RESEARCH ARTICLE

Differential response of Indian mustard (*Brassica juncea* L., Czern and Coss) under salinity: photosynthetic traits and gene expression

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Abstract To explore the effect of salt stress on photosynthetic traits and gene expression in Indian mustard, four genotypes CS 54 (national check for salinity), CS 52-SPS-1-2012 (salt tolerant mutant), CS 614-4-1-4-100-13 (salt sensitive mutant) and Pusa bold (high yielding variety) were evaluated under irrigation water salinity (EC_{iw} 12, and 15 $dS\ m^{-1}$). Results suggest genotype CS 52-SPS-1-2012 followed by CS 54 performed better under imposed salt stress due to differential 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, overexpression of antiporters (*SOS1*, *SOS2*, *SOS3*, *ENH1* and *NHX1*) and antioxidant (*APX1*, *APX4*, *DHAR1* and *MDHAR*) genes in salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 demonstrated their significant role in imparting salt tolerance in Indian mustard.

Keywords Salt tolerant · Salinity · Gene expression · Photosynthetic traits

Abbreviations

P_n	Rate of photosynthesis ($\mu mol\ CO_2\ m^{-2}\ s^{-1}$)
g_s	Stomatal conductance ($mmol\ m^{-2}\ s^{-1}$)
E	Rate of transpiration ($mmol\ H_2O\ m^{-2}\ s^{-1}$)

$iWUE$	Instantaneous water use efficiency [$\mu mol\ (CO_2)\ mmol^{-1}\ (H_2O)$]
C_i/C_a	CO_2 assimilation ($\mu mol\ CO_2\ mol^{-1}$)
PAR	Photosynthetically active radiation
<i>SOS</i>	Salt overly sensitive
<i>ENH</i>	Enhancer of <i>SOS</i>
<i>NHX</i>	Vacuolar Na^+/H^+ antiporters
<i>APX</i>	Ascorbate peroxidase
<i>DHAR</i>	Dehydroascorbate reductase
<i>MDHAR</i>	Mono-dehydroascorbate reductase

Introduction

Gradual and progressive exposure of arid and semi-arid regions to salt stress across the globe, is expected to result in about 30–50% loss of land by the year 2050 (Wang et al. 2003). While, salt affected area is estimated to increase to 16.2 million ha by 2050 against the current 6.74 million ha in different agro-ecological regions of India (CSSRI 2015).

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 brassica 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 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

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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 CO₂ 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 CO₂ assimilation and transpiration which resulted due to plant susceptibility to salinity (Omamt et al. 2006; 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 CO₂ assimilation rate (as a function of C_i). This reduction may be due to reduced stomatal conductance and inhibition of the CO₂ 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 analysed in salt tolerant and sensitive genotypes of Indian mustard to ascertain their association with salt tolerance.

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 comprising of *SOS1*, *SOS2* and *SOS3*, play a major role for salt tolerance in *Brassica crops* (Kumar et al. 2009; Chakraborty et al. 2012). During the stress, *SOS3* (a calcium binding protein) senses increasing concentration of cytosolic calcium and interacts with *SOS2* protein kinase. Then, *SOS2-SOS3* protein complex phosphorylates and activates *SOS1* which is a plasma membrane localized Na⁺/H⁺ antiporter conditioning ion homeostasis (Ji et al. 2013). *NHX1* (vacuolar Na⁺/H⁺ antiporter) play important role in salt tolerance by compartmentalization of Na⁺ into the vacuole and maintain osmotic balance, thus enhancing water uptake and salt tolerance of plants (Blumwald and Poole 1985; Aharon et al. 2003). The *ENH1* gene encodes a chloroplast-localized rubredoxin-like protein involved in the control of ROS detoxification that appears to connect the ion homeostasis function of the SOS pathway via *SOS2* with components of

oxidative stress suppression signalling (Zhu et al. 2007). APXs (Ascorbate peroxidases) are considered major hydrogen peroxide-scavenging enzymes that catalyze the conversion of H₂O₂ to water with generation of two molecules of MDHA (monodehydro-ascorbate). *APX1* is localized in cytoplasm, while *APX4* in chloroplast localization are required for the protection of chloroplasts against reactive oxygen species (Davletova et al. 2005; Panchuk et al. 2005). Genes encoding DHAR 3 (Cytosolic dehydroascorbate reductase) and MDHAR6 (Chloroplast monodehydroascorbate reductase) minimized the oxidative stress with a reduced lipid peroxidation and chlorophyll degradation, induced by the salinity (Miller et al. 2010; Etlilib et al. 2012). DHAR and MDHAR are essential in order to enhance APX activity (Asada 1999). Earlier reports (Sharma et al. 2015; Chakraborty et al. 2012) indicate their association with abiotic stress tolerance in *Brassica juncea*.

