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ORIGINAL ARTICLE

Ectopic overexpression of cytosolic ascorbate peroxidase gene (*Apx1*) improves salinity stress tolerance in *Brassica juncea* by strengthening antioxidative defense mechanism

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Abstract

Salinity stress is considered to be a key constrain that reduces the crop productivity by impairing plant growth and development. During salt stress condition, an underlying mechanism for reduction in crop yield is increase in ROS level that can potentially harm cellular macromolecules, leading to disruption of essential physiological and biochemical processes. Plants possess a complex antioxidative defense machinery for scavenging these ROS. Ascorbate peroxidase (APX, E.C. 1.11.1.11), is a crucial antioxidant enzyme involved in Ascorbate–Glutathione pathway that primarily detoxifies the negative impact of H_2O_2 in cell. The efficient scavenging of H_2O_2 is a prerequisite for enhanced tolerance to salinity stress. Here, we have inspected whether over-expression of APX could provide protection against salinity stress. Cytosolic ascorbate peroxidase (*Apx1*) gene, isolated from *Arabidopsis thaliana*, was chosen as the candidate gene for strengthening the antioxidative defense system of *Brassica juncea*. Physiological parameters were employed to analyze the growth status of transgenic plants. Leaf disc assay was done to evaluate the salinity stress tolerance potential of transgenic plants, using several physiological and biochemical parameters. Under salinity stress, the transgenic plants performed well as compared to their non-transgenic counterparts; as revealed through greater proline accumulation, increased chlorophyll stability index, lower chlorophyll a/b ratio, and higher antioxidative enzyme activities. Further, the lower H_2O_2 levels were well correlated with lesser membrane damage as measured through MDA content. Collectively, our results clearly depicted that ectopic overexpression of *AtApx1* gene was able to confer salinity stress tolerance by strengthening the antioxidative defense system in *B. juncea*.

Keywords Salinity stress · *Brassica juncea* · Reactive oxygen species · Transgenic plants · Antioxidant system

Introduction

Plant's encounter to salt stress adversely affects its growth and productivity worldwide (Wang et al. 2003). Among several abiotic stresses, salinity stress is not only an environmental restrain that reduces the yield potential of a crop plant, but it also imposes a huge negative impact on growth and survival of crop plant. Salinity stress covers a wide range of alteration at physiological, biological and molecular

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levels. It disturbs the cellular homeostasis by disrupting the ionic equilibrium and consequently induces the physiological drought as well as other secondary stresses (Apel and Hirt 2004). In response to these secondary stresses, plants induce the accumulation of osmolytes, anti-stress proteins and trigger the scavenging of reactive oxygen species (ROS) to overcome the devastating milieu. Primarily, accelerating the ROS detoxification system is one of the preferred approaches to ameliorate such adverse effects of salinity, albeit other approaches are also significantly important. The ROS detoxification is sustained by enzymatic and non-enzymatic antioxidant systems (Gill and Tuteja 2010; Miller et al. 2010). Plants have developed several antioxidant enzymes such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX) and peroxidase (POD) that effectively restrict the ROS and hence protect the cell from oxidative injury imposed by ROS accumulation (Mittler and Zilinskas 1992; Noctor and Foyer 1998).

APX enzyme catalyzes the reaction for converting the H_2O_2 into H_2O and O_2, the APX enzymes are further categorized as cAPX (cytosolic), mitAPX (mitochondrial), sAPX (chloroplastic stroma), and tAPX (chloroplast thylakoids) on the basis of their subcellular localization (Shigeoka et al. 2002). Among the different APX isoforms, the cytosolic form is studied extensively and reported to be the most responsive form to environmental constrain (Davletova et al. 2005; Fourcroy et al. 2004; Mittler and Zilinskas 1994). cAPXs are mostly encoded by more than one gene and characterized from several plants including *Arabidopsis thaliana, Oryza sativa, Pisum sativum* and *Lycopersicum esculentum* to name a few (Gadea et al. 1999; Kim and Chung 1998; Kubo et al. 1992; Mittler and Zilinskas 1992). APX activity is known to be enhanced during various environmental stresses, including dehydration (Tanaka et al. 1990), high temperature (Sato et al. 2001), deficiency in microelements and excessive light (Cakmak and Marschner 1992), UV stress (Saxena et al. 2011), and salinity (Hernandez et al. 2000). Cytosolic APX1 provides cross-compartment protection to chloroplastic APX under high light intensity (Davletova et al. 2005). Rice showed the increase in transcript of *cApx* gene by heat stress; interestingly seedlings were having prior exposure to heat stress displayed improved tolerance to cold stress (Sato et al. 2001). Arabidopsis possesses eight *Apx* isoforms including two cytosolic isoforms (*AtApx1* and *AtApx2*) which are reported to be differentially induced under heat and high light stresses (Davletova et al. 2005). *AtApx1*promoter possesses ABA response element (ABRE), heat-shock element (HSE) while *AtApx2* promoter has two HSEs. The presence of different *cis*-regulatory elements *AtApx1* and *AtApx2* is attributed to their differential gene expression under stress (Mittler and Zilinskas 1992).

Engineering crop plants with improved antioxidative enzyme machinery pave the way for the better abiotic stress potential. Here we hypothesize that enhancing the activity of APX enzyme through genetic engineering will strengthen the antioxidative defense system and will enable it to limit the increased production of hydrogen peroxide under salinity stress.

For the present investigations, we have chosen *Brassica juncea* as the target crop, an important oilseed cash crop globally. Brassica is affected by various stresses which translate to the significant loss of quantity and quality produced. *B. juncea* and Arabidopsis belongs to the same family with high similarity in functional genes present between them allows us to study these functionally valuable genes (Anjum et al. 2012). Here, we ectopically expressed *AtApx1* in *B. juncea* and the growth of transgenic plants was analyzed for several physiological parameters under controlled conditions. We have compared the physiological and biochemical markers in transgenic and wild type (WT) plants under salt stress treatment. Transgenic plants were further examined for the ROS scavenging potential by determining the antioxidative enzyme activity of APX, POD, GPX and CAT. Taken together, our data indicated that the potential of *AtApx1* gene, improves the salinity stress tolerance by limiting the ROS-induced damage in *B. juncea*. These findings present a way forward to engineer the salt tolerance potential in agriculturally important crops to minimize the yield loss by salinity stresses.

Materials and methods

Plant materials and growth conditions

Seeds for *B. juncea* variety pusajaikisan were obtained from National Research Centre for Rapeseed and Mustard (NRCRM), Bharatpur (Rajasthan), India and were used in the present study. Brassica were grown in a growth chamber at 22 °C for 16 h light and 15 °C for 8 h dark cycle with 70% relative humidity under white fluorescent light.

