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Constitutive expression of *Brassica juncea* annexin, *AnnBj2* confers salt tolerance and glucose and ABA insensitivity in mustard transgenic plants

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ABSTRACT

Annexins belong to a plasma membrane binding (in a calcium dependent manner), multi-gene family of proteins, which play ameliorating roles in biotic and abiotic stresses. The expression of annexin *AnnBj2* of Indian mustard is tissue specific with higher expression in roots and under treatments with sodium chloride and abscisic acid (ABA) at seedling stage. The effect of constitutive expression of *AnnBj2* in mustard was analyzed in detail. *AnnBj2* OE (over expression) plants exhibited insensitivity to ABA, glucose and sodium chloride. The insensitivity/tolerance of the transgenic plants was associated with enhanced total chlorophylls, relative water content, proline, calcium and potassium with reduced thiobarbituric acid reactive substances and sodium ion accumulation. The altered ABA insensitivity of *AnnBj2* OE lines is linked to downregulation of *ABI4* and *ABI5* transcription factors and upregulation of ABA catabolic gene *CYP707A2*. Furthermore, we found that overexpression of *AnnBj2* upregulated the expression of ABA-dependent *RAB18* and ABA-independent *DREB2B* stress marker genes suggesting that the tolerance phenotype exhibited by *AnnBj2* OE lines is probably controlled by both ABA-dependent and −independent mechanisms.

1. Introduction

Salinity is one of the most important stresses faced by a crop plant, which results in significantly reduced crop productivity. Salinity affects more than 800 million hectares of land world-wide [1]. The annual global losses in agricultural production from salt-affected soils are more than US$12 billion [2] and are expectedly rising. Salinization of agricultural lands is increasing due to intensive agricultural practices and frequent use of ground water for irrigation. Saline soils can be reclaimed either by the use of soil amendments like gypsum, which is rich in calcium; or by leaching down the excess salts from the rooting zone to the deeper layers or by growing salt tolerant crops [3–5]. In line with the scarcity in arable land across the globe, global food production needs to be increased by 70% by the year 2050 to meet the food demands of the estimated 9.3 billion human population [6]. In such a situation, genetic engineering needs to be integrated with the breeding technologies to develop crops, which are climate resilient and could adapt to the changes in environment leading to stress conditions to feed the ever growing population.

Salinity increases the osmotic potential of the soil and hampers water uptake by the roots. Accumulation of Na^+ ions in shoots limits plant growth rate by reducing the photosynthetic rate and changing the plant morphology [1]. Like other abiotic stresses, salt stress generates reactive oxygen species (ROS) that act also as crucial signaling molecules to regulate plant adaptive responses. The alteration in the ROS levels is tightly controlled by keeping a balance in their production and scavenging processes [7–9]. Sudden increase in cellular ROS levels activate a broad range of ROS- sensitive Na^+, K^+ and Ca^{2+} transporters that play crucial role in maintaining plant ionic homeostasis [10–15]. Under severe or prolonged stress conditions, this balance gets perturbed resulting in excessive cellular ROS accumulation leading to uncontrolled oxidation of cellular membranes, DNA damage and impairment of enzymatic activities [16].

Most of the abiotic stresses including salt stress, generate a specific spatio-temporal change in the free cytosolic calcium concentration $[Ca^{2+}]_{cyt}$ known as Ca^{2+} signature. These Ca^{2+} signatures are specific stimuli that are sensed by Ca^{2+} binding proteins, which program the cell to generate a specific response by activating the downstream signaling cascades [17–19]. The change in calcium fluxes is mediated by membrane-localized calcium channels and transporters. Plants regulate

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the calcium fluxes across the membranes through three major classes of calcium transporters: channels, pumps (ATPases) and Ca^{2+}/H^{+} antiporters. Apart from these, other proteins are also involved in transporting Ca^{2+}, which include Cyclic nucleotide gated channels (CNGC), Glutamate receptor homologs (GLR) and annexins [19–22]. The calcium binding property of annexins together with their regulation by different hormones such as abscisic acid (ABA), salicylic acid (SA), methyl jasmonate (MeJA) and ethylene make these proteins key players in the plant signaling processes.

Annexins belong to a multigene family with wide taxonomic distribution across different kingdoms including higher vertebrates, plants, fungi, and protists [23–25]. Their biology has been reviewed recently [26–29]. Expression profiling studies of annexins in different plant species revealed that they are regulated by different signaling molecules and abiotic stress inducers [30–33]. Genetic manipulation of the plants with annexins showed alterations in growth, development and stress responses [34–41].

Brassica juncea (L.) Czern & Coss- (AABB, 2n = 36) is a natural amphidiploid that originated from a natural cross between *Brassica rapa* (genomic constitution AA, 2n = 20) and *Brassica nigra* (BB, 2n = 16). It is grown worldwide as an important oil seed crop. But the productivity of this crop is always under challenge from one or more abiotic stresses such as salinity, drought, and low temperatures during one or more important stages of its life cycle. High yielding varieties developed by conventional plant breeding methods significantly increased the productivity of this crop, but with certain limitations, which can be improved further by resorting to the use of biotechnological tools.

Plant annexin expression is developmentally and tissue-specifically regulated. Previously, our group reported the expression patterns of six annexins of *B. juncea* from 6 to 8 weeks old plants [31,42]. Among these, the role of two annexins, *AnnBj1* and *AnnBj3* were studied in some detail following the gain of function strategy in the heterologous systems. Ectopic expression of *AnnBj1* conferred multiple stress tolerance to the transgenic tobacco and cotton plants [34,36]. The protective role of *AnnBj3* heterologous expression was observed to alleviate oxidative stress in *Arabidopsis* and *Saccharomyces cerevisiae* [37,43].

In the present study, we report on the expression pattern of *B. juncea* annexins at seedling stage (5 d old). Based on these observations on annexin gene expression at seedling stage, we studied the consequence of constitutive expression of *AnnBj2* in the native system, mustard in response to salt stress. The partial promoter of *AnnBj2* isolated by Jami et al. [44] revealed the presence of various *cis*-acting elements that are reportedly involved in abiotic stress signaling. It carries MYB, MYC core and ERD1 elements, which exhibited proven roles in water and dehydration stress [45–47]; GT-1 motif is reported to be involved in pathogen and NaCl-induced gene expression [48] along with CCAAT box present in the promoters of heat shock proteins, CuRE (responsive to copper element), W- box motif related to SA-mediated defense response [49–52]. Neither *AnnBj2* nor its homolog in other species was functionally characterized in detail so far. We show here that the constitutive expression of *AnnBj2* resulted in ABA and glucose insensitivity and improved salt tolerance of the mustard transgenic plants by enhanced proline accumulation and maintaining ion homeostasis in transgenic plants.

2. Materials and methods

2.1. Plant material and growth conditions

Seeds of *B. juncea* (L.) Czern & Coss cultivar 'Pusa Jai Kisan' obtained from National Research Centre for Plant Biotechnology, New Delhi, India was used in the present study. The plants were grown in green house maintained at 26 ± 2 °C for seed collection. *In vitro* experiments were conducted in a tissue culture room maintained at 25 ± 2 °C with 16/8 h, light/dark period.

2.2. Preparation of AnnBj2-pCAMBIA2300 constitutive expression cassette

For constructing the expression cassette for *AnnBj2*, it was PCR amplified from the cDNA with *Nco*I and *Xba*I restriction sites in the forward (5′CGGGATCCATGGCGTCTCTCAAAAGTCCC3′) and reverse (5′GCTCTAGATCAGACATCCCCATGTCCGAG3′) primers respectively. The PCR amplification product was double-digested with these two restriction enzymes and cloned into the corresponding sites of pRT100 vector for the incorporation of CaMv35S promoter and Termination signal in the expression cassette. This cassette was released from the pRT100 by *Pst*1 restriction digestion and cloned into the corresponding site of binary vector pCAMBIA2300, which was designated as *AnnBj2*-pCAMBIA2300. The construct was then mobilized in the *Agrobacterium tumefaciens* EHA105 by the standard freeze- thaw method of transformation. The recombinant bacterial strain was characterized using standard molecular methods.

2.3. Generation of AnnBj2 overexpression lines of mustard, B. juncea

To generate *AnnBj2* overexpression lines, both cotyledonary petiole and hypocotyl explants were used from one week old mustard seedlings grown on half strength Murashige and Skoog (MS) medium without any growth regulators. The explants were precultured on the regeneration medium (MS + 2.0 mg/l 6-aminobenzyl amino purine (BAP), 0.05 mg/l naphthaleneaceticacid (NAA), 3.0 mg/l silver nitrate) for two days before co-cultivation on the same medium without silver nitrate. *Agrobacterium* strain harboring the overexpression construct was grown in Luria-Bertani (LB) medium supplemented with the necessary antibiotics until the O.D reached 0.5 at 28 °C. The cells were then pelleted at 5000 rpm (4 °C) and resuspended in liquid MS medium and the final O.D was adjusted to 0.2. After co-cultivation for 48 h, the explants were washed with sterile double distilled water containing 500 mg/l cefotaxime after which the explants were dried on sterile tissue paper and kept on the post-culture medium (MS + 2.0 mg/l BAP, 0.05 mg/l NAA, 1.0 g/l casein acid hydrolysate, 3.0 mg/l silver nitrate and 500 mg/l cefotaxime) for five days following which they were transferred to the shoot induction- selection medium, which is same as the one used for post-culture but augmented with 15 mg/l kanamycin for selecting the transformed shoots. The regenerated green shoots were transferred to the shoot elongation medium (MS + 1.0 mg/l BAP + 3.0 mg/l silver nitrate + 500 mg/l cefotaxime). Healthy shoots were shifted to the rooting medium containing 0.5 mg/l NAA. The plantlets with well-developed roots were then transferred to the soil and vermiculite mix (1:3) and acclimatized in the culture room before shifting to the green house.

2.4. Screening and molecular confirmation of the putative transgenic lines

The transgenic plants were screened by PCR using CaMv35S forward and *AnnBj2* gene specific reverse primers. The seeds of putative T_0 transgenics, which were raised as described earlier, were screened by growing them on ½ MS medium supplemented with 150 mg/l kanamycin as a selective agent. The seedlings, which remained green for two weeks were transferred to soil to obtain the T_1 plants. Southern blotting was performed to estimate copy number and stable integration of transgenes in the transgenic mustard lines in T_2 generation.

For Southern blotting, 10 μg of DNA was digested with 50 units of the restriction enzyme *Eco*RI, which has a single site within the T-DNA region of *AnnBj2*-pCAMBA2300 construct and blots were probed with DIG-labeled *npt*II gene (700 bp) following the manufacturer's protocol (DIG DNA Labeling and Detection kit, Cat no. 11093657910, Roche Biochemicals, Germany).

2.5. Seed germination assays

The surface sterilized T_2 seeds of mustard *AnnBj2* OE transgenics

and a null segregant line (NS) were incubated on germination media comprising ½ MS supplemented with various concentrations of NaCl (0, 100 and 200 mM), mannitol (200 mM), ABA (4 and 8 µM) or glucose (6%). Germination bottles were kept in dark for 24 h and shifted to culture room with 16/8, dark/light conditions at 26 ± 2 °C. Protrusion of the radicle out of the seed coat was used as a scorable marker for germination. Data were recorded on a daily basis. Root and shoot length of the seedlings were measured after 5 d of germination using a ruler. The germination assays were performed with three technical replicates of 100 seeds each to ensure reproducibility of data.

2.6. Chlorophyll content

Total chlorophyll content was estimated from the leaves of two months old mock and NaCl treated plants following the protocol of Hiscox and Israelstam [53]. Briefly, 100 mg of the sample was extracted in 4 ml of DMSO and the absorbance was recorded at 645 and 663 nm against DMSO as a control. Total chlorophyll content was calculated according to Arnon [54] equation and expressed as mg g^{-1}FW.