This study aimed to explore the role of major salt responsive genes/sequences and photosynthetic traits in the development of salt tolerant mustard genotypes.

Materials and methods

Plant material

The experimental material comprised of four Indian mustard genotypes; CS 54 (national check for salt tolerance) and Pusa Bold (high yielding national Check) and two mutants CS 52-SPS-1-2012 (highly salt tolerant) and CS 614-4-1-4-100-13 [highly salt sensitive, developed by gamma rays irradiation treatment and stabilised for six generations (Sharma et al. 2008)].

Experimental setup

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, Na₂SO₄ and CaCl₂, maintaining Na:Ca and Cl:SO₄ 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, compartmentalisation 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 rate of photosynthesis (P_N), transpiration (E), stomatal conductance (g_s), intracellular CO₂ concentration and leaf to air vapour pressure deficit (VPD_{leaf-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 $\mu\text{mol m}^{-2} \text{s}^{-1}$, relative humidity ~ 70%, temperature 25 ± 1 °C and air CO₂ 355 $\mu\text{mol mol}^{-1}$. Further, iWUE ($\mu\text{mol mol}^{-1}$) was calculated as P_N/E , and the CO₂ assimilation ($\mu\text{mol CO}_2 \text{mol}^{-1}$) was calculated as the ratio between internal CO₂ (C_i) and ambient CO₂ (C_a) concentration.

Measurement of ion concentration

The ion concentrations in shoot (mid and top shoot) and roots were estimated using di-acid method (Piper 1942) containing HNO₃ and HClO₄ 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 (ANOVA) was done using the SAS 9.3 software (SAS Institute Inc., Cary, USA).

Quantitative reverse transcription-PCR (qRT-PCR) analyses

Complete RNA was extracted from shoot tissues of control and salt-stressed plants using TRIzol reagent (Invitrogen, USA). Contaminating DNA was removed by further treatment with DNase I of *Sigma-Aldrich* following the manufacturer's instructions. RNA was treated with 1 unit of RNase inhibitor (RNase K; *Sigma*) and stored at - 80 °C till further use. It was run on 1% agarose gel to verify quality and integrity of the RNA samples, and the 28S and 18S bands were confirmed. Next, 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 Table S1) 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, 1 h 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.

The expression analysis of nine salt responsive genes (Supplementary Table S1) was carried out with qRT-PCR 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 (three technical replicates) using 10 independent biological sample of each genotype per salinity treatment (10 biological replicates) 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. Cycle threshold (C_T) values from each reaction were generated, and the relative changes in gene expression were calculated using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001), where fold change in gene expression is $\log 2^{-\Delta\Delta C_T}$, in which $\Delta C_T = (C_T \text{ of target gene} - C_T \text{ of reference gene})$, and $\Delta\Delta C_T = (\Delta C_T \text{ of salt treatment sample} - \Delta C_T \text{ of reference sample})$. For expression analysis results, Heatmapper (Babicki et al. 2016) was used for generating heat map.

Results

Analysis of variance

Significant mean square of genotypes indicating significant genetic variation was detected for photosynthetic rate (P_n), stomatal conductance (g_s), transpiration rate (E), instantaneous water use efficiency ($iWUE$), 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 indicating significant differences of these traits for control and salinity. The significant interaction effect of salinity levels $\times$ genotypes revealed the variable response of genotypes by expression of traits over the salinity (Table 1).

Effects of irrigation water salinity on photosynthesis and associated traits

The net photosynthesis, stomatal conductance, water use efficiency and transpiration rate decreased substantially in the all genotypes evaluated at higher salinity (EC_{iw} 15 $dS\ m^{-1}$) as compared to control (Table 2). 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 (Table 3).

Salt stress significantly reduced transpiration rate in all the genotypes and the rate of reduction increased with increasing salinity stress (Table 4). 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 (Table 5).

Salt stress significantly reduced CO_2 assimilation rate in all the genotypes and the rate of reduction increased with increasing salinity stress (Table 6). The 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 displayed the lowest (26.74%), compared to control.

Seed yield under salinity

Reduction in seed yield was recorded in the four genotypes of mustard under increasing salinity (Table 7). 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.