Plant transformation

Agrobacterium tumefaciens strain C58C1rifR (pGV2260) harboring *AtApx1* (TAIR: AT1G07890) was used for plant transformation. Construct was generated by cloning the *Arabidopsis* cytosolic ascorbate peroxidase cDNA (*AtApx1*) with EcoRV and XhoI sites (Primers are listed in Table S3) under the control of a constitutive promoter (CaMv35S) and was transferred to *B. juncea* by *Agrobacterium*-mediated transformation. For plant transformation, hypocotyls from 6 day old seedlings were co cultivated with *Agrobacterium* culture for 24 h and further washed and selected from MS medium supplemented with kanamycin (30 mgL^{-1}). Selected explants were regenerated on shoot regenerating

medium (MS medium supplemented with combination of growth hormones such as 2.5 mgL^{-1} N^6-benzylaminopurine and 1.0 mgL^{-1} 1-naphthaleneacetic acid). Well-developed shoots were shifted to root regeneration medium (MS medium supplemented with 0.5 mgL^{-1} indole butyric acid). Plantlets were grown at 22 °C under white fluorescent light and 16-h photoperiod. Completely developed plants were hardened and were grown to maturity. The progeny of the primary transformants were multiplied by selfing and T_2 generation seeds were used for further experiments.

Confirmation of transgenic plants

Genomic DNA (gDNA) was isolated from one-month old transgenic plants along with WT plants using modified CTAB method (Augustine et al. 2013). For PCR amplification, genomic DNA was used as the template and amplified using gene specific primers and *nptII* primers (Primers are listed in Table S3). For southern blot, 10 µg of gDNA was digested with EcoRV and the samples were electrophoresed on agarose gel. After electrophoresis, the gel was treated with depurination, denaturation and neutralization solutions followed by hybridization. After hybridization and washing, the blot was exposed to X-ray film and the film was developed. We have used three independent transgenic lines for further experiments which are designated in figure and/or graphs as follows: L1 represents *AtApx1* transgenic line 3; L2 represents *AtApx1* transgenic line 4; L3 represents *AtApx1* transgenic line 5.

Quantitative real-time PCR

For isolation of total RNA from transgenic and WT leaf sample, TRI reagent (Sigma) was used. RNA (2 µg) was reverse transcribed, used for cDNA synthesis (using ABI cDNA synthesis kit). qPCR was performed with *AtApx1* primers (listed in Table S3) and endogenous control (*ACT*) along with negative control (lacking cDNA sample) (Petla et al. 2016). Three biological replicates were used with triplicate reaction in each assay. Primers used here are listed in Table S3.

Measurement of physiological growth parameters

To know the physiological and growth status of transgenic plants, rate of photosynthesis was assessed using Infrared gas analyzer (IRGAs) following the manufacturer's instruction. The flow rate of air through the sample chamber was (11 cm^2) set at 0.4 LPM and leaf temperature was maintained at 24 ± 0.8 °C and measured at a time interval of 15/20 s that was kept constant for all samples. Fully expanded leaves in triplicates (transgenic and WT) were used for analysis and five recurrent readings were obtained each time.

Chlorophyll fluorescence measurement

To estimate photosynthetic activity in WT and transgenic plants, we measured chlorophyll fluorescence (Fv/Fm) with a handy chlorophyll fluorescence measuring device (Handy PEA, Hansatech, UK) following the manufacturer's instruction. Measurements for Fv/Fm were recorded in the forenoon hours and for fluorescence level (F_0) in modulated light. The maximum fluorescence level (Fm) was obtained by 1.5 s saturating pulse at 300 mol m^{-2} s^{-1} on dark adopted leaves (20 min).

Soil plant analysis development (SPAD) measurements

SPAD values were recorded in sunlight following the manufacturer's instruction (SPAD meter, Opti Science, CMM-200, USA). The instrument calculates the transmission of red light and infrared light at 650 nm and 940 nm, respectively. Leaves were randomly sampled from matured transgenic and WT plants. Five readings obtained by portable SPAD meter from individual leaves were averaged and calculated.

Leaf disc assay

To examine the morphological difference under salt stress, leaf disc assay was performed on transgenic along with WT plants using varied saline concentrations (NaCl-100, 150 and 200 mM) to the half MS medium (Singla-Pareek et al. 2008). Leaf discs (1 cm) were cut from independent transgenic lines of *B. juncea* and WT plants and floated on 4 ml half MS medium supplemented with different concentrations of NaCl and kept for 48 h at 22 °C under a 16-h photoperiod. Finally, 200 mM concentration of NaCl was chosen for performing further assays on the basis of morphological assessment of leaf discs. The chlorophyll content of the leaf discs was determined for getting information regarding salt stress-induced chlorophyll loss in the transgenic and WT plants. Leaf discs floated on half MS medium without salt served as the experimental control. Leaf samples from control and salt-treated matured plant (both WT and transgenic) were used for further physiological analysis.

Seed germination study and salinity stress treatment

For seed germination study and stress experiments, seeds from both WT and transgenic *B. juncea* were germinated on filter paper (Whatman no. 2) water-logged with 4 mL of the ½ MS medium supplemented with and without 200 mM of NaCl. Seed germination was evaluated after every 12 h. For stress experiments, seeds were germinated on germination

paper with 4 mL of ½ MS medium supplemented with and without 200 mM of NaCl and allowed to grow for 10 days. Ten day old seedlings were chosen for measuring stress markers and antioxidant enzyme activity.

Chlorophyll and carotenoid content

For determining chlorophyll content, the method given by Hiscox and Israelstam (1979) is followed. Fresh leaves (200 mg) were extracted in 5 ml dimethylsulfoxide (DMSO) and incubated at 65 °C for 40 min. After cooling, a 2 ml aliquot of the chlorophyll content was taken into a cuvette and measurement was done spectrophotometrically at 470, 645, 663 nm using DMSO as blank. Respective absorption was used to measure the Chlorophyll *a*, *b*, and total chlorophyll (Chl $a+b$), based on the equations reported by (Joshi et al., 2013). Carotenoid content was measured using the method of (Terzi and Kadioglu 2006). Chlorophyll stability index was determined using ratio of chlorophyll content of stressed leaf to control leaf sample (Terzi and Kadioglu 2006). All quantifications were performed in triplicate.

Proline content

Free proline content was measured using the method reported by Bates et al. (1973). Proline concentration was calculated with the help of calibration curve made using L-proline. Further, the final values were expressed as µg gm^{-1} FW^{-1}. All quantifications were performed in triplicate.

Malondialdehyde and hydrogen peroxide content

MDA content was estimated using thiobarbituric acid (TBA) method reported by Heath and Packer (1968). The MDA concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-1}. Values are presented from the measurements of three replicates. Hydrogen peroxide (H_2O_2) level was determined following potassium iodide (KI) method spectrophotometrically as described by Salvi et al.(2016). All quantifications were performed in triplicate.

Antioxidative assay ofAPX, GPX, POD and CAT

For enzyme assay, protein was extracted in protein extraction buffer containing 100 mM sodium phosphate buffer (pH 7.0), 5 mM ascorbate, 10% glycerol and 1 mM EDTA (Ghosh et al. 2020). Bradford reagent was used to determine the protein concentration (Bradford 1976).

