} s^{-1}); E=Transpiration rate (mmol H_2O m^{-2} s^{-1}); $_iWUE$=Instantaneous water use efficiency [μmol (CO_2) $mmol^{-1}$ (H_2O)]; C_i/C_a=CO_2 assimilation (μmol CO_2 mol^{-1}).

*, ** = Significant at P≤0.05 and P≤0.01 level of significance, respectively.

Differential expression of salt responsive genes under salinity

Plants generally adapt transcriptional regulation salt responsive genes to mitigate the harmful effects of high salt concentration by restoration of cellular ion homeostasis and osmotic balance (Mallikarjuna *et al.,* 2011). Transcript abundance study in response to salt stress could also be provided an estimate of specific gene activation or down regulation (Liao *et al.,* 2016; Singh *et al.,* 2018b). The levels of transcription of nine major genes and their roles in stress signalling network was studied under salinity using qRT-PCR to corroborate the differential responses of physiological and ionic parameters in the salt tolerant (CS 54 and CS 52-SPS-1-2012) and salt sensitive (Pusa bold and CS 614-4-1-4-100-13) genotypes under imposed salinity stress. The genes under study were categorized into two groups as Group I (*SOS1, SOS2, SOS3, ENH1* and *NHX1*) pertaining to ionic and Group II (*APX1*, *APX4*, *DHAR3* and *MDHAR6*) pertaining to oxidative modules of salt stress tolerance, respectively (Fig. 3). The expression of genes *SOS1, SOS2, SOS3, ENH1* and *NHX1*, was found higher in salt tolerant genotypes compared to salt sensitive genotypes under salt stress (EC_{iw} 12 and 15 dS m^{-1}). However *SOS3, ENH1* and *NHX1* showed higher expression at EC_{iw} 15 dS m^{-1}. The salt sensitive genotypes are slow to respond and to maintain homeostasis while this process is faster in the salt tolerant genotypes. Similarly, the expression of *APX1*, *APX4* and *MDHAR6* was assessed higher in salt tolerant genotypes compared to salt sensitive genotypes.

The expression of genes pertaining to ionic module was found higher in the salt tolerant genotypes CS 54 and CS 52-SPS-1-2012 than salt sensitive genotypes Pusa bold and CS 614-4-1-4-100-13 under salt stress (EC_{iw} 12 and 15 dS m^{-1}). We also observed higher expression of *ENH1* and *NHX1* showed higher expression in the salt tolerant genotype at 15 dS m^{-1}. Although, *ENH1* showed high transcript levels in *B. juncea* even

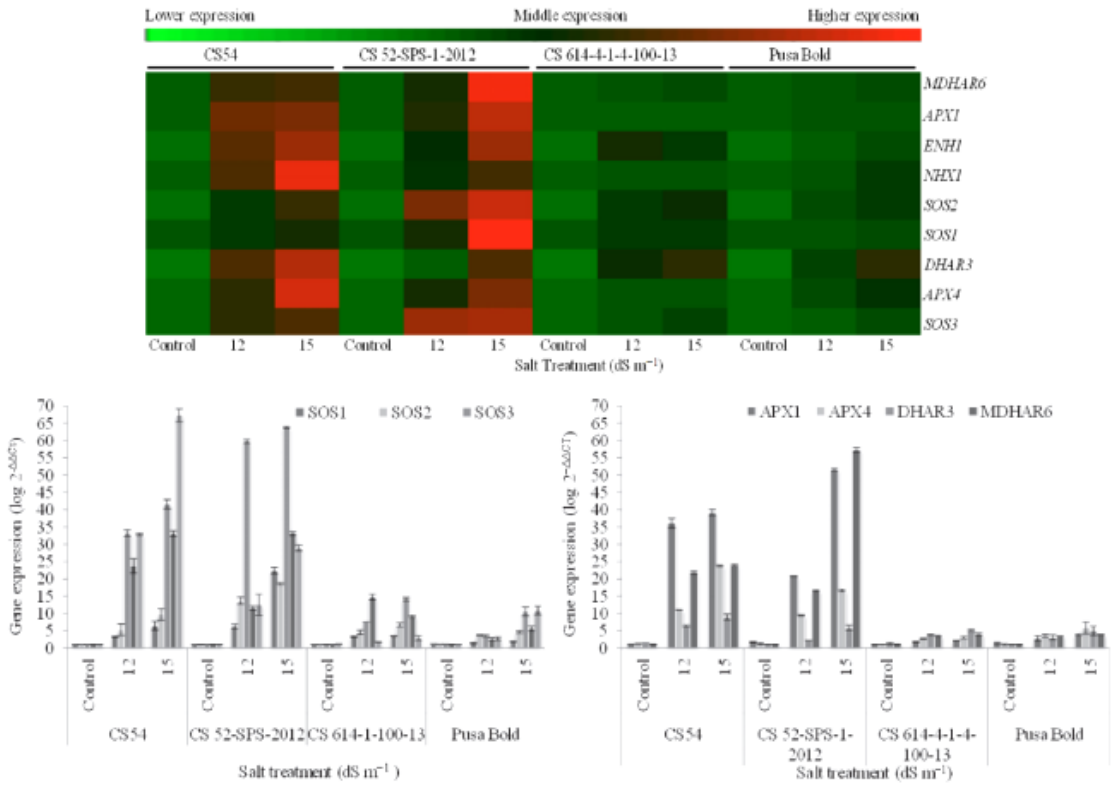

Fig. 3 Expression profiling of salt responsive genes/sequences in Indian mustard: SOS1, SOS2, SOS3, ENH, NHX1, APX1, APX4, DHAR3 and MDHAR6. Plotted values are the average of 10 independent biological samples per genotype from each salinity level with standard error