Effect of irrigation water salinity on ionic accumulation in different plant parts

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.

Expression pattern of nine genes under varying irrigation water salinity

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 levels of transcription of nine major genes and their roles in stress signalling network was studied under salinity using qRT-PCR. 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*, *DHAR1* and *MDHAR3*) pertaining to oxidative modules of salt stress tolerance, respectively (Fig. 2).

Table 1 Summary of analysis of variance for ionic ratio, photosynthetic traits and yield under saline environments

Source of variation	df	Parameters	Photosynthetic rate (P_n) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Stomatal conductance (gS) (mmol $\text{CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Transpiration rate (E) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$)	Instantaneous water use efficiency (iWUE) ($\mu\text{mol mol}^{-1}$)	CO_2 assimilation ($\mu\text{mol CO}_2 \text{ mol}^{-1}$)	Yield/plant (g)	Shoot Na/K	Root Na/ K
Replication	2	MSS F_{Ca} F_{Cr}	5.28 2.85 $P \leq 0.05 = 3.25$; $P \leq 0.01 = 5.21$	0.11 1.38 $P \leq 0.01 = 5.21$	0.28 1.12 $P \leq 0.01 = 5.21$	0.86 1.51 $P \leq 0.01 = 5.21$	0.02 0.07 $P \leq 0.01 = 5.21$	13.28 2.98 $P \leq 0.01 = 5.21$	3.33 2.56 $P \leq 0.01 = 5.21$	10.04 2.58 $P \leq 0.01 = 5.21$
Genotypes	3	MSS F_{Ca} F_{Cr}	252.23** 136.34 $P \leq 0.05 = 2.85$; $P \leq 0.01 = 4.34$	0.53** 6.63 $P \leq 0.01 = 4.34$	1.34** 5.36 $P \leq 0.01 = 4.34$	41.47** 72.75 $P \leq 0.01 = 4.34$	1.19** 4.41 $P \leq 0.01 = 4.34$	732.25** 164.55 $P \leq 0.01 = 4.34$	218.12** 167.78 $P \leq 0.01 = 4.34$	3812.3** 980.03 $P \leq 0.01 = 4.34$
Salinity	4	MSS F_{Ca} F_{Cr}	855.88** 462.64 $P \leq 0.05 = 2.62$; $P \leq 0.01 = 3.86$	0.37** 4.63 $P \leq 0.01 = 3.86$	21.58** 86.32 $P \leq 0.01 = 3.86$	13.89** 24.37 $P \leq 0.01 = 3.86$	0.30** 4.81 $P \leq 0.01 = 3.86$	873.96** 196.40 $P \leq 0.01 = 3.86$	730.62** 562.02 $P \leq 0.01 = 3.86$	3863.6** 993.22 $P \leq 0.01 = 3.86$
Interaction (salinity $\times$ genotype)	12	MSS F_{Ca} F_{Cr}	16.62** 8.98 $P \leq 0.05 = 2.02$; $P \leq 0.01 = 2.69$	0.71** 8.88 $P \leq 0.01 = 2.69$	3.15** 12.60 $P \leq 0.01 = 2.69$	3.63** 6.37 $P \leq 0.01 = 2.69$	0.97** 3.59 $P \leq 0.01 = 2.69$	36.88** 8.29 $P \leq 0.01 = 2.69$	78.37** 60.28 $P \leq 0.01 = 2.69$	1087.3** 279.52 $P \leq 0.01 = 2.69$
Error	38	MSS	1.85	0.08	0.25	0.57	0.27	4.45	1.30	3.89

df degree of freedom, MSS mean sum of squares, F_{Ca} calculated F value, F_{Cr} critical F value, F ratio of two variances*, ** Significant at $P < 0.05$ and $P < 0.01$ levels respectively

Table 2 Effect of different salinity levels on photosynthesis rate (P_n) ($\mu\text{mol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})			
	Control	12	15	Mean
CS 54	23.09	18.14	9.45	16.89
CS 52-SPS-1-2012	27.57	19.68	12.17	19.81
Pusa bold	22.86	16.80	7.97	15.88
CS 614-4-1-4-100-13	22.86	4.80	0.97	9.54
C.D. ($P \leq 0.05$)				
Genotypes (G)	0.48			
Salinity (S)	0.53			
Interaction (G $\times$ S)	1.06			