2.7. Proline estimation

Proline content was estimated using the standard procedure described by Bates et al. [55]. Leaf samples (100 mg) from mock and NaCl treated plants (two months old) were extracted in 2 ml of 3% of sulfosalicylic acid. The homogenate was centrifuged and 100 µl of supernatant was treated with 100 µl of 3% of sulfosalicylic acid, 200 µl glacial acetic acid and 200 µl acid ninhydrin mixture by boiling at 100 °C for 1 h. The reaction was stopped by keeping the samples on ice and 1.2 ml of toluene was added to the samples. The chromophore containing toluene was transferred to fresh tubes. The absorbance of the chromophore was read at 520 nm using toluene as a blank. Proline concentration was determined from a standard curve and expressed as µg g^{-1} FW.

2.8. Lipid peroxidation

Lipid peroxidation of the samples was estimated by measuring thiobarbituric acid reactive substances (TBARS) following the protocol described by Heath and Packer [56]. Briefly, 100 mg tissue samples were homogenized in 0.5 ml of 0.1% TCA and the homogenate was centrifuged at 12,000 rpm (4 °C) for 10 min. The supernatant (0.5 ml) was mixed with 1.5 ml of 0.5% (w/v) TBA in 20% TCA (w/v) and incubated at 95 °C for 30 min. The reaction was stopped by keeping the tubes on ice followed by centrifugation for 5 min at 12,000 rpm (4 °C). The absorbance of the resultant supernatant was measured at 532 and 600 nm. The OD_{600} values were subtracted from the MDA-TBA complex values at 532 nm, and MDA concentration was calculated using the Lambert-Beer law with an extinction coefficient εM = 155 mM-1cm-1. Results were presented as nmol MDA g^{-1} FW.

2.9. Relative water content

Relative water content (RWC) was measured using the following equation as per the protocol of Weatherley [57]. One-week old seedlings grown on ½ MS medium were transferred to liquid ½ MS medium supplemented with 100 and 200 mM NaCl for 72 h. Fresh weight (W) of the seedlings was taken after which the samples were hydrated completely for 12 h to obtain turgid weight (TW). Samples were oven dried for 48 h at 60 °C to determine its dry weight (DW). RWC was calculated using the following formula:

$$RWC\ (\%) = [(W\text{-}DW)/(TW\text{-}DW)] \times 100$$

2.10. Salinity tolerance at whole plant level

To study the salinity tolerance in pot grown plants in the green house, Zhang et al. [58] method of salt treatment was followed with minor modifications. Thirty seeds of each genotype were sown in the pots (30 cm diameter in size with 5 holes at bottom), each of which was fed with 200 ml of 100 mM NaCl solution prior to sowing. After germination, the seedlings were watered with 100 ml of 100 mM NaCl once in a week and with 200 ml of tap water every alternate day. The control set of plants were watered with the same volume of water without any NaCl. After two months of growth, fully expanded fourth leaf from the top of the plants was collected to estimate chlorophyll, proline and MDA contents. Seed germination percentage in the pots was calculated after five days of sowing. To check the Na^+, K^+ and Ca^{2+} accumulation in the shoots, freshly harvested leaves were washed properly with deionised water to clean the leaf surface and dried in hot air oven at 65 °C for four days. Samples of 100 mg (DW) of air dried leaf tissues were acid digested with a mixture of nitric acid (HNO_3) and perchloric acid ($HClO_4$, 3:1, v/v) mixture following the protocol of Munns et al. [59] with minor modifications. Ionic estimations were done using Atomic Absorption Spectrophotometer (Perkin Elmer).

2.11. ROS detection

ROS production in the roots of mustard seedlings under salt stress has been detected using 2',7'-dichlorodihydrofluoresceindiacetate (H_2DCFDA) dye and visualized using confocal microscopy. Briefly, 5 d old seedlings were subjected to 100 mM NaCl stress. After 24 h of NaCl treatment, roots of the seedling were incubated with 10 µM H_2DCFDA for 20 min in dark. After incubation, roots were washed with double distilled water to remove excess dye and visualized under a Leitz confocal scanning microscope ((λex–488 nm and λem–530 nm). Quantification of images was done using ImageJ 1.42 software (NIH, USA) by selecting similar areas of pigmentation.

In situ localization of H_2O_2 production under mock and salt stress has been detected by 3,3-Diaminobenzidine (DAB) staining. Two weeks old *in vitro* grown seedlings were treated with 100 mM NaCl for 48 h and followed by incubation in 1 mg/ml DAB solution (pH = 3.8) overnight. The excess DAB stain was removed by transferring the samples to absolute ethanol and boiling for 10 min. The samples were then kept on Whattman filter paper pre-soaked with 10% glycerol and images were captured.

2.12. Stress treatments for gene expression analysis

To study the tissue specific expression of annexin genes at seedling stage, 5 d old seedlings grown on ½ MS medium solidified with 0.8% agar were used. To study the induction of annexins in response to NaCl and ABA, 5 d old mustard WT seedlings were treated with 100 mM of NaCl or 100 µM ABA separately and the samples were collected at 0, 1, 3 or 12 h post treatment. To compare the gene expression of salt stress marker genes, 5 d old mustard seedlings of control and *AnnBj2* OE lines were treated with 100 mM NaCl solution for six hours and the samples were quick-frozen in liquid Nitrogen (LN_2) for RNA isolation. To study the gene expressions at seed germination stage, sterilized seeds of control and *AnnBj2* OE lines were kept on ½ MS + 8 µM ABA to undergo germination. After 24 h of incubation, seeds were harvested, quick-frozen in LN_2 for RNA isolation.

2.13. RNA isolation, semi-quantitative and quantitative real time PCR

Total RNA was isolated using Trizol reagent (Sigma-Aldrich) as per the manufacturer's instructions and 2 µg of the total RNA was reverse transcribed to obtain the first strand of complementary DNA with MMLV-Reverse transcriptase (Sigma-Aldrich) and oligo $dT_{(18)}$ primer following the manufacturer's protocol. The resultant cDNA samples

were diluted 2.5 X and 1 µl of diluted cDNA was used as a template in a total 10 µl reaction volume for the PCR amplification using program; initial denaturation at 95 °C for 5 min followed by 40 cycles of denaturation at 95◦C for 15 s, annealing at 58◦C for 30 s and extension at 72◦C for 30 s. Primers used in Real-Time PCR are listed in STable 1. *B. juncea actin2* and *EF1α* were used as reference genes for normalizing the gene expression levels. The relative gene expression was analyzed using the Livak and Schmitzen's $\Delta\Delta C_T$ method [60]. The expression levels of the *AnnBj2* in the putative transgenic lines were determined by semi-quantitative RT-PCR to identify the high expression lines. *Actin2* was used as the internal control. The qRT-PCR was performed using 2X Fast start SYBR green PCR master mix (Roche GmbH, Germany) in a 96 well plate using Realplex Ep4 system (Eppendorf GmbH, Germany).

2.14. Statistical analysis

All graphs were plotted using SigmaPlot-11 scientific data analysis and graphing software. Data were analyzed by one way ANOVA (analysis of variance) with Duncan's Multiple Range Test (DMRT) to determine the significant difference between the null and transgenic lines at $p \leq 0.05$ (indicated by single asterisk mark) or $p \leq 0.01$ (indicated by double asterisk mark).

3. Results

3.1. Tissue-specific expression of AnnBj2 and its induction by NaCl and ABA at seedling stage

In an earlier study, our group has reported earlier on the tissue-specific expression of six annexin genes from samples collected from two month old mustard plants [31] and their induction in response to various abiotic stresses and signaling molecules from leaves of six week old plants. In the present investigation, we studied the expression of annexins at 5 d old seedling stage. We now observed that transcript levels of *AnnBj1* and *AnnBj2* are most abundant followed by *AnnBj4* and *AnnBj7*. *AnnBj3* and *AnnBj6* are the least expressing annexin genes at this stage. The transcript levels of *AnnBj1* were almost the same in all the three tissues, whereas *AnnBj2* has maximum expression in roots followed by hypocotyl and cotyledonary leaves (Fig. 1a). We checked for the expression of all the six annexins in response to NaCl stress (100 mM) at seedling stage (5 d old) and found that the transcripts of *AnnBj1*, *AnnBj2* and *AnnBj4* were strongly upregulated in response to NaCl treatment (Fig. 1b). Among these three annexins, transcripts of *AnnBj2* and *AnnBj4* were strongly induced as early as 1 h of treatment, which were maintained up to 3 h and then decreased at 12 h post NaCl treatment. Transcripts of *AnnBj1* increased gradually and reached maximum at 12 h post treatment. Message levels of *AnnBj3*, *AnnBj6* and *AnnBj7* did not show any change in response to NaCl treatment (Fig. 1b). ABA treatment also upregulated *AnnBj2* transcripts within 1 h treatment and the transcript levels were maintained up to 12 h treatment (S Fig. 3).

3.2. Development and molecular confirmation of AnnBj2 transgenic plants

To functionally characterize the role of *AnnBj2* in plant abiotic stress tolerance, we generated ten transgenic plants of mustard over-expressing *AnnBj2* gene. These plants were screened by PCR using *nptII* and gene specific primers (Primer list is provided in supplementary information STable 1). PCR confirmation of the transgenic plants has been shown in supplementary information SFig. 1. The mustard transgenic plants were analyzed for the expression of *AnnBj2* gene by semi-quantitative RT-PCR, and the high expression plants were selected for further analysis. To generate homozygous population, T_1 transgenic seeds from T_0 plants were germinated on ½ MS plates supplemented with 150 mg/l kanamycin to screen the positive plants, which were further grown in the soil to get the T_1 plants. Homozygosity of the

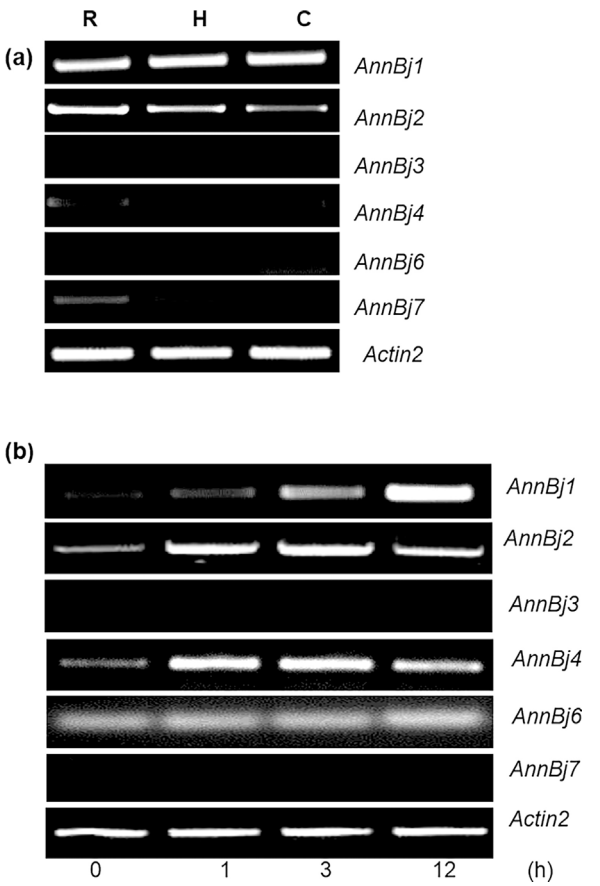

Fig. 1. (a) Tissue specific expression of annexins in root, hypocotyl and cotyledons of five day old *Brassica juncea* seedlings. (b) Effect of 100 mM NaCl on the expression of *B.juncea* annexins at seedling stage (5 d old). R represents root, H represents hypocotyl, and C represents cotyledonary leaf.

transgenic lines was confirmed by 100% seed germination in the presence of kanamycin (150 mg/l) in T_2 generation. Southern analysis showed that most of the transgenic mustard OE lines exhibited single copy independent integrations. Some plants with multiple insertions were obtained, but were not included in the further experiments. Null segregant (NS) line from one of the segregating families did not show any hybridization signal (data shown in supplementary S Fig. 1c).