Specific APX activity was measured following the method reported by Nakano and Asada (1981). Briefly, ascorbate oxidation by H_2O_2 was measured in 1.5 ml assay mix containing 50 mM phosphate buffer (pH 6.0), 0.1 µM EDTA, 0.5 mM ascorbate, and 1.0 mM H_2O_2 with the addition of 50 µl of leaf extract at 290 nm. APX activity was calculated using extinction coefficient of 2.8 mM^{-1} cm^{-1}. GPX assay was performed by the method reported in Urbanek et al. (1991). The tetraguaiacol formed was calculated with molar extinction coefficient (26.6 mM^{-1} cm^{-1}). The enzyme activity was expressed in terms of µmol min^{-1} mg^{-1} protein. For POD activity, the method described in Siegel and Galston (1967) was followed. For CAT activity, we used the method reported by Beers and Sizer (1952) with few modifications. In this method, the reaction mixture of 1.5 ml contains 50 µl of the enzyme extract, 100 mM phosphate buffer (pH 7.0), 0.1 µM EDTA, and 20 mM H_2O_2. The decline in H_2O_2 content was observed at 240 nm, immediately and 1 min after H_2O_2 addition. The difference in absorbance (ΔA_{240}) was divided by the H_2O_2 molar extinction coefficient (36 M^{-1} cm^{-1}) and the enzyme activity was expressed as µmol of H_2O_2 min^{-1} mg^{-1} protein.

Statistical analysis

Data presented in this study are mean ± standard deviation (SD). For statistical analysis (one-way analysis of variance) we used Duncan's multiple range test (DMRT, $\alpha=0.05$) using SPSS program (SPSS, Chicago, IL, USA). Letters mentioned on the graph designate the similarities or differences between mean values.

Results

Molecular analysis of transgenic lines expressing *AtApx1*

The C58C1Rif^R (pGV2260) strain harboring *AtApx1* construct (described in material and methods Sect. 2.2) (Fig. S1) was transformed into the *B. juncea* using *Agrobacterium*-mediated genetic transformation (Fig. 1a). To optimize the regeneration frequency, different combinations of growth hormones were used, and the appropriate concentration was selected (Table S1). Among different tissues, the segment of hypocotyl has been used as a source of explants to achieve the successful transformation. After transformation several transgenic lines were obtained, later on five independent transgenic lines (L1–L5) were analyzed. To validate the AtApx1 integration, we carried out PCR using *AtApx1* (898 bp) and *nptII* (Kan) (734 bp) gene specific primers in the T_0 generation plants indicating the integration of the *AtApx1* gene (Fig. S2A). For confirming the single transgene integration, southern hybridization was performed. Transgenic lines L3, L4 and L5 were shown to have single copy insertion while WT did not show hybridization signal (Fig. S2B). Further, T_2 plants were raised from the seeds of T_1

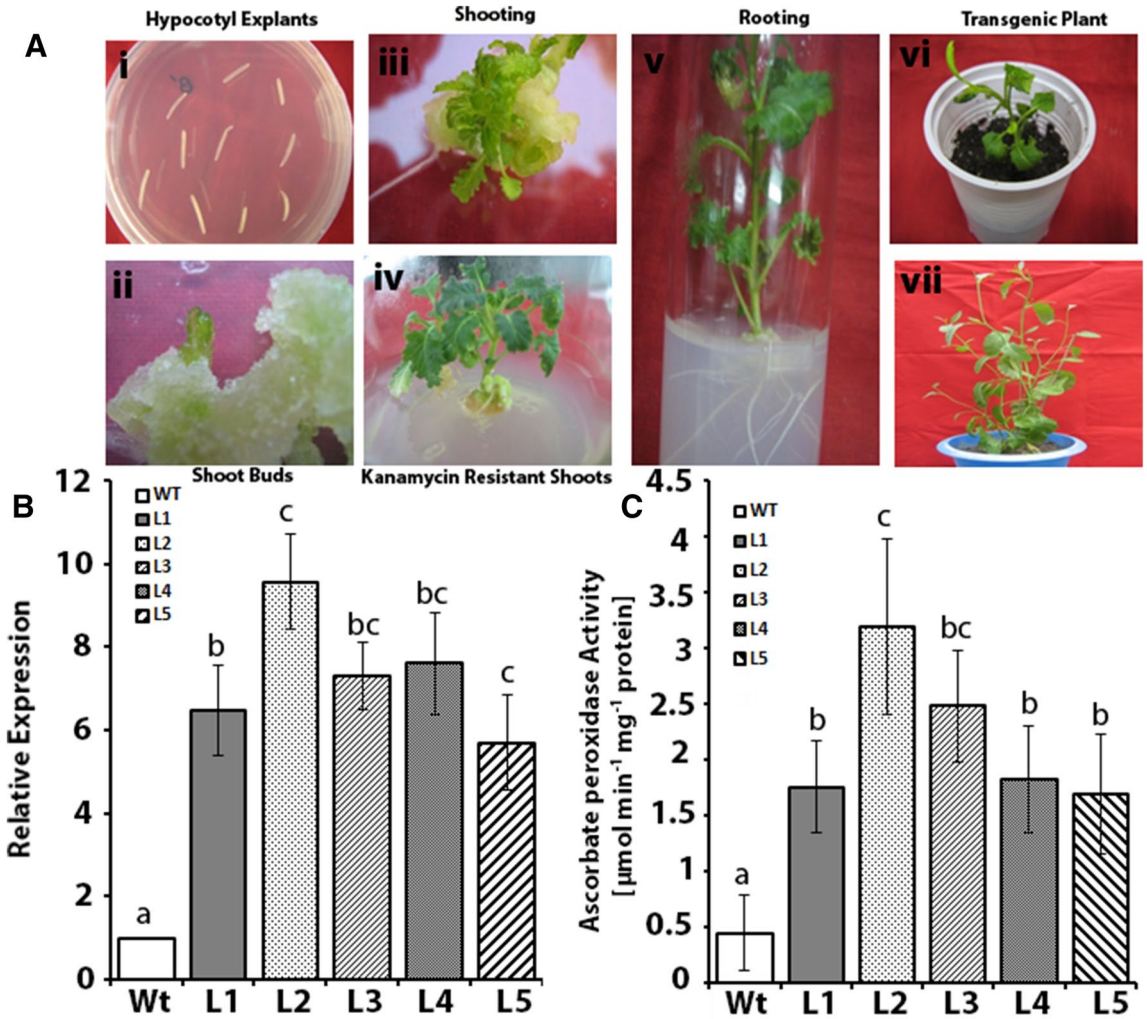

Fig. 1 Generation of transgenic *Brassica juncea* and their analysis. **a** Different stages of *Agrobacterium*-mediated genetic transformation of *Brassica juncea* (I–VI). **I** Hypocotyl explants, **II** shoot buds, **III** shooting. **IV** kanamycin-resistant shoots, **V** rooting, **VI**, **VII** transgenic plant. **b** qRT-PCR of transgenic lines. The relative expression values of each gene were normalized to an endogenous control *ACTIN2* and calculated using ΔΔCT method. Values are means of three biological replicates each with three technical replicates. **c** APX activity in transgenic lines. Protein extract (50 µl) was assayed in the reaction mix as described in materials and methods. Error bars indicate the standard deviation. Significant differences among means ($\alpha = 0.05$) are denoted by the different letters

generation (via self-pollination). In control condition, transgenic plants (at T_1 and T_2) appeared similar to WT in general morphology. We measured *AtApx1* transcript as well as APX activity under control conditions in the selected transgenic lines and WT plants. Each transgenic line displayed significant elevation of *AtApx1* transcript and activity (Fig. 1b, c).