Table 3 Effect of different salinity levels on stomatal conductance (g_s) ($\text{mmol CO}_2 \text{ m}^{-2} \text{ s}^{-1}$) of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})			
	Control	12	15	Mean
CS 54	0.53	0.35	0.30	0.39
CS 52-SPS-1-2012	0.57	0.48	0.38	0.48
Pusa bold	0.46	0.27	0.19	0.31
CS 614-4-1-4-100-13	0.57	0.15	0.10	0.27
C.D. ($P \leq 0.05$)				
Genotypes (G)	0.02			
Salinity (S)	0.02			
Interaction (G $\times$ S)	0.04			

Table 4 Effect of different salinity levels on transpiration rate (E) ($\text{mmol H}_2\text{O m}^{-2} \text{ s}^{-1}$) of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})			
	Control	12	15	Mean
CS 54	4.23	3.00	2.03	3.09
CS 52-SPS-1-2012	4.98	3.13	2.58	3.56
Pusa bold	4.05	2.99	1.90	2.98
CS 614-4-1-4-100-13	4.35	2.29	1.40	2.68
C.D. ($P \leq 0.05$)				
Genotypes (G)	0.13			
Salinity (S)	0.14			
Interaction (G $\times$ S)	0.28			

The expression of genes *SOS1*, *SOS2*, *SOS3*, *ENH1* and *NHX1*, was found higher in salt tolerant genotypes (CS 54 and CS 52-SPS-1-2012) compared to salt sensitive genotypes (Pusa bold and CS 614-4-1-4-100-13) under salt stress (EC_{iw} 12 and 15 dS m^{-1}). However *SOS3*, *ENH1*

Table 5 Effect of different salinity levels on instantaneous water use efficiency (iWUE) [$\mu\text{mol (CO}_2\text{) mmol}^{-1} \text{ (H}_2\text{O)}$] of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})			
	Control	12	15	Mean
CS 54	5.46	6.05	4.66	5.39
CS 52-SPS-1-2012	5.54	6.29	4.72	5.51
Pusa bold	5.64	5.62	4.19	5.15
CS 614-4-1-4-100-13	5.26	2.10	0.69	2.68
C.D. ($P \leq 0.05$)				
Genotypes (G)	0.30			
Salinity (S)	0.33			
Interaction (G $\times$ S)	0.67			

Table 6 Effect of different salinity levels on CO_2 assimilation ($\mu\text{mol CO}_2 \text{ mol}^{-1}$) of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})			
	Control	12	15	Mean
CS 54	0.82	0.70	0.56	0.69
CS 52-SPS-1-2012	0.86	0.70	0.63	0.73
Pusa bold	0.85	0.67	0.53	0.68
CS 614-4-1-4-100-13	0.81	0.56	0.47	0.61
C.D. ($P \leq 0.05$)				
Genotypes (G)	0.01			
Salinity (S)	0.01			
Interaction (G $\times$ S)	0.02			

Table 7 Effect of different salinity levels on Yield per plant (g) of mustard genotypes

Genotypes	Salinity levels (EC_{iw} dS m^{-1})			
	Control	12	15	Mean
CS 54	28.95	16.51	10.37	18.61
CS 52-SPS-1-2012	35.99	25.56	14.27	25.27
Pusa bold	23.14	14.52	6.61	14.76
CS 614-4-1-4-100-13	21.49	3.64	2.97	9.37
C.D. ($P \leq 0.05$)				
Genotypes (G)	0.68			
Salinity (S)	0.76			
Interaction (G $\times$ S)	1.53			

and *NHX1* showed higher expression at EC_{iw} 15 dS m^{-1} . The 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

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

(CS 54 and CS 52-SPS-1-2012). Similarly, the expression of *APX1*, *APX4* and *MDHAR6* was assessed to be higher in salt tolerant genotypes (CS 54 and CS 52-SPS-1-2012) compared to salt sensitive genotypes (Pusa bold and CS 614-4-1-4-100-13).