We determined the expression of *AnnBj2* in the different AnnBj2 transgenic lines at seedling stage by quantitative PCR. We found that the relative expression of *AnnBj2* was in the range of 4–48 fold higher than the WT in the different transgenic lines (S Fig. 1d). On the basis of quantitative expression analysis, we selected three transgenic lines OE 2.2, OE 3.3 and OE 5.5 as high AnnBj2 expressing lines, which exhibited a relative expression of 48 fold, 32 fold and 38 fold respectively.

Further, we examined the levels of expression of *AnnBj2* in root and shoot tissues of these three OE lines at seedling and maturity stage. The expression of AnnBj2 was higher in the roots compared to shoots at both the developmental stages (S Fig. 2). In both root and shoot tissues, *AnnBj2* expression was significantly higher in *AnnBj2* OE lines compared to the NS line at seedling and maturity stages.

3.3. Salinity tolerance of mustard plants overexpressing AnnBj2 at seed germination stage

Seed germination is one of the critical stages that are prone to salt stress. Mustard seeds of null (NS) and *AnnBj2* transgenic lines (OE 2.2, OE 3.3 and OE 5.5) were germinated on media supplemented with different NaCl concentrations and the germination pattern was monitored daily. *AnnBj2* transgenic lines showed higher seed germination

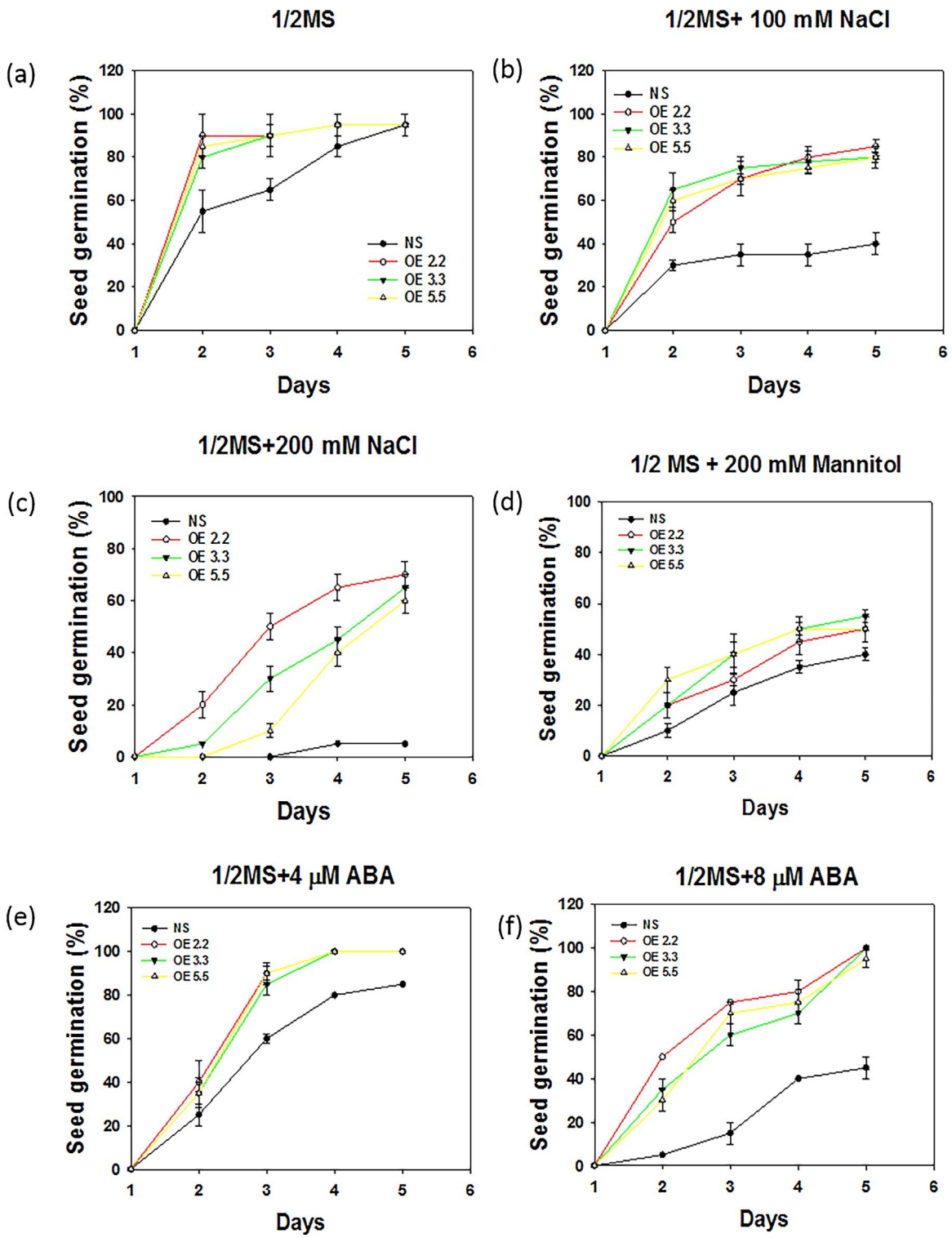

Fig. 2. Seed germination pattern of Null (NS) and *AnnBj2* OE *B. juncea* lines. (a) 0 NaCl (b) 100 mM NaCl (c) 200 mM NaCl (d) 200 mM Mannitol (e) 4 µM ABA (f) 8 µM ABA. Seed germination percentages were recorded daily over 5 d time period. Experiments were carried out with three technical replicates and repeated with at least three biological replicates. Error bar represents mean ± SD of three biological replicates. Statistical analysis was done by one way ANOVA (Analysis of variance) with Duncan's multiple range test (DMRT) to determine the significant difference between null and the transgenic lines. Single asterisk (*) indicates $p \leq 0.05$, double asterisk represents $p \leq 0.01$.

percentage even in the absence of NaCl in the germination medium compared to that of the NS line (Fig. 2a). With an increase in NaCl concentration in the germination medium, a gradual decrease in the seed germination pattern was observed. But, the decrease in the germination rates was higher in the NS seeds compared to the *AnnBj2* transgenic lines OE 2.2, OE 3.3 and OE 5.5 (Fig. 2b & c). The transgenic lines (OE 2.2. OE 3.3 and OE 5.5) germinated faster than NS at all the NaCl concentrations used in our study. Under 100 mM NaCl stress, OE 2.2 and OE 3.3 maintain almost similar seed germination as under control conditions (70–80%) whereas severe reduction was observed in NS seeds (40%). With 200 mM NaCl stress, only 5% of NS seeds germinated as against 50–60% germination in OE 2.2 and OE 3.3 (Fig. 2c).

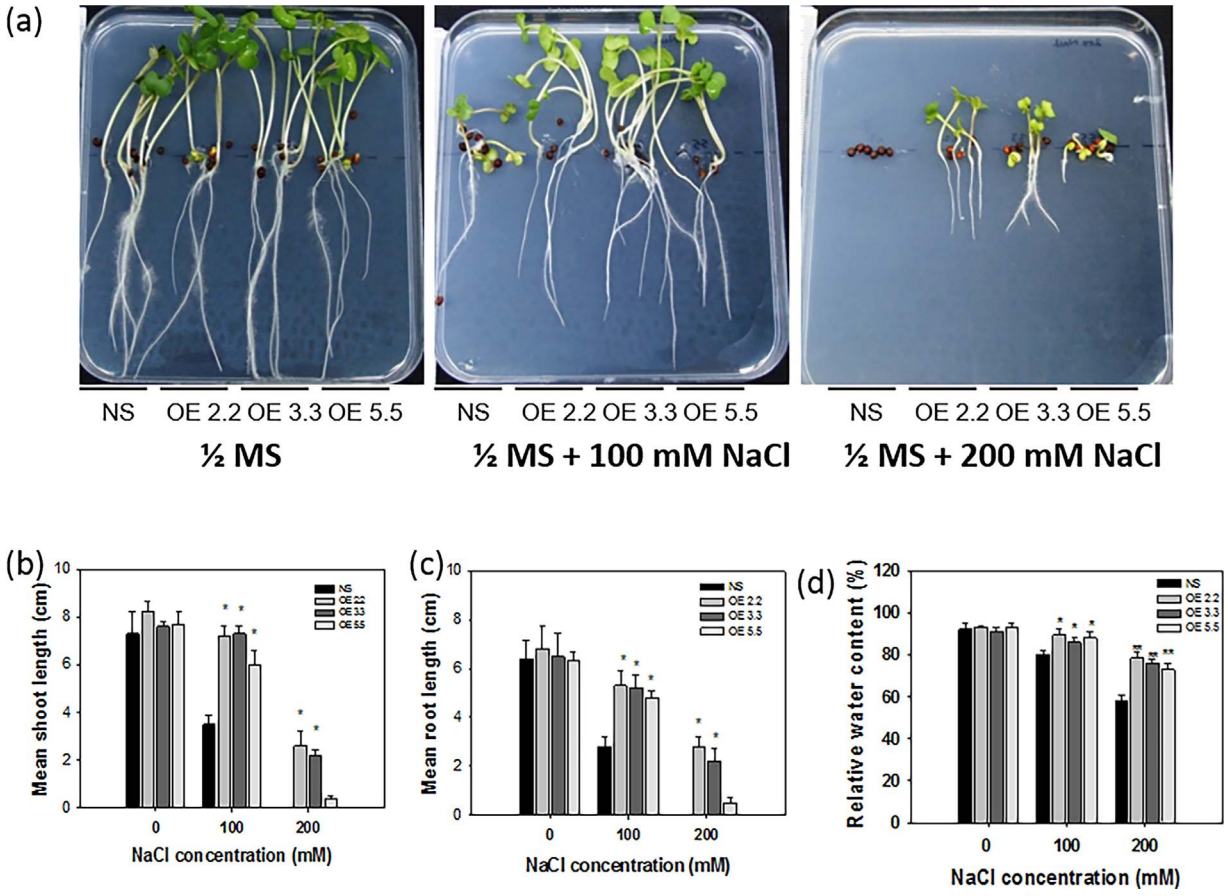

Fig 3. *In vitro* salinity tolerance and relative water content (RWC) of Null and *AnnBj2* OE *B. juncea* lines. (a) Growth responses of Null (NS) and *AnnBj2* OE transgenic seedlings after 5 d of incubation on NaCl stress media (b) Mean shoot length (c) Mean root length (d) Relative water content. Data recorded after 5 d germination. Salt tolerance assays were repeated at least three times with independent biological replicates. Error bar represents mean ± SD (n = 3). Data were analyzed by one way ANOVA with DMRT to determine the significant difference between the null and transgenic lines. Single asterisk (*) indicates $p \leq 0.05$ and double asterisk (**) represents $p \leq 0.01$.

Phenotypic differences in the growth of seedlings are shown in Fig. 3a. On 100 mM NaCl stress medium, *AnnBj2* transgenic lines did not show any significant difference in the root growth compared to their counterparts grown on a medium without any stress. In contrast, 33% reduction in the root growth was observed in NS seedlings relative to those germinated without NaCl stress (Fig. 3c). While 200 mM stress severely restricted seed germination and root growth of NS line, *AnnBj2* transgenic lines (OE 2.2 and OE 3.3) showed better root and shoot growth compared to the NS line (Fig. 3b & c). We observed seed germination pattern of the *AnnBj2* OE lines in the presence of non-ionic osmotic stress. After 5 d of incubation on 200 mM mannitol, *AnnBj2* OE lines exhibited 50–55% of germination in contrast to 40% of NS line.