AtApx1 overexpression does not alter normal physiological parameters and displays comparable growth with WT plants

Physiological status and growth were compared to rule out the occurrence of any alteration in the physiology of the transgenic plant because of genomic alteration (overexpression of *AtApx1* gene). Gas exchange was measured at the vegetative stage with the young fully expanded leaves of WT as well as transgenic plants under normal growth conditions. During gas exchange measurement, we did not observe significant variation in net photosynthetic rate and stomatal conductance of transgenic leaves than WT leaves (Fig. 2a, b) that revealed their normal photosynthetic efficiency. To assess the health integrity of photosynthetic apparatus, we further determine the cholorophyll fluroscence (Fv/Fm). Photosynthetic efficiency of transgenic plants was comparable to the WT plants under control conditions (Fig. 2c) and the architecture of the photosynthetic apparatus of transgenic plants was found to be normal. In addition to the above parameters, SPAD value was also examined which gives an indication of the nitrogen status and relative greenness of leaves. The results suggested that the relative greenness

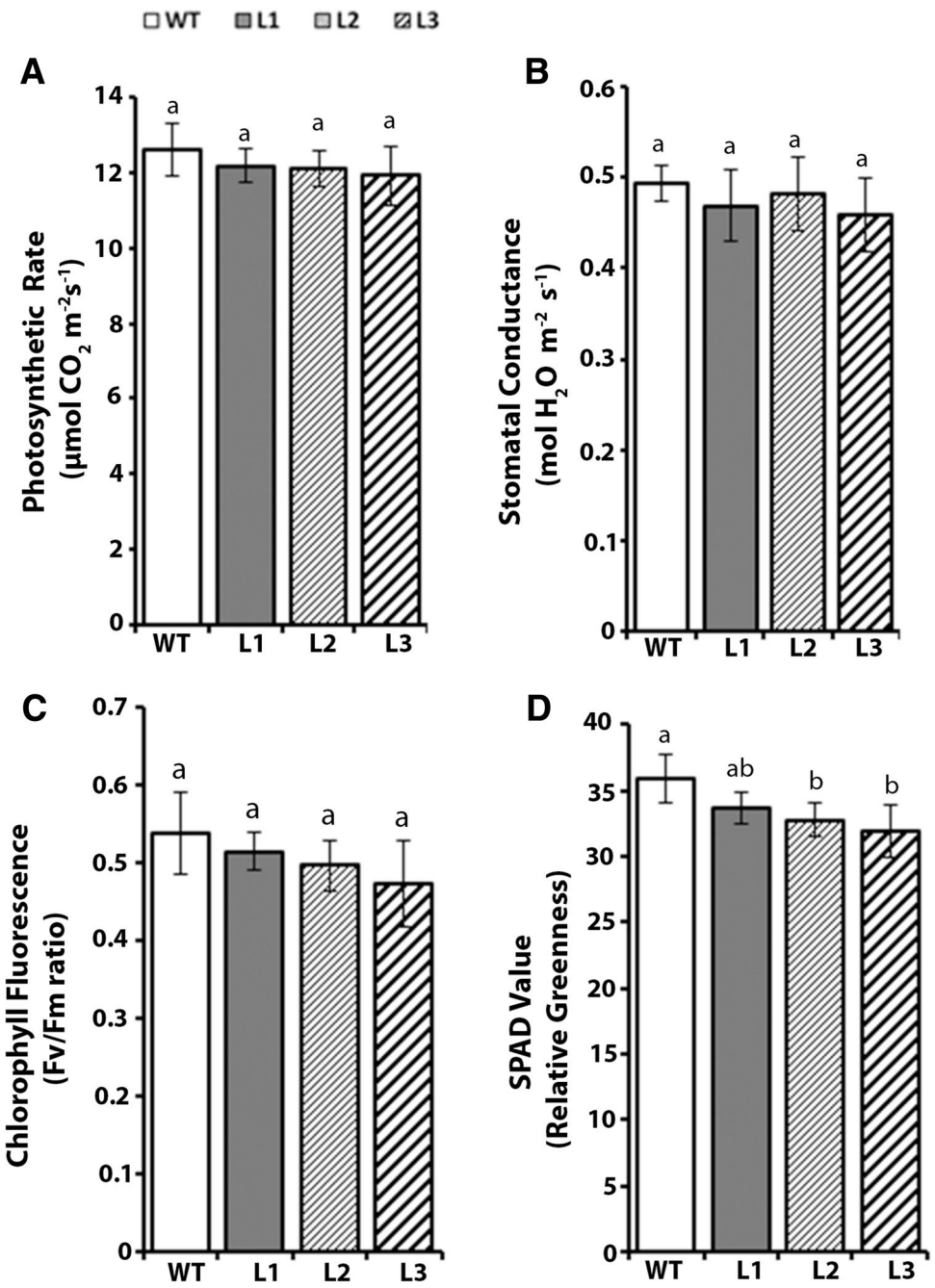

Fig. 2 Physiological attributes of wild type (WT) and *AtAPX1* transgenic plants under control conditions. **a** Photosynthetic rate, **b** stomatal conductance, **c** chlorophyll fluorescence, **d** soil plant analysis development (SPAD) value. Measurements were taken in one-month old pot grown plant under normal growth conditions. (*WT* wild Type, L1 represents *AtApx1* transgenic line 3; L2 represents *AtApx1* transgenic line 4; L3 represents *AtApx1* transgenic line 5). Error bars indicate the standard deviation. Significant differences among means ($\alpha = 0.05$) are denoted by the different letters

was slightly better in WT than the transgenic under normal growth conditions (Fig. 2d).

AtApx1 overexpression leads to higher chlorophyll retention and enhanced salt tolerance

As shown in Fig. 1b, c the transgenic *B. juncea* plant overexpressing *AtApx1* gene had a higher transcript and APX activity than WT plants. The homozygous lines were used to test, if the increased APX activity would render the plants an advantage in tolerating the salinity stress. As the bleaching in the leaves is considered as a good indicator of salinity-induced damage, the pot grown transgenic *B. juncea* plants were first evaluated for the leaf disc assay (Fig. 3a).

Under control condition, we did not observe evident difference between WT and transgenic plants. Whereas under leaf disc assay, WT leaf disc treated with salt stress (200 mM for 24 h) showed substantial damage as compared to transgenic leaves. Further during chlorophyll and carotenoid content measurement, we found that chlorophyll *a*, chlorophyll *b* and total chlorophyll concentrations were significantly declined in all the plants exposed to