Discussion

The key factor that determines plant growth and productivity is photosynthesis. Decline in productivity in many plant species is often associated with reduction in its photosynthetic ability under salinity stress (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 might be due to the stomatal closure in sensitive mustard genotypes induced by ABA accumulation in response of salinity, which limit the CO₂ assimilation (Noreen et al. 2010), transpiration and stomatal conductance (Lu et al. 2009), stomatal or non-stomatal factors or both (Maxwell and Johnson 2000). Stomatal conductance drastically declined 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 increasing salinity stress. This decline in stomatal conductance is largely due to build-up of salts in the leaves of salt sensitive mustard genotypes under higher salinity. Increased ionic accumulation cause a decrease in leaf turgor and changes in leaf morphology and stomatal aperture of salt sensitive genotypes which in turns leads to partial closing of stomata to minimize water loss

Fig. 2 Gene expression of salt responsive genes/sequences in Indian mustard: *SOS1*, *SOS2*, *SOS3*, *ENH*, *NHX1*, *APX1*, *APX4*, *DHAR3* and *MDHAR6*. The relative changes in gene expression (fold change) were calculated using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen

2001). Heatmapper (Babicki et al. 2016) was used for generating heat map. Plotted values are the average of 10 independent biological samples per genotype from each salinity level with standard error

by transpiration and hence reduced stomatal conductance (Chaves et al. 2009; James and Sirault 2012). Further, reduced transpiration in salt sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold compared to salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 under increasing salinity might be due to the toxicity caused by excess amount of Na⁺ in the transpirational path causing cells damage in the transpiring leaves resulting in reduce growth (Munns et al. 2006). 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 CO₂ 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 than 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 CO₂ availability for carboxylation leading to acceleration of senescence at moderate and severe stress (Chaves et al. 2009). The reduction in CO₂ assimilation rate may be due to reduced stomatal conductance and limited availability of CO₂ 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 CO₂ assimilation under salinity (Saleem et al. 2011). The lower P_n values, under salt stress,

were positively related to decrease in g_s 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).

Moreover, the subsequent reduction in seed yield was observed for all genotypes of mustard with increasing salinity levels. 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 Dhingra 2007).

All the genotypes exhibited significant differences in ion accumulation (both Na^+ and K^+) in shoot and roots under different salinity treatments. 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 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). In the vacuoles, the harmful ions are compartmentalized for intracellular ion homeostasis which is necessary for cytoplasmic metabolic activity and there is an increase in cellular osmolality to counter osmotic stress (Wang et al. 2007).

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. Higher salinity stress caused high sodium uptake and increased Na/K ratio values cause sodicity, which increases soil resistance, reduces root growth, and reduces water movement through the root with a decrease in hydraulic conductivity (Rengasamy and

Olsson 1993), and its reduction depends upon the salt content of the irrigation water used, thus, affecting the level of water absorption (Jonathan et al. 2006; Zobel et al. 2007). Maintaining ion homeostasis by ion exclusion and compartmentalization is an essential process for growth during salt stress (Serrano et al. 1999; Hasegawa 2013). Plants do not tolerate high salt concentration in their cytoplasm. Therefore, either the excess salt is transported to the vacuole or is sequestered in older tissues, thereby protecting the plant from salinity stress (Reddy et al. 1992; Zhu 2003). Previous reports (Kumar et al. 2009) also enunciated that salt tolerant genotypes of *B. juncea* tolled least penalty in terms of growth reduction and, electrolyte leakage and higher maintenance of K^+/Na^+ ratio at seedling stage compared to other non salt tolerant cultivars. 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 et al. 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, roots of salt stressed plants had the highest Na^+ concentrations than shoots. The change in ion ratios might be caused by the Na^+ influx through the K^+ acquisition pathways (Blumwald et al. 2000; Almeida et al. 2017).

To mitigate the harmful effects of high salt concentration by restoration of cellular ion homeostasis and osmotic balance, plant generally adapt transcriptional regulation salt responsive genes (Mallikarjuna et al. 2011). Studying transcript abundance 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). Under salt stress (EC_{iw} 12 and 15 $dS\ m^{-1}$), 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. In agreement with the previous studies, 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* in non-stress conditions and is further overexpressed in response to salinity (Kumar et al. 2009; Sharma et al. 2015). When Na^+ ion enters into cytoplasm subsequently transported to the vacuole via membrane localized *NHX1* encoded Na^+/H^+ antiporter (Fukuda et al. 2011). Under stressed condition, the survivability of the plant depends upon the activity of vacuolar Na^+/H^+ antiporter (Dietz et al. 2001). Tonoplast

localized NHXs proteins are essential for active K^+ uptake at the tonoplast, for turgor regulation, and removing toxic concentration of Na^+ through sequestration into the vacuole for stomatal function (Barragán et al. 2012). 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 removal of 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.