3.4. *AnnBj2 overexpressing mustard transgenic lines show reduced sensitivity to ABA at seed germination stage*

ABA plays an inhibitory role in seed germination and the expression of AnnBj2 was observed to be induced by ABA. We found that the germination percentage of NS seeds was severely affected with an increase in the ABA concentration in the germination medium. In contrast to this, seed germination of *AnnBj2* OE 2.2, OE 3.3 and OE 5.5 showed reduced sensitivity to ABA (Fig. 2e & f.). Day wise germination pattern revealed 3rd d as the critical point where major difference in seed germination percentage was observed between the genotypes. At the end of 3rd day, 90% of the *AnnBj2* OE seeds germinated in contrast to 60% of the NS seeds in the presence of 4 μM ABA. The difference in the seed germination percentage became more pronounced with an increase in the ABA concentration to 8 μM with only 10% of NS seeds germinating as against 50–60% germination in *AnnBj2* OE lines (Fig. 2e & f). Phenotypic difference between the NS and *AnnBj2* OE lines were shown in Fig. 4a.

3.5. *AnnBj2 overexpressing transgenic lines of mustard show reduced sensitivity to glucose*

ABA insensitive phenotype is often associated with glucose insensitive phenotype [61,62]. To study the effect of glucose on seed germination and seedling growth of *AnnBj2* overexpressing transgenic lines of Mustard, seeds were germinated in the presence of different concentrations of glucose and their seed germination and seedling growth were monitored up to 7 d. Cotyledon greening and expansion were used in assessing glucose tolerance. Glucose showed a detrimental effect on root growth and cotyledon greening and expansion in NS compared to that of the *AnnBj2* transgenic lines OE 2.2, OE 3.3 and OE 5.5 (Fig. 5a). In the presence of 6% glucose, NS germinated seeds showed only 10% cotyledon greening while OE 2.2, OE 3.3 and OE 5.5 exhibited 40%, 80% and 60% respectively (Fig. 5b). Seed germination pattern of NS and OE lines is shown in Fig. 5d. At the end of 5 d, only 65% of NS seeds were germinated whereas the OE lines exhibited 80–90% germination. Root length was measured at the end of 5th day and there was significant increase in root growth in the transgenic lines on glucose containing medium as shown in Fig. 5c. Glucose induced delay in seed germination may be due to its role as an osmotic stress inducer or as a signaling molecule. We used 200 mM mannitol as osmotic stress control to study its effect on the seed germination percentages of NS and *AnnBj2* OE lines. As shown in Fig. 2d, *AnnBj2* OE lines exhibited 50–55% of germination after 5 d of incubation on 200 mM mannitol stress medium in contrast to 40% of NS line. In

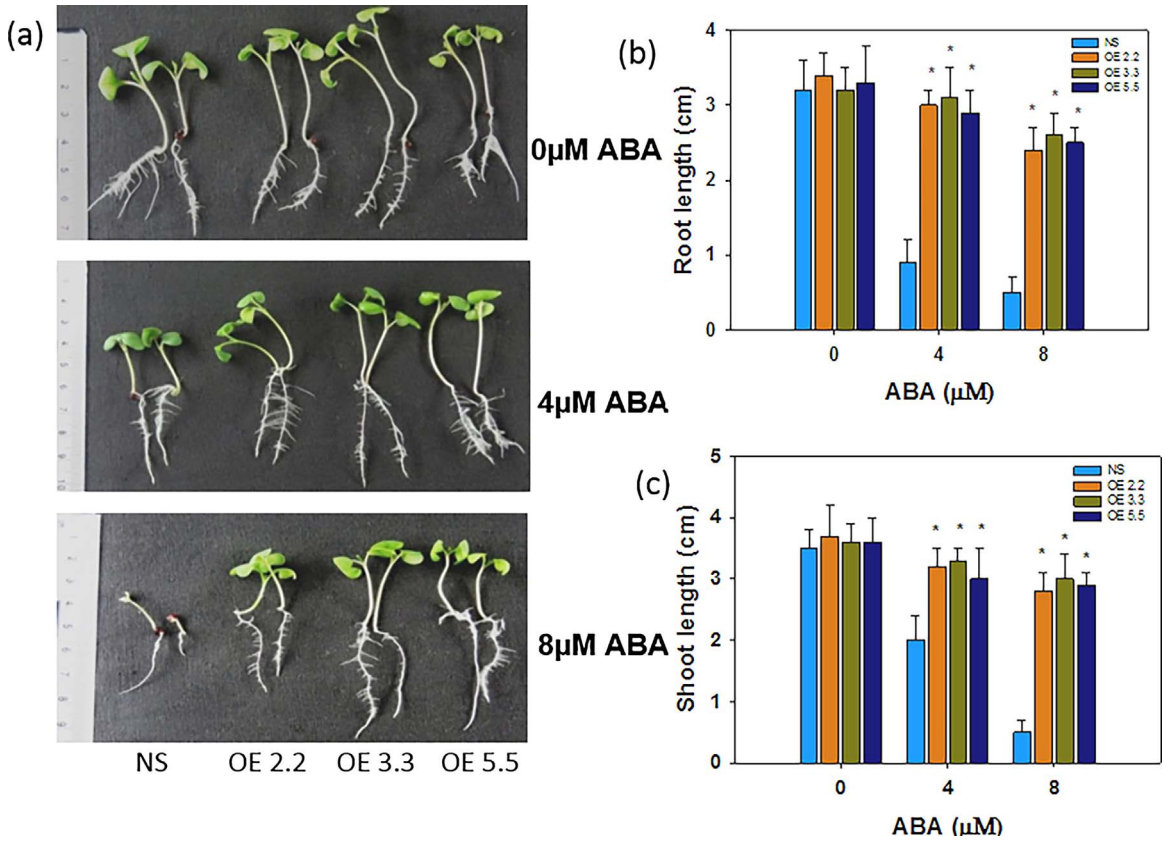

Fig. 4. Response of null (NS) and *AnnBj2* transgenic seeds to ABA at seed germination stage. (a) Seedling growth in presence of 0, 4 and 8 μM ABA. (b) Mean shoot length (c) Mean root length. Data shown here are recorded after 5 d germination. The experiment was carried out with three technical replicates and repeated with three biological replicates to ensure reproducibility of data. Error bar represents mean ± SD (n = 3). Data were analyzed by one way ANOVA with DMRT to determine the significant difference in shoot and root length of *AnnBj2* OE seedlings to that of the NS seedlings at $p \leq 0.05$ (indicated by single asterisk mark).

contrast to this, 6% glucose has more pronounced difference in seed germination of NS and *AnnBj2* OE lines suggesting that the inhibition of seed germination on glucose containing medium is not a simple effect of enhanced osmoticum in the culture medium.

3.6. AnnBj2 overexpressing mustard seedlings maintained higher relative water content under NaCl stress

Relative water content (RWC) is considered as a measure of plant water status as well as osmotic adjustment under abiotic stress conditions. To assess RWC in *AnnBj2* OE transgenics, one week old seedlings were subjected to different NaCl concentrations. We found that there was no significant difference in the RWC of NS and *AnnBj2* OE seedlings without any stress treatment. When RWC was calculated after NaCl stress treatments, a significant reduction in the RWC of NS was observed compared to that of the *AnnBj2* OE 2.2 and OE 3.3 lines. The 200 mM NaCl stress reduced the RWC of NS line to 58 ± 2% whereas the transgenic lines maintained it at 75–78% (Fig. 3d).

3.7. Overexpression of AnnBj2 increases ROS scavenging capacity in transgenic plants

To assess the effect of *AnnBj2* overexpression on ROS scavenging in *AnnBj2* OE lines, we performed DAB and H_2DCFDA staining experiments in transgenic plants after stress treatments. Under mock treatment, DAB staining results showed almost similar levels of H_2O_2 in NS and *AnnBj2* OE lines. However, salt stress induced higher H_2O_2 accumulation (as depicted by intense brown color) in NS seedlings compared to *AnnBj2* OE seedlings (Fig. 6a). Similar results were obtained by H_2DCFDA staining of roots under salt stress. NS line exhibited higher accumulation of ROS than the *AnnBj2* OE 2.2 & 3.3 lines (Fig. 6b.).

3.8. AnnBj2 overexpressing transgenic mustard plants show salinity tolerance at whole plant level

Transgenic lines overexpressing *AnnBj2* showed higher seed germination percentage in pots treated with NaCl solution compared to that of the control (NS) genotype. Salt treatment reduced seed germination in NS plants to 40% whereas the transgenic lines OE2.2 and OE 3.3 maintained 80 ± 6 and 63 ± 5% respectively (data shown in supplementary information SFig. 4). Further, differences in growth and survival of control and transgenic lines were observed under NaCl treatment. NS line had stunted growth compared to *AnnBj2* transgenic lines (Fig. 7a). Transgenic line OE 2.2 showed vigorous growth both in unstressed and stressed condition compared to others. After two months of growth, leaf samples were collected from the non-stressed and stressed samples to compare the chlorophyll, proline and MDA contents. Total chlorophyll content of NS and *AnnBj2* transgenic lines was almost similar under non-stressed condition; but under salt stress, transgenic lines retained two-fold (approx.) higher chlorophyll content compared to that of controls (Fig. 7b). Under NaCl stress, proline content of transgenic lines was significantly higher than that of the NS plants (Fig. 7c). Lipid peroxidation levels estimated by measuring MDA levels also revealed higher membrane damage in the controls compared to the transgenic lines (Fig. 7d).

3.9. Ion estimation

Salt stress is known to disrupt ion homeostasis in plants. To gain an insight into how *AnnBj2* overexpression affected the ion content under

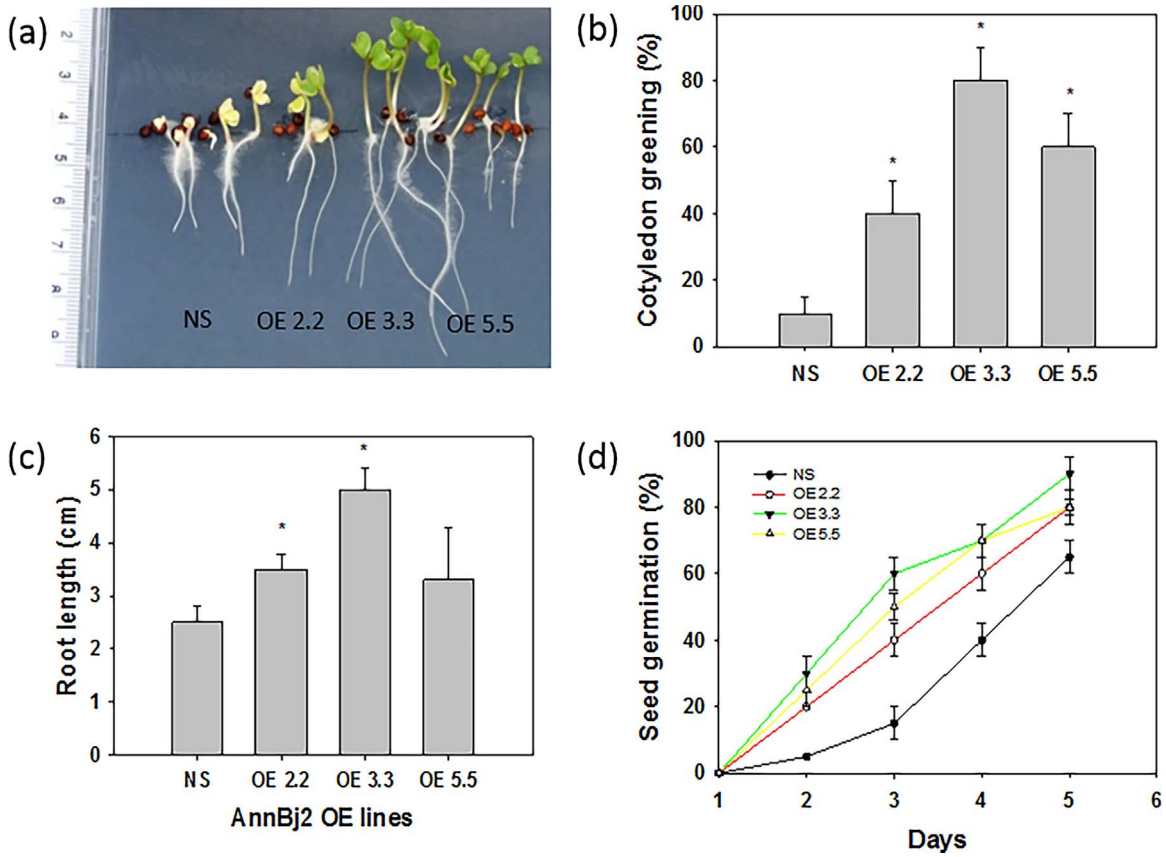

Fig. 5. Glucose tolerance of *AnnBj2* OE lines. (a) Seedling growth in the presence of 6% glucose. Image was taken on the 5 d of germination (b) cotyledon greening (c) Mean root length (d) Seed germination pattern of NS and *AnnBj2* OE lines in the presence of 6% glucose. Error bar represents mean ± SD of three biological replicates. Single asterisk mark (*) indicates significance level at $p \leq 0.05$.