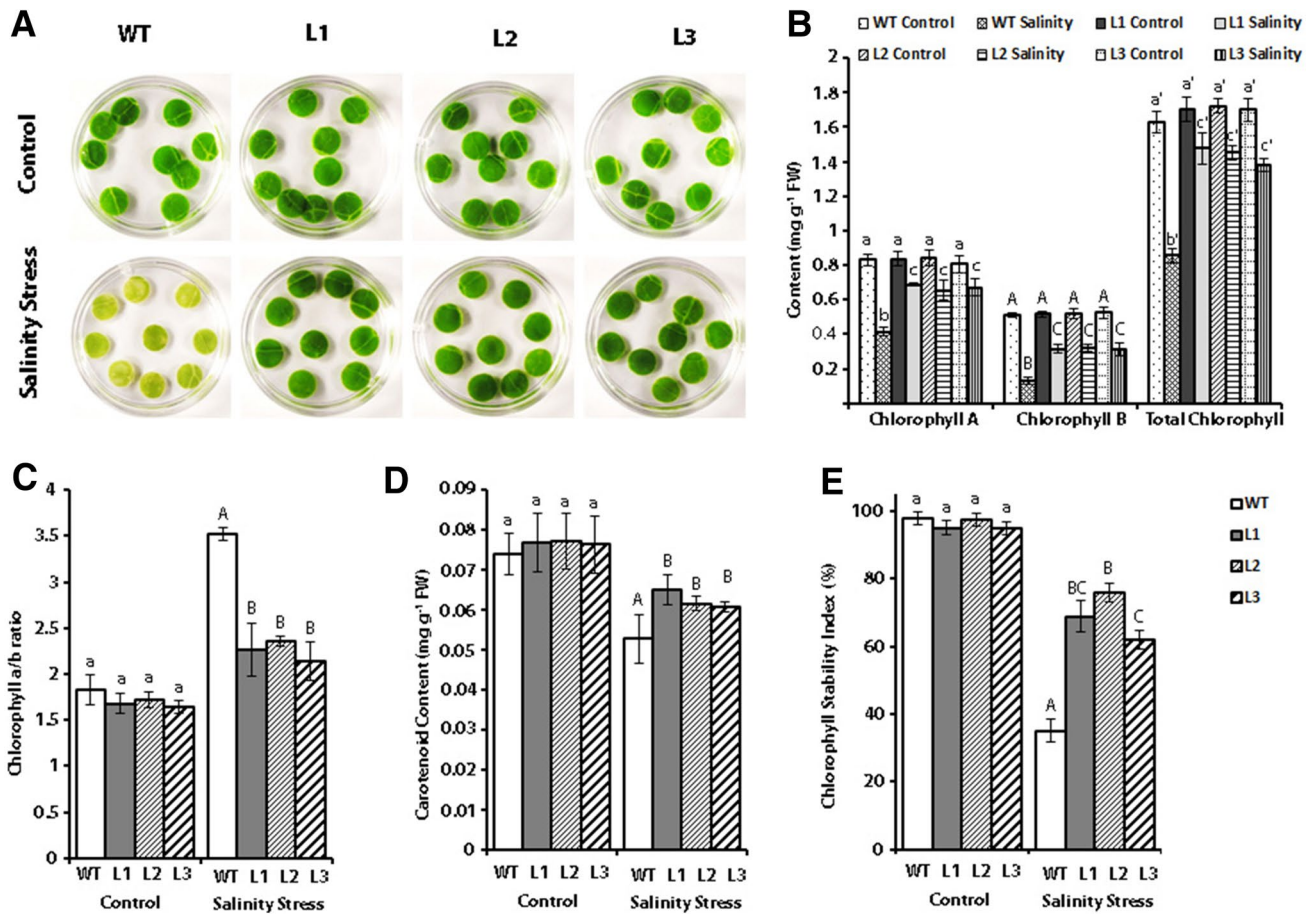

Fig. 3 Salt stress tolerance assay of *AtApx1 Brassica juncea* transgenic plants. Retardation of salt stress promoted senescence in one-month old transgenic *Brassica* plant overexpressing *AtApx1*, indicating the tolerance at cellular level towards toxic levels of 200 mM salt after 48 h of treatment. Leaf disc floating in MS medium without salt served as the experimental control. **a** Comparative phenotype analysis in WT and transgenic *Brassica* leaf disc under control (upper panel) and salt treated (lower panel) conditions, **b** chlorophyll content, **c** chlorophyll *a*/*b* ratio, **d** carotenoid content, **e** chlorophyll stability index (%). (*WT* wild Type, L1 represents *AtApx1* transgenic line 3; L2 represents *AtApx1* transgenic line 4; L3 represents *AtApx1* transgenic line 5). Error bars indicate the standard deviation. Significant differences among means ($\alpha = 0.05$) are denoted by the different letters. Values are means of three biological replicates each with three technical replicates

salinity stress; however, such a decrease was more profound in WT (Fig. 3b). The total chlorophyll content decreased by 48% in WT plants under 200 mM NaCl stress as compared to the transgenic leaf discs which recorded about 15% decrease under salinity stress, over that of their respective control. (Fig. 3b). Chlorophyll *a*/*b* ratio was enhanced in both WT and transgenic after salt stress treatment, however, the increase in WT was 2-fold while it was 1.3-fold in transgenic over their respective control (Fig. 3c).

Total carotenoid content decreased in leaves of WT and transgenic plants after salinity stress; however, the decrease was lesser in transgenic plants as compared to WT (Fig. 3d). These results were also supported by the chlorophyll stability index which is a measure of stress tolerance potential of plants (Fig. 3e). Chlorophyll stability index was 1.9-fold higher in the leaves of transgenic plant than the WT (Wanichananan 2003).

AtApx1 overexpression lines accumulate higher proline, lower H_2O_2 and MDA content under salinity stress

Plants exposed to salt stress preferentially accumulate higher concentrations of proline that acts as a rescue molecule against oxidative injury (Jain et al. 2001; Jebara et al. 2005; Koca et al. 2007). Therefore, WT and transgenic plants were evaluated for the accumulation of proline content after salinity stress. As anticipated, after stress exposure proline content accumulated in both WT and transgenics; however, net proline accumulation was 1.8-fold higher in the transgenic as compared to WT (Fig. 4a).