Evidence of increasing *SOS1*, *SOS2* and *SOS3* stress signaling pathway demonstrates their roles in ion homeostasis and salt tolerance. *SOS1*, encoding plasma membrane Na^+/H^+ antiporter, is essential in regulating Na^+ efflux at cellular level and facilitating long distance transport of Na^+ from root to shoot (Shi et al. 2000; 2002; Ji et al. 2013). *SOS2* gene encoding protein kinase, is activated by salt stress elicited Ca^{2+} signals (Liu et al. 2000). The *SOS3* encodes myristoylated Ca^{2+} binding protein (Ishitani et al. 2000). The *SOS3-SOS2* complex is loaded onto plasma membrane where it phosphorylates *SOS1*. The phosphorylated *SOS1* results in the increased Na^+ efflux, reducing Na^+ toxicity (Martínez-Atienza et al. 2007). 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 CS 54 and CS 52-SPS-1-2012. Our experimental finding are in agreement with previous reports (Sharma et al. 2015; Kumar et al. 2017) enunciating that overexpression of *SOS* complex confers salt tolerance in mustard by increased Na^+ efflux and reducing Na^+ concentration in tissues of salt tolerant genotypes of mustard.

For detoxification of sodium ions through sequestration into the vacuole, *NHX* proteins are essential whereas for the export of Na^+ outside the cell, *SOS* signaling pathways are important. The expression pattern of these transporters is modulated by the salt tolerant genotypes that resulted in a minimum accumulation of Na^+ in the salt tolerant cultivars. Further, salt sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold were not able to regulate these pathways as efficiently as salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 (Chakraborty et al. 2012; Ji et al. 2013; Ma et al. 2006).

Salt stress causes deregulation and/or disruption of electron transport chains in chloroplasts and mitochondria of plants. Under these conditions, molecular oxygen acts as an electron acceptor, giving rise to the accumulation of ROS which is severely harmful for cell integrity causing lipid peroxidation, protein denaturing and DNA mutation (Groß et al. 2013). Antioxidant enzymes like APX, DHAR and MDHAR play important role in detoxifying ROS with increased antioxidant activities induced by salinity stress (Yoshimura et al. 2000; Candan and Tarhan 2003; Thounaojam et al. 2012). There is a positive correlation between salinity tolerance and the activity of antioxidant enzymes (Asada 1999; Gupta et al. 2005; Panchuk et al. 2005). The genes *APX1*, *APX4* and *MDHAR6* pertaining to oxidative module also showed a higher induction in the salt tolerant genotypes CS 54 and CS 52-SPS-1-2012 than salt sensitive genotypes. Further, higher accumulation of Na^+ in salt sensitive genotypes CS 614-4-1-4-100-13 and Pusa bold tissues cause more oxidative stress as well as ionic stress which can be correlated with the lower expression of *APX1*, *APX4*, *DHAR3* and *MDHAR6* genes in these genotypes (Diaz-Vivancos et al. 2013) and higher levels of ROS accumulation (Buchanan et al. 2005; Chawla et al. 2013; Martinez et al. 2018). Overexpression of *APX1*, *APX4* and *MDHAR6* in the salt tolerant genotypes CS 52-SPS-1-2012 and CS 54 compared to salt sensitive one at higher salt stress in agreements with previous findings (Eltelib et al. 2012; Kumar et al. 2009; Martinez et al. 2018) 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. Our findings on expression of salt responsive genes, are corroborative with the previous reports (Taji et al. 2004; Kumar et al. 2009; Gill and Tuteja 2010; Ji et al. 2013) showing that the major factor contributing to stress tolerance in mustard was their constitutive expression in tolerant genotypes.

Conclusions

The salt tolerance of Indian mustard genotypes CS 52-SPS-1-2012 and CS 54 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, possibly by limiting Na^+ influx into shoots and maintaining the higher net photosynthetic traits under stress compared to salt susceptible genotypes Pusa bold and CS 614-4-1-4-100-13. Furthermore, the existence of a more efficient salt scavenging system in CS 52-SPS-1-2012 and

CS 54 genotypes is the evident of overexpression of antiporters (*SOS1*, *SOS2*, *SOS3*, *ENH1* and *NHX1*) and antioxidant (*APX1*, *APX4*, *DHAR1* and *MDHAR*) defense genes compared to salt susceptible genotypes. The manipulation of some of these genes in Indian mustard might help to mitigate the ionic toxicity effects and cellular ionic homeostasis along with conditioning of photosynthetic attributes leading to a promising yield under salinity stress. Thus, with the genetic improvement of agro-morphological traits for salt tolerance in Indian mustard, 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.

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

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