though in non-stress conditions and is further over-expressed in response to salinity (Kumar *et al.*, 2009; Sharma *et al.*, 2015). The Na^+ exclusion mechanism of *NHX1*encoded Na^+/H^+ antiporter from the transpiration path is an important trait in salt tolerance of mustard genotypes CS 54 and CS 52-SPS-1-2012. In fact, our results can be complemented with the Na^+ and K^+ concentrations estimated in the root and shoot of mustard genotypes and established roles of *NHX1* in ion homeostasis by active K uptake and removes excess Na^+ from xylem through sequestration into the vacuole; thus, protecting the tissues of photosynthetic leaf from the toxic effect of Na^+ in the tolerant plants (Singh *et al.*, 2019). The better performance of CS 54 and CS 52-SPS-1-2012 under salt stress was associated with the *SOS* protein complex phosphorylation and activation of plasma membrane localized Na^+/H^+ antiporters that enhanced sequestration of Na^+ in roots and reduced toxic Na^+ transport to shoots. This suggested that salt sensitive genotypes (Pusa bold and CS 614-4-1-4-100-13) are slow to respond and to maintain homeostasis while this process is faster in the salt tolerant genotypes. The expression pattern of these transporters is modulated by the salt tolerant genotypes resulted a minimum accumulation of Na^+ in the salt tolerant cultivars. Further, salt sensitive genotypes were not able to regulate these pathways as efficiently as salt tolerant (Chakraborty *et al.*, 2012; Ji *et al.*, 2013; Singh *et al.*, 2019).

The genes *APX1*, *APX4* and *MDHAR6* pertaining to oxidative module also showed a higher induction in the salt tolerant genotypes than salt sensitive. Further, higher accumulation of Na^+ in salt sensitive genotypes tissues cause more oxidative stress as well as ionic stress which can be correlated with their lower expression in these genotypes (Diaz-Vivancos *et al.*, 2013) and higher levels of ROS accumulation (Chawla *et al.*, 2013; Martinez *et al.*, 2018; Singh *et al.*, 2019). Over-expression of *APX1*, *APX4* and *MDHAR6* in the salt tolerant genotypes than sensitive one at higher salt stress implies the efficient detoxification of reactive oxygen species (ROS) in these genotypes and reduced the sodium accumulation which results in low electrolytic leakage and lipid peroxidation compared to control plants under salinity stress and helps in mitigating the adverse effects of salinity stress in mustard and promotes plant recovery from the stress.

Based on our findings on we have developed a model predicted the salt tolerance mechanism in Indian mustard and conditioning the differential functions of antiporter and antioxidant transcripts in the mitigation of detrimental effect of salt stress (Fig. 4).

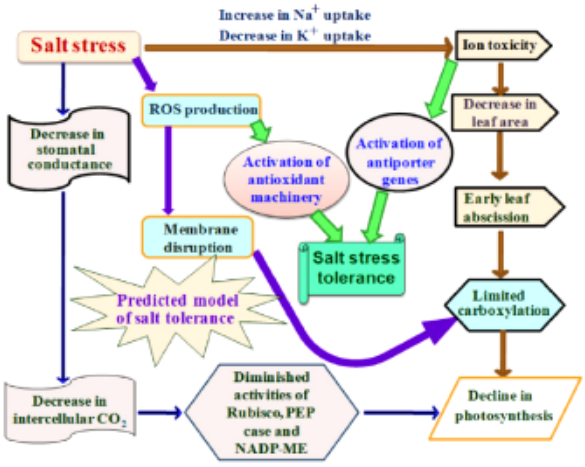

Fig. 4 Model suggested the three-way effect of salt stress on mustard plants; (i) Decreasing stomatal conductance results in the decreased intercellular CO_2 which caused diminishing activities of photosynthetic enzymatic machinery and decline in net photosynthesis rate. (ii) Production of reactive oxygen species (ROS) which disrupt the membrane system and limited the carboxylation process results in the least photosynthesis. (iii) Imbalance in the cellular ionic concentrations due to increased uptake of Na^+ and decreased K uptake which caused ion toxicity. This ion toxicity leads to decrease in leaf area and early leaf fall down and limited carboxylation results in declined photosynthesis rate. The salt tolerant mustard genotypes counteract on these toxic paths by activation of antioxidant gene network for ROS scavenging and antiporter gene complex that enhanced sequestration of Na^+ in roots and reduced toxic Na^+ transport to shoots, hence, makes mustard plant tolerant to salt stress

Conclusions

The salt tolerance in the Indian mustard under imposed salinity stress might be the function of Na^+/H^+ antiporters that enhanced sequestration of Na^+ in roots and restricted transport of toxic Na^+ to shoots and from older leaves to metabolically active younger leaves thus maintaining the higher net photosynthetic traits under stress compared to salt susceptible genotypes. This research will not only help

researchers in determining relative importance of different components of salt tolerance mechanism but will also facilitate genes that can then be used to screen mustard germplasm for salt tolerance. The manipulation of some of these genes might help to conditioning of photosynthetic attributes leading to a promising yield under salinity stress. Thus, breeders should pay equal attention to the photosynthetic traits along with pyramiding of antiporters and antioxidant defence genes for higher economic yield under salinity stress.

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Received: August 2018; Accepted: November 2018