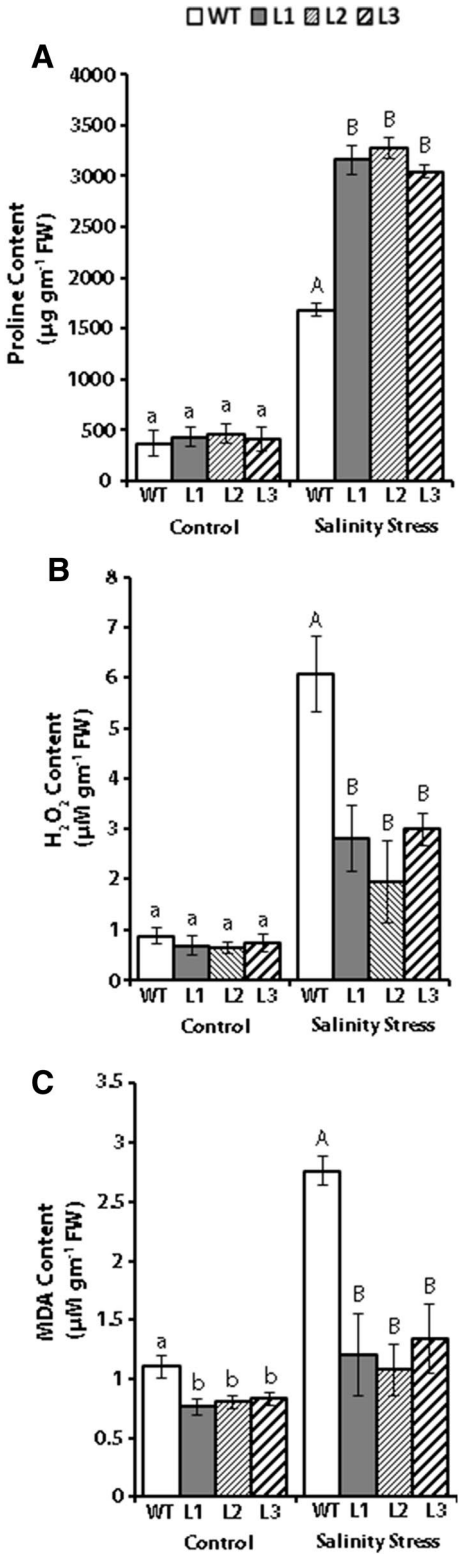

Fig. 4 *Brassica juncea AtApx1* transgenic lines exhibit improved salt stress tolerance by reduced ROS accumulation. Quantitative analysis of **a** proline content, **b** hydrogen peroxide (H_2O_2) content, **c** malondialdehyde (MDA) content. Quantifications were done in control and after salt stress treatment as described in the materials and methods section. (*WT* wild Type, L1 represents *AtApx1* transgenic line 3; L2 represents *AtApx1* transgenic line 4; L3 represents *AtApx1* transgenic line 5). Error bars indicate the standard deviation. Significant differences among means ($\alpha = 0.05$) are denoted by the different letters. Values are the means of three biological replicates each with three technical replicates

The cellular H_2O_2 level in WT plant was about 2.8-fold higher in contrast to transgenic plant under salinity stress (Fig. 4b). To further extend this analysis, we evaluated malondialdehyde (MDA) content to evaluate H_2O_2-mediated damage. MDA content was found to increase drastically by almost 2.5-fold in WT plants when challenged to salt stress, whereas its level in the transgenic plants increased only by 1.5-fold, indicating that WT plants experienced higher loss in membrane integrity (Fig. 4c). These results clearly depicted that under similar stress condition transgenic plants hold better capacity to detoxify ROS in comparison to WT.

AtApx1 overexpression resulted in improved germination rate after salinity stress

Under salinity stress treatment, transgenic lines showed better % germination rate (Fig. 5a and b). After 48 h of stress treatment % germination was recorded to be just 50% in WT. However, the germination rate was similar in both WT and transgenic seeds under control condition (without NaCl). Seeds were counted as germinated just after the radicle production from the seeds.

AtApx1 overexpression confers salinity stress tolerance and improves antioxidant enzyme activities in *B. juncea*

We analyzed the activity profile of different enzymes (APX, GPX, CAT and POD) associated with ROS scavenging pathway (Fig. 6a–d). The activity profile of all plants was similar under control condition while an evident upregulation of APX, GPX and POD was observed in transgenic plants (Fig. 6a–c).

The APX specific activity was found to be 1.9-fold higher in transgenic than the WT after salt stress treatment (Fig. 6a). It is clear that APX activity is significantly induced under salinity stress, although a distinctly higher APX activity in the transgenics is attributed to the over-expression of *AtApx1* gene. Additionally, we also estimated the GPX and POD activity to explore the antioxidative machinery of transgenic plants under salinity stress. The results revealed that under salinity stress transgenic plant possess 1.5-fold higher GPX activity than that of WT (Fig. 6b). Similarly, total peroxidase activity also showed a marked increase in transgenic plant (twofold) than WT (Fig. 6c). To our surprise, catalase activity was found to decrease in WT as well as the transgenic

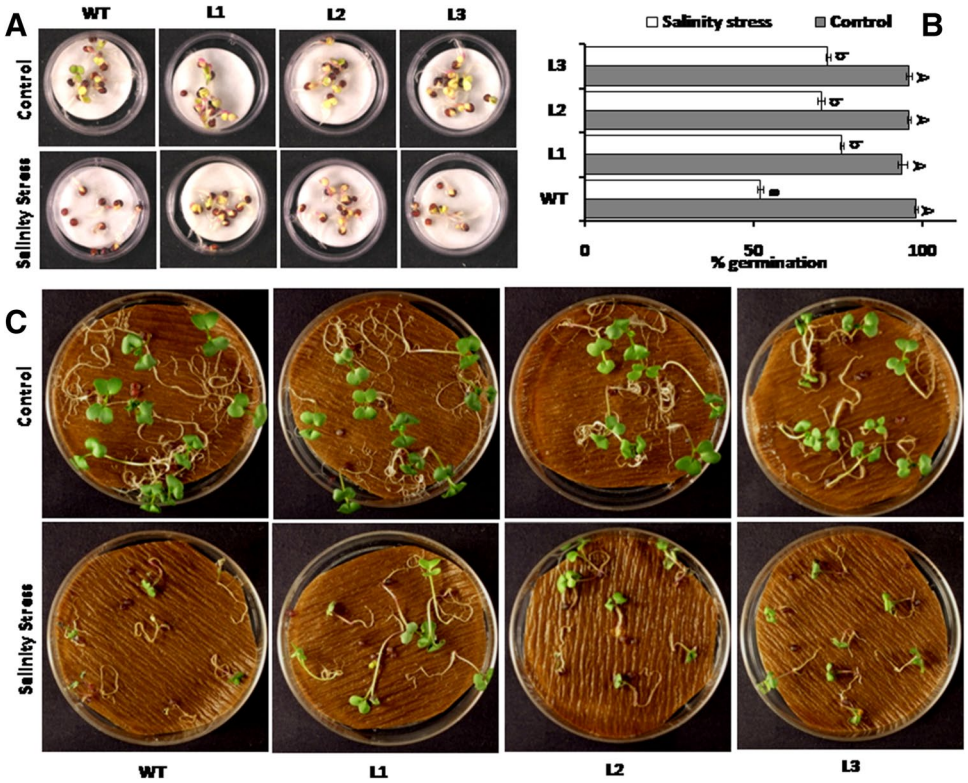

Fig. 5 *Brassica juncea AtApx1* transgenic lines show better germination rate under salt stress treatment. **a** Comparative seed germination study in WT and transgenic *Brassica* under control conditions (upper panel) and salt treated (lower panel). **b** Graph showing seed germination rate in WT and transgenic lines after 48 h under control conditions and salt treatment. **c** Stress treatment to WT and transgenic *Brassica* seedlings (10 days old) (*WT* wild Type; L1 represents *AtApx1* transgenic line 3; L2 represents *AtApx1* transgenic line 4; L3 represents *AtApx1* transgenic line 5). Error bars indicate the standard deviation. Significant differences among means ($\alpha = 0.05$) are denoted by the different letters. Values are means of three biological replicates each with three technical replicates

after salt stress (Fig. 6d). These results lead us to speculate that the ectopic overexpression of *AtApx1* activated several other enzymes associated with the ascorbate–glutathione cycle in the transgenic plants under salinity stress.

Discussion

The salinity stress is inevitably associated with enhanced ROS accumulation and oxidative stress which drastically reduces the crop yield. Among several adaptive strategies, activating the antioxidative system to limit the detrimental effects of ROS is a preferred choice for plants (Foyer and Noctor 2003; Mittler and Zilinskas 1994). A positive correlation has been reported between the antioxidant enzyme activity of enzymes such as APX, POD and SOD and detoxification of ROS under salinity stress (Cakmak and Marschner 1992; Jebara et al. 2005). APX is a key antioxidative enzyme which detoxifies H_2O_2 to H_2O and O_2 (Davletova et al. 2005; Kubo et al. 1992; Nakano and Asada 1981).