NaCl stress, we measured the Na^+, K^+ and Ca^{2+} ion accumulation in the leaves of two-month-old NS and *AnnBj2* OE lines (OE2.2 and OE 3.3) under control and salt stress conditions (Fig. 8). We observed an increase in Na^+ ion content and a decrease in K^+ ion content in all the genotypes under NaCl stress when compared to the control condition. But, this relative increase in Na^+ ion content or decrease in K^+ ion content differed among the NS and OE lines. We did not find any significant difference in the Na^+/K^+ ion content between control and OE lines under control conditions. But, under salt stress, sodium ion accumulation was higher in control (58 mg/g DW) compared to that of the *AnnBj2* OE lines (32 and 40 mg/g DW). The OE lines (OE 2.2 and OE 3.3) maintained higher K^+ content compared to the NS line (Fig. 6). There was a decrease in K^+/Na^+ ratio of both control and OE lines under NaCl stress; OE 2.2 maintained highest K^+/Na^+ (1.25) whereas the NS exhibited the lowest (0.43). We observed, a decrease in the Ca^{2+} of all the three genotypes under salt stress compared to that of the control condition, but the relative decrease is significantly less in the *AnnBj2* OE lines (Fig. 8). Further, we compared Na^+ ion content at anatomical level in the root outer surface and inside the root tissue after subjecting mustard seedlings to salt stress. FESEM- EDX analysis showed higher content of Na^+ inside the roots in NS line compared to *AnnBj2* OE lines (SFig. 5).

3.10. Altered expression pattern of ABA signal related genes at seed germination stage

We observed that the constitutive expression of *AnnBj2* in mustard reduced ABA sensitivity at the seed germination stage. ABA signaling plays a crucial role in seed germination. To gain further insight into the role of *AnnBj2* in ABA signaling, we studied the relative expression of some important genes (*ABI3*, *ABI4*, *ABI5*, *AAO3*, *NCED6* and *CYP707A2*), whose action controls seed germination under ABA treatment (Fig. 9). We did not find any significant differences between control and *AnnBj2* OE lines in the expression of two important ABA biosynthetic genes *AAO3* (Abscisic Aldehyde Oxidase3) and *NCED6* (9-*cis*-Epoxycarotenoid Dioxygenase6) during seed imbibition. The expression level of *CYP707A2* (Cytochrome P450, Family 707, Subfamily A, Polypeptide 2), which is involved in ABA degradation was expressed to significantly higher levels in both the *AnnBj2* OE lines compared to the NS line (Fig. 9). ABI4 (Abscisic acid insensitive 4) and ABI5 (Abscisic acid insensitive 5) are positive regulators of ABA biosynthesis at seed germination stage. We found a significant reduction in the expression level of *ABI4* and *ABI5* in both OE 2.2 and OE 3.3 compared to the NS line. The expression of *ABI3* did not show any significant differences between control and the transgenic lines. Similar pattern of expression of these genes was observed even in the absence of ABA except for *NCED6*. In the absence of ABA, transcript levels of *NCED6* were significantly lower in the *AnnBj2 OE lines* compared to NS line while in the presence of ABA, their transcript levels did not show any significant difference.

3.11. Overexpression of AnnBj2 in mustard activated the expression of both ABA dependent and ABA independent stress marker genes

AnnBj2 expressing transgenic plants showed salt tolerance and ABA insensitivity. To investigate whether the salt tolerance phenotype exhibited by *AnnBj2* OE lines follow ABA dependent or independent signaling pathway, we compared the expression of a few candidate ABA dependent and ABA independent stress marker genes in mock and salt treated seedlings of these genotypes. We found relative abundance of the transcripts of *ERF5*, *RAB18*, *DREB2B,* and *P5CS1*, which were higher in the *AnnBj2* OE lines (OE 2.2 and OE 3.3) compared to control

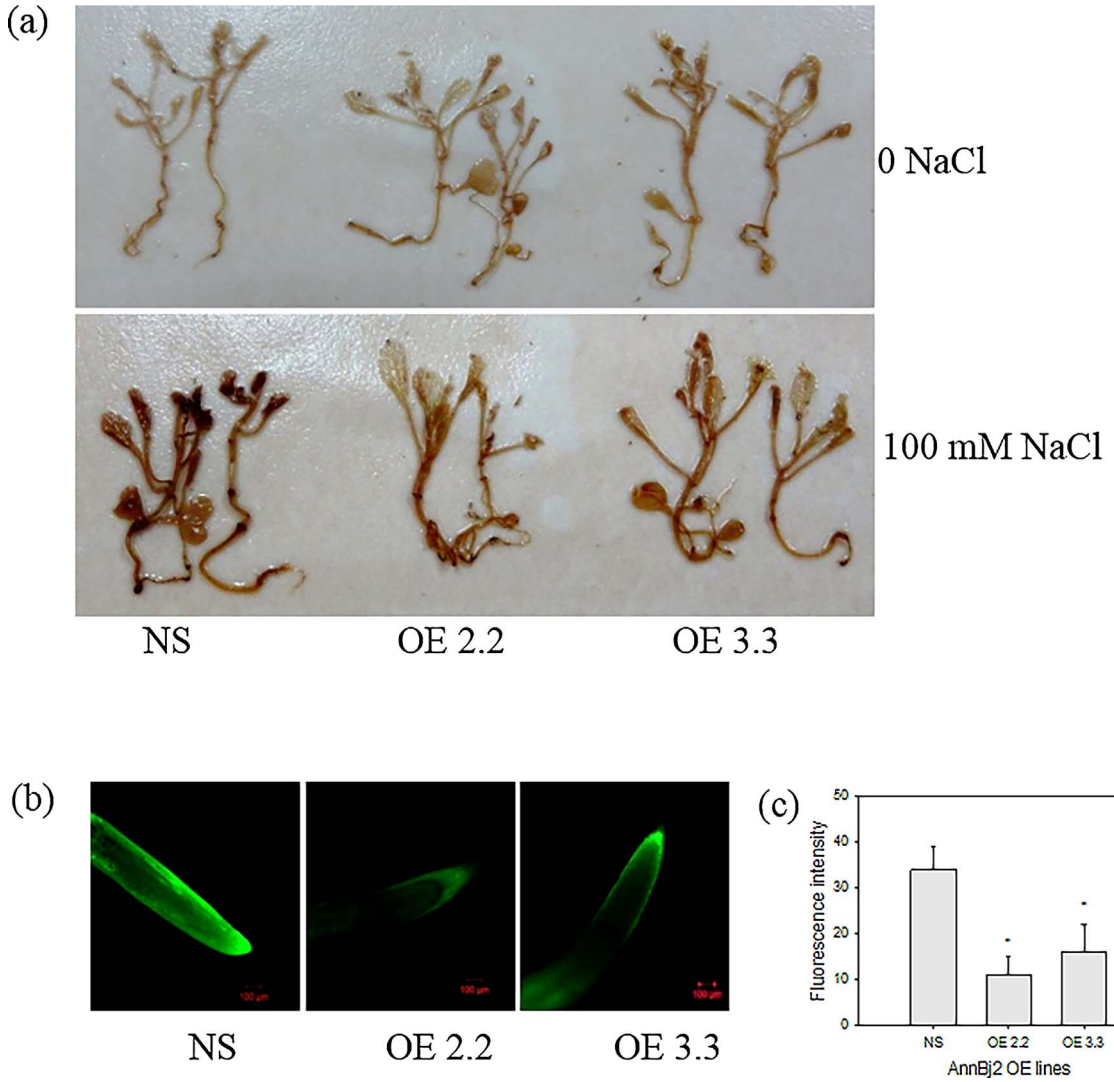

Fig. 6. *In situ* ROS measurements in NS and *AnnBj2* OE lines under salt stress (a) DAB staining for detection of H_2O_2 accumulation. Two week old seedlings were given 100 mM NaCl stress for 48 h. Brown color represents level of H_2O_2 accumulation (b) *in situ* ROS accumulation in roots detected by H_2DCFDA staining. 5 d old seedlings were incubated in 100 mM NaCl stress for 24 h followed by H_2DCFDA staining for 20 min (c) Quantification of ROS production in roots by H_2DCFDA staining using Image-J Software. These experiments were repeated three times with independent biological replicates. Error bars represents mean ± SD (n = 3). Single asterisk mark (*) indicates significance level at $p \leq 0.05$.

seedlings (Fig. 10). *RD29A* did not show any significant difference between the control and *AnnBj2* OE lines.

3.12. Expression of selective transporter genes involved in maintaining ion homeostasis under salt stress

We found that *AnnBj2* OE lines were salt tolerant and accumulated less Na^+ and more K^+ under salt stress. To assess how this ion homeostasis is reestablished in the transgenic lines, we studied the expression levels of the some selective transporters such as *GORK1*, *HKT1*, *NHX1*, *SOS1*, *AKT1*, *Ca^{+2}-ATPase* and *SOS1,* which are involved in maintaining Na^+, K^+ and Ca^{2+} ion homeostasis (Fig. 11). HKT1 is involved in retrieval of Na^+ ions from the xylem sap. Salt stress increased the relative abundance of *HKT1* transcripts, which were ~10 fold higher in *AnnBj2* OE lines compared to the NS line. The quantitative PCR data showed enhanced expression of *SOS1* in OE 2.2 and OE 3.3 compared to NS line under salt stress. AKT1 transporter is involved in the acquisition of K^+ in the cytosol during stress to maintain K^+ homeostasis in the cell. Under normal conditions (without stress), *AKT1* relative expression did not show any significant difference between NS line and AnnBj2 OE lines, but salt stress increased the relative abundance of *AKT1* transcripts ~40 fold and ~20 fold higher in OE 2.2 and OE 3.3 respectively, compared to the NS line. Although salt stress increased the transcripts of *NHX1* and *GORK1*, no significant difference was found between NS and *AnnBj2* OE lines. *Ca^{2+}-ATPase* upregulation has also been observed in the *AnnBj2* overexpressing mustard transgenic plants in the present study in comparison to the NS lines.