Since, H_2O_2 is easily diffusible through biological membranes, it accumulates excessively in the cytosol. Incidentally, cytosolic isoform of APX is the one which responds maximally to externally imposed stress (Davletova et al. 2005). Therefore, we attempted to genetically engineer *B. juncea* (var. pusajaikisan) by overexpressing a cytosolic APX so as to strengthen the antioxidative defense system of the plants.

In this study, we have demonstrated that ectopic expression of ascorbate peroxidase gene (*AtApx1*) in the economically important oilseed crop *B. juncea* could improve salinity stress tolerance. These findings are well correlated with other reports which suggested that increased APX activity plays a promising role under salinity stress tolerance (Gossett et al. 1994; Jebara et al. 2005).

To analyze the growth status of transgenic *B. juncea* several physiological parameters were examined viz photosynthetic rate, stomatal conductance, chlorophyll fluorescence and SPAD values. Stomatal conductance is well correlated with the gas exchange and CO_2 assimilation (Lincoln and Zeiger 2006; Salisbury and Ross 1992) which ultimately affect photosynthetic rate and in turn is linked with the productivity of plant. Chlorophyll fluorescence is an important criterion to determine the photosynthetic efficiency. The Fv/Fm ratio is used to measure the quantum efficiency of the electron transport in photosystem II (PSII) (Maxwell and Johnson 2000). Higher the variable fluorescence, higher is the photosynthetic capacity of the leaf. Thus, the parameters, maximal fluorescence (Fm), variable fluorescence (Fv) and the Fv/Fm ratio characterize the functional state of PSII (Moradi and Ismail 2007). Similarly, chlorophyll content in terms of relative greenness measured by SPAD meter indicates the growth status of plants. The lower SPAD values for transgenic plants might result through the channeling of carbon and metabolic energy towards the continuous synthesis of APX and

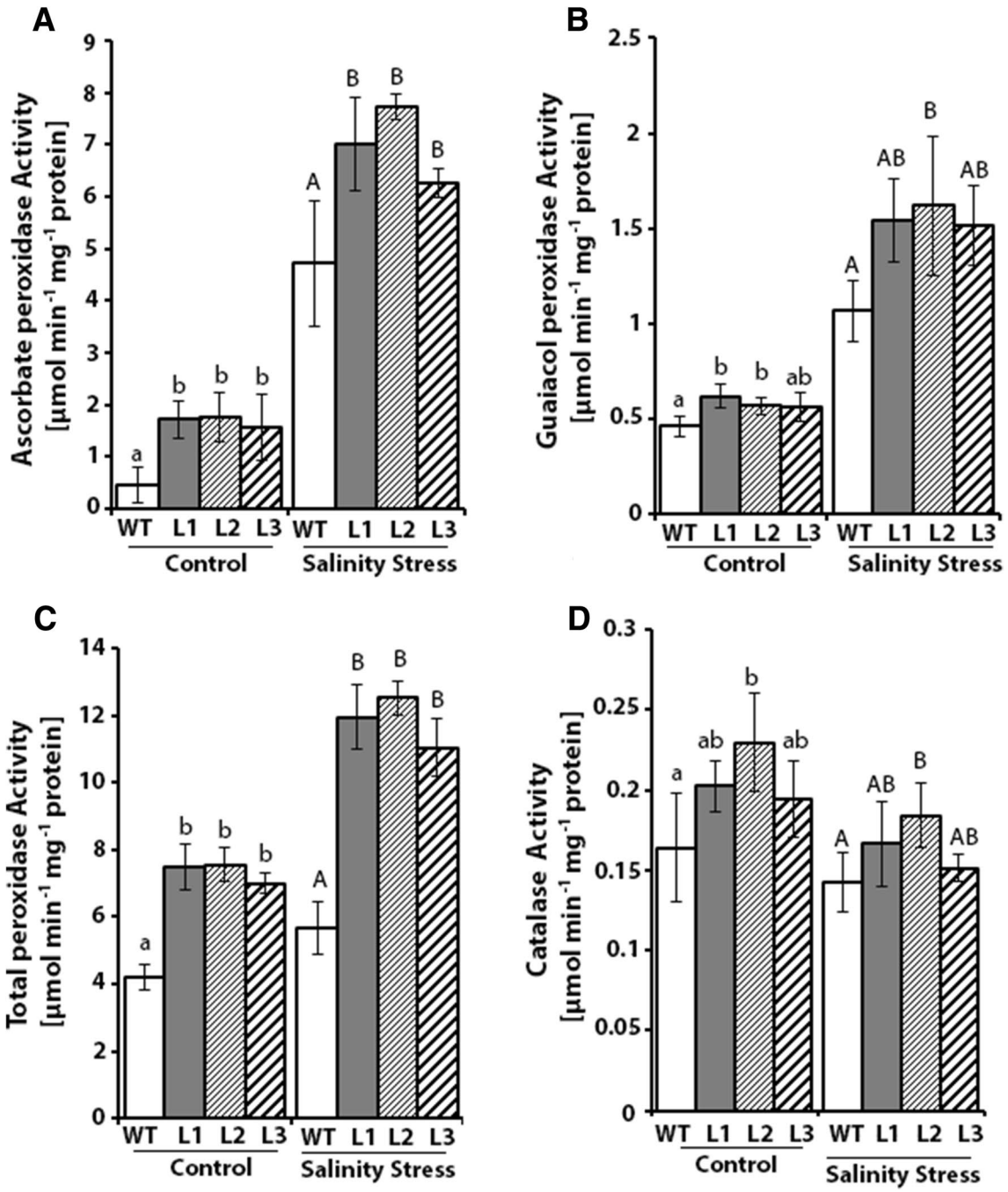

Fig. 6 *Brassica juncea AtApx1* transgenic lines maintain higher antioxidative potential after salt stresses. Quantitative measurement of antioxidative activity of **a** ascorbate peroxidase, **b** guaiacol peroxidase, **c** total peroxidase, **d** catalase activity. Quantifications were done in control and after salt stress treatments in one-month old plant as described in the materials and methods section. (*WT* wild Type; L1 represents *AtApx1* transgenic line 3; L2 represents *AtApx1* transgenic line 4; L3 represents *AtApx1* transgenic line 5). Error bars indicate the standard deviation. Significant differences among means ($\alpha = 0.05$) are denoted by the different letters. Values are means of three biological replicates each with three technical replicates

possible changes in the flavonoid and carotene contents of plants. On comparative analysis, these parameters which inferred the constitutive expression of *AtApx1* did not obtrude the photosynthetic rate, stomatal conductance and chlorophyll fluorescence of transgenic plants under normal growth conditions (Fig. 2).This avails us to ascertain about the uniformity of further experimental analysis.