4. Discussion

Ectopic or overexpression studies of annexins showed that they play a protective role in plants, while the loss of function of some annexins compromised in drought, salt, heat or oxidative stresses with reduced survival of mutants when challenged with corresponding stresses showing that these proteins are involved in stress alleviation [34–41]. In the present study, we observed that *AnnBj2* exhibited higher expression in roots compared to other tissues at the seedling stage and is induced by ABA and NaCl in mustard. We observed that AnnBj2 has more pronounced effect on ionic stress than non-ionic osmotic stress (mannitol and PEG as shown in SFig. 5). Hence, we mainly focused on the characterization of the *AnnBj2* expressing mustard plants under salt stress. We observed that the overexpression of *AnnBj2* conferred enhanced salinity tolerance on mustard transgenic plants by enhancing proline accumulation and maintaining ion homeostasis.

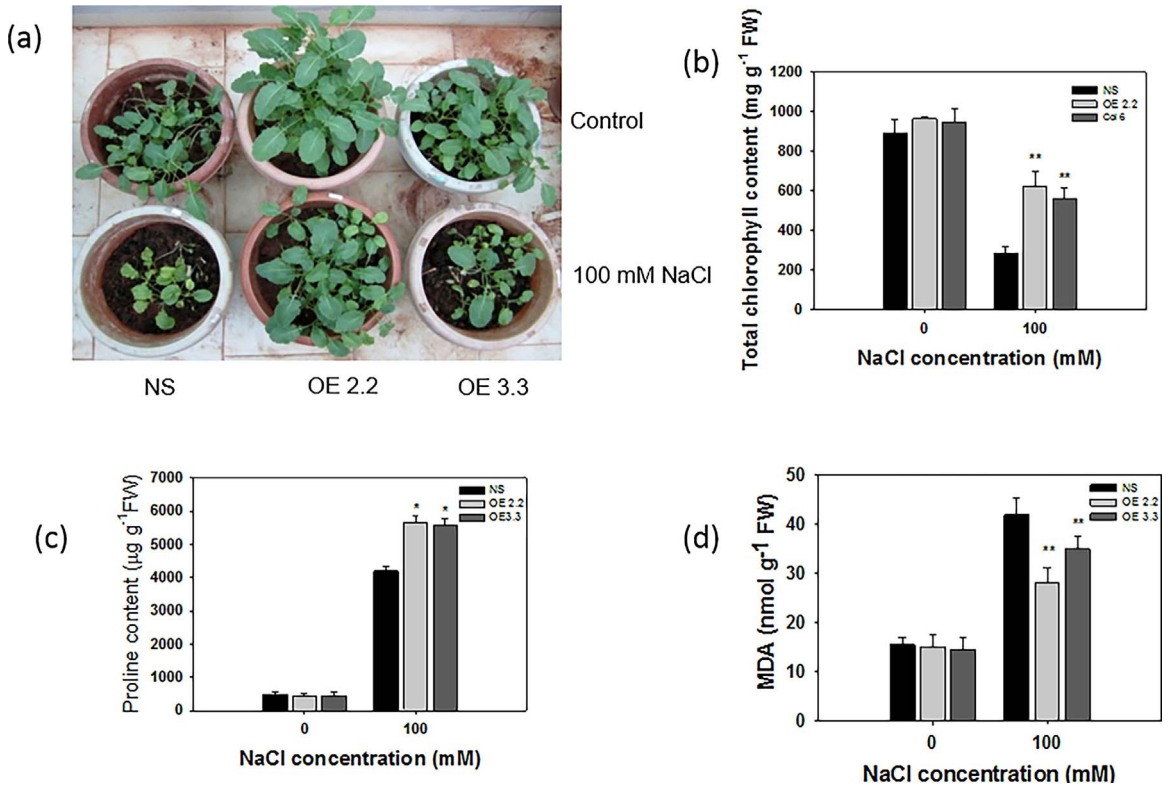

Fig. 7. Salinity tolerance of *AnnBj2* transgenic lines at whole plant level. (a) Phenotypic differences in the growth of null and *AnnBj2* OE lines under control and NaCl treatment in soil. (b) total chlorophyll content (c) proline content (d) MDA content. Picture showing the phenotypic difference was taken after eight weeks of sowing the seeds. Error bar represents mean ± SD of three biological replicates. Single asterisk mark (*) indicates significance level at $p \leq 0.05$.

AnnBj2 of mustard shared 97% similarity with its *Arabidopsis* homolog AnnAt2 at amino acid level (Supplementary information SFig. 6) and the tissue specific expression pattern of *AnnBj2* at seedling stage is analogous to that of its homologs in *Arabidopsis* and *Brassica rapa* [63,64]. In the present investigation, we found contrasting difference in the induction of *AnnBj2* in response to NaCl to that of our earlier report [31]. This may be due to the difference in the age of the plant samples and the method of salt treatment used in the two studies. Earlier, our group reported that 200 mM NaCl stress given to mature leaves through petiole feeding did not induce *AnnBj2* expression, but in the present investigation, NaCl stress induced the expression of *AnnBj2* at the seedling stage. This is in line with the previous reports of spatio-temporal regulation of annexins. Earlier reports suggested that the tissue specific expression of certain annexins may be associated with their specific functions [65,66]. The proteomic data of its homolog in Arabidopsis (AnnAt2) also confirmed its upregulation under salt stress [67].

AnnBj2 OE lines of mustard exhibited better root growth than controls under salt stress. Laohavisit et al. [68] reported earlier the role of AnnAt1 in salinity-induced root cell adaptation. The present study showed that AnnBj2 is also involved in maintaining sustained root growth in salt treatments. Previously, our group reported that the ectopic expression of *AnnBj1* conferred multiple stress tolerance in tobacco and cotton [34,36]. However, *AnnBj2* expressing mustard transgenic plants exhibited salt stress tolerance only in present study with no phenotypic difference in osmotic stress (mannitol) and PEG. We found differential expression pattern of these two genes under salt stress and in different tissues at seedling stage. AnnBj1 and AnnBj2 share 62% similarity at amino acid level. To know whether these two genes have similar functions under salt stress, a detailed comparative study of gain of function and/or loss of function mutants is needed.

Salt stress leads to the disruption of osmotic and ionic homeostasis of cells [1,69,70]. Proline has been regarded as an important osmolyte and its accumulation under salt stress proved to be important in imparting plant salt tolerance [71]. It was previously reported that the overexpression of *P5CS1* gene, a rate-limiting enzyme in the biosynthesis of proline enhanced osmotic stress tolerance in transgenic plants [72]. In our present study, we found *AnnBj2* constitutive expression in mustard lead to enhanced proline accumulation and salt stress tolerance. Quantitative PCR data evidenced significantly higher expression of *P5CS1* in *AnnBj2* OE lines compared to null segregant under salt stress.

Several studies showed a strong correlation between salt exclusion and salt tolerance in many plant species [70,73,74]. Although there are a few reports, which confirm that overexpression of annexins conferred salinity tolerance, their role in maintaining ionic homeostasis has not been reported in detail yet, though Divya et al. [36] reported some analysis on ion accumulation in cotton transgenic plants expressing *AnnBj1*. In the present study, we found that the *AnnBj2* OE mustard lines exhibited comparatively less Na^+ accumulation and maintained higher K^+/Na^+ ratio than the NS under salt stress with associated expression of several genes involved in ion homeostasis. Maintenance of higher K^+/Na^+ ratio in the cytoplasm is associated with salinity tolerance [1].

To gain an insight into how AnnBj2 is involved in maintaining ionic homeostasis, we studied the expression of some key transporters involved in the regulation of Na^+, K^+ and Ca^{2+} under salt stress. Na^+ toxicity of plants is often correlated with its hyper accumulation in the shoot, which interferes in shoot photosynthesis related functions. Plants with reduced Na^+ in the shoots exhibit enhanced salinity tolerance [1]. The *AnnBj2* OE lines exhibited higher transcript levels of *SOS1* and *HKT1* compared to the of NS line. *SOS1* encodes a plasma membrane Na^+/H^+ antiporter, whose enhanced expression conferred salt tolerance in transgenic plants [75]. HKT1 is involved in the retrieval of Na^+ ions from the xylem sap and restricting its translocation from root to shoot [76,77]. Previously, it was shown that K^+ retention ability has a

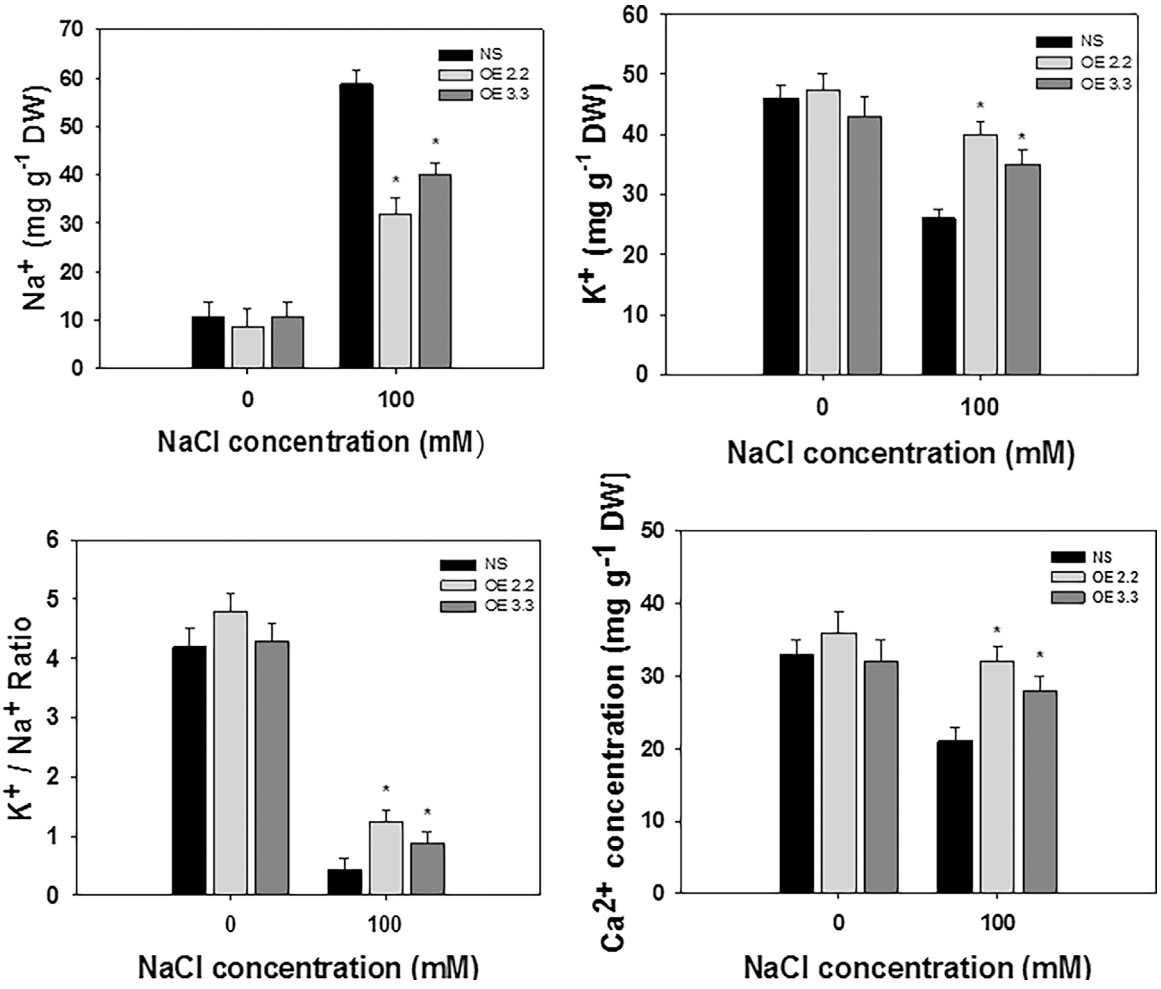

Fig. 8. Ion estimation from the leaves of NS and *AnnBj2* OE plants harvested after eight weeks of treatment NaCl treatment (a) Na^+ content (b) K^+ content (c) K^+/Na^+ ratio (d) Ca^{2+} content. Error bar represents ± S.D (n = 3). Data were analyzed by one way ANOVA with DMRT to determine the significant difference between null and the transgenic lines with $p \leq 0.05$ (indicated by single asterisk mark).

positive correlation with plant salt tolerance ability [78–80]. In cereals, high-affinity K + transporter 1;5 (HKT1;5) facilitates Na^+ exclusion from root xylem vessels to reduce shoot accumulation, whereas HKT1;4 partitions Na^+ from the root xylem stream to leaf sheaths thereby reducing movement of the cytotoxic ion to photosynthetically active leaves [81]. In line with this, we found higher K^+ content in *AnnBj2* OE lines with reduced Na^+ in shoots and enhanced salt tolerance with associated upregulation of HKT1. *Nax1* and *Nax2* loci are involved in salinity tolerance in durum wheat and these proteins are involved in the salt tolerance mechanism in different ways. HKT1;4 and HKT1;5 are shown to be the targets for these genes. HKT proteins were shown to limit the Na^+ transport from root to shoot, which is supposed to enhance salt tolerance [82]. *SOS1* transcripts were, however, lower in *Nax* lines showing that the durum wheat exhibits a dual mechanism for salt tolerance for greater versatility to adapt to the continuously changing environments and limit the Na^+ transport to the shoot resulting in stress tolerance [82]. Our observations on significantly higher expression of *HKT1* and salt tolerance are in line with the published results.

AKT1 is involved in the acquisition of K^+ ions in the cytosol to maintain K^+ homeostasis [83,84]. *OsAKT1* overexpression in rice led to reduction in sensitivity to osmotic/drought stress in transgenic plants and improved rice osmotic and drought stress tolerance by increasing tissue levels of K^+ especially in the root [85]. In the present study, we found higher expression of *AKT1* in *AnnBj2* OE lines and this may be responsible for higher K^+ content in *AnnBj2* OE leaves and the differential expression pattern of these transporters could be the possible reason for higher K^+/Na^+ ratio in *AnnBj2* OE lines.

Some previous reports suggested ion transport activity of annexins [28,86]. AnnAt1 was earlier reported to restrict the entry of Na^+ fluxes across the root epidermal cells, and the knock out mutant *annAt1* exhibited higher Na^+ influx when challenged with NaCl stress [68]. Recently, Jia et al. [67] reported that SCF E3 ligase overexpression restricted Na^+ ion accumulation in the plant tissues and played a positive role in response to salt stress. SCF overexpression upregulated AnnAt1, AnnAt2, and AnnAt3 at the protein level [67]. Further electrophysiological studies are needed to confirm ion transport activity of AnnBj2 across the lipid bilayer. Gene expression analysis under salt stress in the present study to investigate further into the role of AnnBj2 in transport revealed that *AnnBj2* OE lines have relatively higher transcript levels of *SOS1* compared to the control. *SOS1* encodes a Na^+/H^+ exchanger on the plasma membrane that modulates the movement of ions across the cell membrane [87]. This suggests that AnnBj2 appears to restrict the entry of Na^+ inside the cells by stabilizing the integrity of membrane-bound SOS1 Na^+ antiporter. Also, the stress tolerance exhibited by the current transgeincs could be due to interplay of several transporters and Ca^{2+}-ATPase pump.

Ca^{2+} also plays a protective role in alleviating salt stress by competing with Na^+ at plasma membrane [88,89]. More recently, Wang et al. [90] found a significant decrease in the Ca^{2+} content of *annAt1* loss of function mutants. We found significantly higher expression of *Ca^{+2}-ATPase* also in *AnnBj2* OE lines under salt stress. Ca^{2+}-ATPase pump plays an important role in restoring $[Ca^{2+}]_{cyt}$ to basal level under

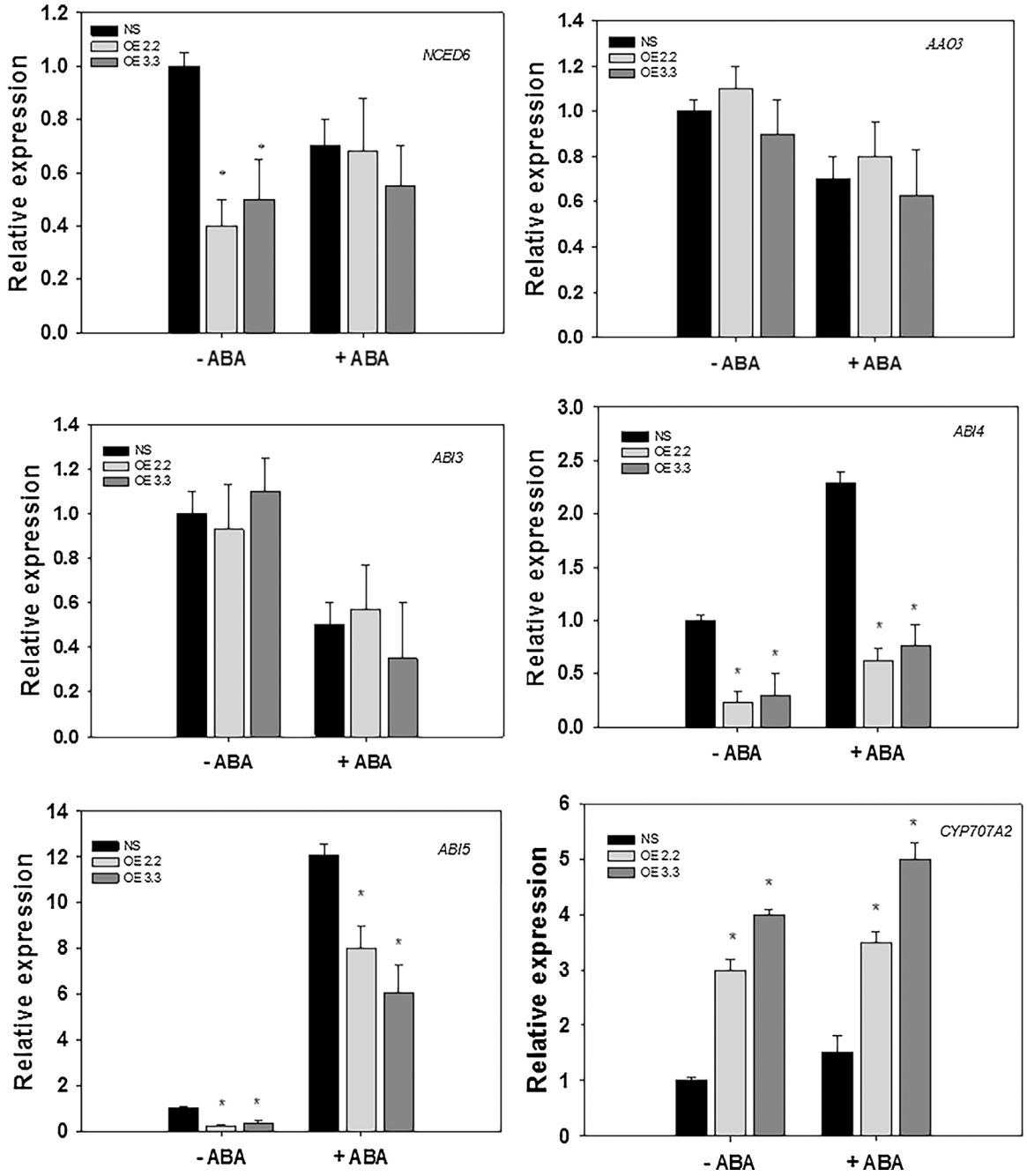

Fig. 9. Expression of genes involved in ABA regulation during seed germination. Sterilized seeds were kept on ½ MS media or supplemented with 8 μM ABA for 24 h under 16/8 light/dark period. After 24 h, seeds were collected and used for RNA isolation to study the relative expression of genes involved in seed germination. The transcript levels were normalized using reference gene *actin2* and *EF1α*. Expressions levels were represented using Livak and Schmittgen's $\Delta\Delta C_T$ method. Experiment was repeated with three biological and three technical replicates. Error bar represents mean ± SD (n = 3). Data were analyzed by one way ANOVA with DMRT to determine the statistical difference between null and the transgenic lines at $p \leq 0.05$ (indicated by single * mark).

different stresses. Ca^{+2}-ATPases impart stress tolerance by sensing calcium levels in the cells and modulating the downstream signaling cascades and activation of specific target genes and their encoded proteins in transport systems and stress signaling, which finally result in stress tolerance in plants [91,92]. *OsACA6*, a P-type IIB Ca^{2+}-ATPase promoted salinity and drought stress tolerance in tobacco upon heterologous expression through ROS scavenging and upregulation of stress-responsive genes [93]. Thus, significantly coordinated expression of several genes appears to work in maintaining ionic homeostasis in *AnnBj2* OE plants and consequential stress tolerance.

The *AnnBj2* OE lines 2.2, OE 3.3 and OE 5.5 of mustard showed enhanced seed germination pattern even in the absence of any stress. The role of annexins in seed germination and vigor was reported [94,95].

ABA plays an important role in seed germination [96]. We observed ABA insensitivity in the *AnnBj2* OE lines at seed germination stage. This intrigued us to study some of the crucial genes, which regulate seed germination process at the transcription level. During seed germination, NCED6 catalyzes the cleavage of 9-*cis*-neoxanthin to xanthoxin, the rate limiting step in ABA biosynthesis [97] and *CYP707A2* encodes an ABA 8-hydroxylase that catalyzes the hydroxylation of ABA for its conversion into phaseic and dihydro-phaseic acids [98]. The relative expressions of these two genes regulate ABA content of the seeds during imbibition [99,100]. During germination, ABA content of the seeds