Salinity stress is known to impair the biosynthesis of enzymes involved in the maintenance of photosynthetic apparatus and may also trigger chlorophyll degradation

which precisely reduced the chlorophyll content in salt-stressed plant (El-Tayeb 2005; Fang et al. 1998). Chlorophyll content can be precisely correlated with the cellular metabolic status; thus, its reduction indicates oxidative damage in tissues due to accumulation of ions and other active oxygen species. Loss of chlorophyll pigment under salt stress was observed for some salt-sensitive plant species (Fang et al. 1998; Saxena et al. 2013). In our investigation, leaf disc senescence assay displayed higher degree of bleaching in WT and turned yellow in the presence of 200 mM NaCl as compared to the transgenic. The reduced chlorosis in the leaf discs of transgenics were well correlated with the quantitative measurement of chlorophyll content in salt-treated matured plant (Fig. 3). Likewise, the lower chlorophyll content is possibly associated with its impaired biosynthesis due to salinity-induced ROS attack. The higher activity of APX in transgenics could protect chlorophyll biosynthesis or curtail its degradation (Maruta et al. 2010; Stenbaek and Jensen 2010). Reduced sensitivity to salinity stress in *AtApx1* overexpressing transgenic leaf is similar to the earlier observation (Badawi et al. 2004; Jebara et al. 2005; Moradi and Ismail 2007). In addition, the chlorophyll stability index and carotenoid content (Fig. 3d, e) were higher in the transgenic plants compared to WT plants, indicating a more efficiently functioning photosynthetic apparatus. The higher chlorophyll stability index (CSI) is considered as a potential indicator of stress tolerance capacity of plants. Higher CSI is associated with the better availability of chlorophyll, thus generating a higher photosynthetic rate (Moradi and Ismail 2007). Also, the chlorophyll *b* which showed higher reduction than chlorophyll *a* indicates that chlorophyll b is more sensitive than chlorophyll a under stress conditions (Netondo et al. 2004). Further, the higher chlorophyll *a*/*b* ratio has been associated with the alteration of pigment composition of photosynthetic system which possesses lower level of light harvesting chlorophyll proteins (LHCPs) following abiotic stress treatment (Loggini et al. 1999; Nakano and Asada 1987).

Due to excess ROS, the proper functioning of macromolecule present in cytosol as well as sub cellular organelles such as chloroplasts and mitochondria are severely hampered. Enhanced salt tolerance accompanies the accumulation of a high level of proline content during stress conditions. Being a cytosolic osmolytica, proline not only offers osmotic balance, rather it may also stabilize the structure and function of other cellular macromolecules such as regulatory proteins and nucleic acid (Jain et al. 2001; Kubo et al. 1992; Saxena et al. 2011; Strizhov et al. 1997). Higher proline content in transgenic leaves under control conditions could be attributed to the changes in the normal biosynthetic reactions in the cells, which are being forced to continuously express *AtApx1* gene introduced under the control of a constitutive promoter. However, under salt stress treatment a significantly higher proline accumulation in transgenic seedlings could be attributed to the protective role of proline as an osmoprotectant as well as a ROS scavenger (Matysik et al. 2002). Higher accumulation of free proline in salinity stress tolerance has been well documented (Jebara et al. 2005). It has been reported that overexpression of proline biosynthesis enzymes endows reduced oxidative damage and improved photosynthesis (Strizhov et al. 1997). H_2O_2 is a detrimental ROS, which imposes severe effects on cellular machinery (Salvi et al. 2017, 2020). Salinity treatments caused significant increase in H_2O_2 content and thus lipid peroxidation. MDA content reflects on the degree of lipid peroxidation and thus indicates ROS-triggered cellular damage (Salvi et al. 2017). Relatively higher H_2O_2 accumulation and lipid peroxidation has been documented in salt stress sensitive varieties as compared to resistant variety (Gossett et al. 1994). The apparently lower level of MDA, a lipid peroxidation product and consequently reduced membrane damage in transgenic plants can be explained by lower H_2O_2 level in respective plants. Thus, the enhanced tolerance observed is likely to be a result of lower H_2O_2 level that is attributed to significant protection against lipid peroxidation and eventually protects the cellular integrity under oxidative injury. H_2O_2 imposes toxic effects on cell metabolism which eventually impair the growth and development of plant, thus cells require a fast and efficient way of minimizing H_2O_2 toxicity and the formation of the highly toxic hydroxyl radicals (Tanaka et al. 1985).

It is well known fact that salt stress can cause excessive accumulation of ROS; including hydrogen peroxide (H_2O_2) resulting in oxidative damage of cells (Miller et al. 2010). The H_2O_2 formed as a product of superoxide dismutase activity is a potential damaging agent under different abiotic stresses (Cakmak and Marschner 1992).The lower accumulation of H_2O_2 content in transgenic plants with salinity treatment can be directly correlated with ectopic overexpression of *AtApx1*. Induction in enzyme activity of antioxidative pathways is positively correlated with reduced ROS and enhanced stress tolerance capacity in plants. In *Mesembryanthemum crystallinum* cytosolic *Apx* transcripts and its enzyme activities are found to be induced under salt stress treatment. Being an intricate system, different enzymes of antioxidative pathways are interrelated, thus co-regulated. So, the over-expression of one enzyme can also influence the activity of other enzymes (Gossett et al. 1994; Loggini et al. 1999). Similarly, the induction in the enzyme activity of peroxidase and guaiacol peroxidase was observed in this study which may be due to the overexpression of APX activity. Though guaiacol peroxidase and total peroxidase activity induced in both WT and transgenic, however net GPX activity was higher in the transgenic than the WT in salinity stress. Kim et al. (2005) documented the induction in GPX activity, along with APX and catalase activity under

salinity stress in barley shoots and roots. A consequently increased activity of total peroxidase and guaiacol peroxidase in transgenics, under salt treatment, indicate the existence of an intricate cross-talk mechanism among different peroxidases in the cell. In contrary, catalase activity did not exhibit significant difference, under control and salt-stressed conditions. The decrease in catalase activity under stress conditions might be due to inhibition of biosynthesis or inactivation of catalase activity due to stress (Shim et al. 2003). A similar decline in CAT activity has been reported in rice subjected to salt stress (Lee et al, 2001). Shi et al. (2001) also found a decline in CAT activity under heat stress and salt stress in *Arabidopsis* plants overexpressing peroxisomal APX. Protective roles of APX and related antioxidant enzymes including CAT, GPX and POD in mitigating salt stress have been reported (Gossett et al. 1994; Mittler and Zilinskas 1992).Taken together our results indicated that *AtApx1* overexpressing plants are more tolerant to salinity than WT plants, with transgenic plants having higher chlorophyll retention, antioxidant enzyme activities, proline content and better net photosynthesis than WT plants.

Conclusion

Collectively, in the present studies a successful attempt has been made to strengthen the antioxidative defense potential of *B. juncea* (*var*. pusajaikisan), through overexpression of cytosolic ascorbate peroxidase gene. The over expressed enzyme could substantially improve the salinity stress tolerance profile of the host plant, as assessed through various physiological and biochemical parameters. Thus, *AtApx1* in *B. juncea* plays a pivotal role in preventing the excess accumulation of ROS and helps in enhancing the stress tolerance potential of host plant. APX along with the coordination of other antioxidative enzymes like GPX, catalase and POD maintains the ROS homeostasis and provides tolerance to the cell.

Author contribution statement SS conceptualized the idea, performed major experiments and drafted the manuscript. PS contributed to preparation of the manuscript, conducted some biochemical experiments and data analysis. NUK conducted southern blotting and some biochemical analysis. PJ performed some physiological measurement and data collection. SA conceptualized the design of the work. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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