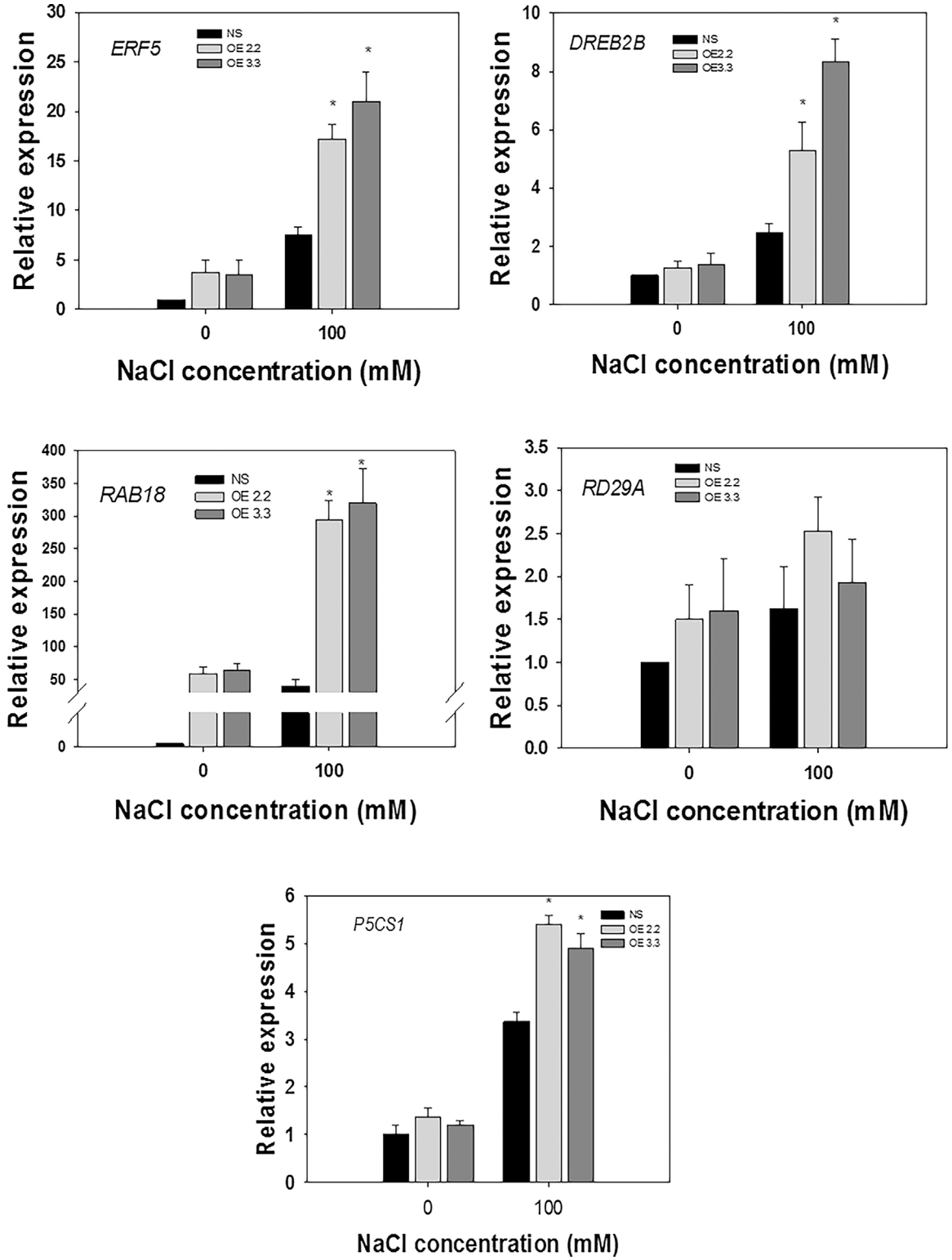

Fig. 10. Expression of salt stress induced marker genes in control and *AnnBj2* OE lines. Five days old seedlings were treated either with mock or 100 mM NaCl for six hours and the samples were immediately freezed for RNA isolation. The relative expression of the *ERF5*, *DREB2B*, *RAB18*, *RD29A*, and *P5CS1* were normalized using the reference gene *actin2* and *EF1α*. Expressions levels were represented using Livak and Schmittgen's $\Delta\Delta C_T$ method. The experiment was repeated with three biological and three technical replicates. Error bar represents mean ± SE (n = 3). Data were analyzed by one way ANOVA with DMRT to determine the statistical difference between null and the transgenic lines at $p \leq 0.05$ (indicated by single * mark).

decreases while there is an increase in GA level [101,102]. Our results show that *AnnBj2* OE lines have increased transcript levels of *CYP707A2* that is involved in ABA degradation, while there was no significant difference in ABA biosynthesis gene, *NCED6*. Also, *ABI4* and *ABI5*, which are positive regulators of seed dormancy, have significantly decreased transcript levels in the *AnnBj2* OE transgenic plants. Shu et al. [103] reported that ABI4 acts a central factor regulating GA/ABA homoeostasis and *ABI4* overexpression in Arabidopsis

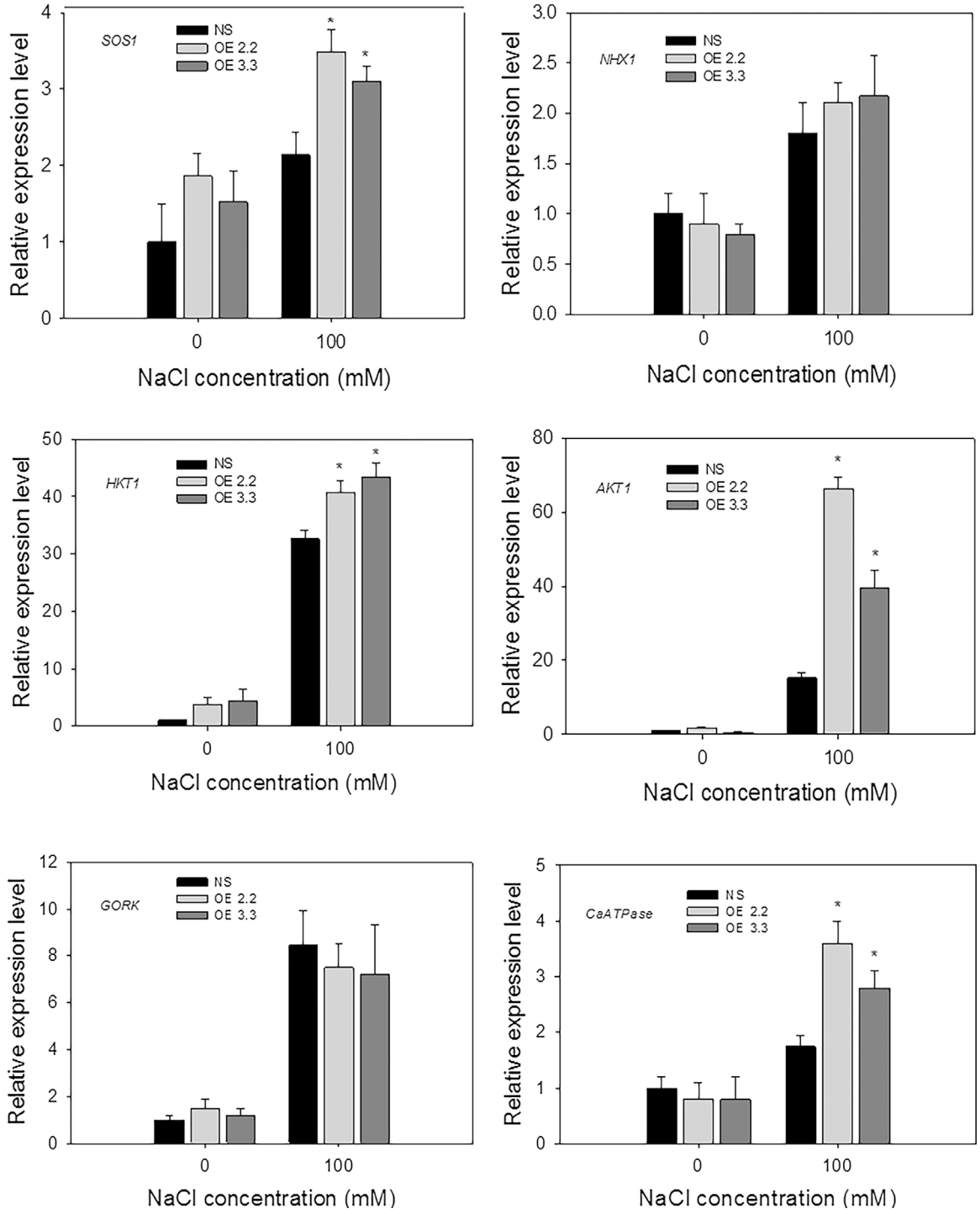

Fig. 11. Expression analysis of transporter genes under salt stress. Five days old seedlings were treated either with mock or 100 mM NaCl for six hours and the samples were immediately quick frozen for RNA isolation. The relative expression of the genes was normalized using the reference gene *actin2* and *EF1α* Expressions levels were represented using Livak and Schmittgen's $\Delta\Delta C_T$ method. The experiment was repeated with three biological and three technical replicates. Error bar represents mean ± SE (n = 3). Data were analyzed by one way ANOVA with DMRT to determine the statistical difference between null and the transgenic lines at $p \leq 0.05$ (indicated by single * mark).

leads to poor seed germination. Another positive regulator of seed dormancy, *ABI5* had significantly lower expression in the *AnnBj2* OE lines compared to that of the controls. *ABI5* overexpressing Arabidopsis seeds showed hypersensitive response to ABA during seed germination and early seedling growth [104]. *AnnBj2* transgenic plants exhibited enhanced levels of *CYP707A2* with reduced expression of *ABI4* in a significant contrast to controls. Kushiro et al. [98] demonstrated that all *CYP707A* genes got upregulated in drought stress treatments and subsequent rehydration of the treated plants. In *Glycine max* also, most of the *CYP707A* genes got upregulated in drought stress and salinity. Overexpression of *CYP707A1* in *atcyp707a2* insertion mutant resulted in its decreased sensitivity to ABA showing that it functions as an ABA hydroxylase [105]. The constitutive expression of *CYP707A3* restored growth retardation induced by the exogenous application of ABA with increased transpiration and reduced endogenous ABA levels in both turgid and dehydrated plants [106]. Together, these gene expression studies suggest that AnnBj2 is involved in the regulation of ABA signaling during seed germination.

High levels of glucose (6%) delay seed germination either due to its osmotic effect or by acting as a signaling molecule to enhance the ABA content of the imbibed seeds [62,107–109]. It has also been shown that the ABA-insensitive mutants exhibit glucose insensitive phenotype [110,111]. Interestingly, the transgenic mustard plants expressing *AnnBj2* exhibited glucose insensitive phenotype. Gene expression study of the imbibed seeds revealed higher expression of ABA catabolic gene, *CYP707A2* in *AnnBj2* OE lines compared to NS line. ABA signaling genes *ABI4* and *ABI5* act as negative regulators of seed germination in the presence of glucose [107,112,113]. Reduced expressions of *ABI4* and *ABI5* in the *AnnBj2* OE lines could be attributed to the glucose insensitive phenotype of *AnnBj2* OE transgenic lines.

In our study, we also observed that the expression of *AnnBj2* conferred ABA insensitivity at seed germination stage and salt tolerance at seedling and adult stages. It is a well-known fact that accumulation of ABA induces stomatal closure in the leaves to maintain water balance during stress conditions [114]. Previously, several groups have reported that the constitutive expression of certain genes like *WRKY20*, *GmbZIP62*, *GsSKP21* and *OsPP108* conferred ABA insensitivity at seed germination stage along with abiotic stress tolerance such as drought, salt and alkalinity too [115–118]. This suggests that ABA signaling regulates seed germination and stomatal closure by different mechanisms.

The *AnnBj2* mustard transgenics exhibited strong expression of ABA responsive genes like *RAB18* and genes like *DREB2B*, *ERF5* that have been shown to act in ABA-independent pathway. This phenomenon suggests that the salt tolerance phenotype observed in the mustard transgenics is controlled by both ABA-dependent and ABA-independent pathways, which may cooperatively cross talk with each other. RD29A is a possible convergence point in the cross talk as the promoter of *RD29A* carries both ABRE and DRE/CRT elements [119]. ABA dependent and independent stress signaling might not occur as a parallel process, but can cooperate and converge in activating stress genes. The enhanced expression of *RD29A* could be because of the activation of either ABRE or DRE elements [120]. Enhanced expression of *RAB18* has been observed in the salt tolerance observed in *LcSAINS1* expressing Arabidopsis and rice plants [121]. Reduced expression of *RAB18* and *RD29A* has been explained as the operation of ABA-independent pathway in *OSPP2C* expressing Arabidopsis transgenics [118]. Recent studies suggest that there is a cross talk between ABA-dependent and ABA-independent pathways in abiotic stress and the global transcriptional network activated in response to osmotic stress is not specifically involved in any one of the two pathways, but is controlled cooperatively [122].

In conclusion, we have studied the role of *AnnBj2* in salt stress by overexpressing it in mustard, which is the first report of functional characterization of any mustard annexin in the native system. Overexpression of *AnnBj2* conferred salt tolerance by maintaining improved physiological indices and ionic homeostasis in the transgenic plants. Gene expression studies suggested the possible function of this gene in regulating the ABA content of seeds by regulating the expression of genes involved in ABA metabolism conferring on them ABA and glucose insensitivity.

Author contributions

IA, TV, PCS and PBK planned and designed the research. IA, DY and PS performed the experiments. IA, TV, PCS and PBK analyzed data. IA and PBK wrote the manuscript. All authors read and approved the manuscript.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.plantsci.2017.09.010.

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