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Research article

Aldo-keto reductase-1 (AKR1) protect cellular enzymes from salt stress by detoxifying reactive cytotoxic compounds

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ABSTRACT

Cytotoxic compounds like reactive carbonyl compounds such as methylglyoxal (MG), melandialdehyde (MDA), besides the ROS accumulate significantly at higher levels under salinity stress conditions and affect lipids and proteins that inhibit plant growth and productivity. The detoxification of these cytotoxic compounds by overexpression of NADPH-dependent Aldo-ketoreductase (AKR1) enzyme enhances the salinity stress tolerance in tobacco. The *PsAKR1* overexpression plants showed higher survival and chlorophyll content and reduced MDA, H2O2, and MG levels under NaCl stress. The transgenic plants showed reduced levels of Na^+ levels in both root and shoot due to reduced reactive carbonyl compounds (RCCs) and showed enhanced membrane stability resulted in higher root growth and biomass. The increased levels of antioxidant glutathione and enhanced activity of superoxide dismutase (SOD), ascorbate peroxidase (APX) and glutathione reductase (GR) suggest AKR1 could protect these enzymes from the RCC induced protein carbonylation by detoxification process. The transgenics also showed higher activity of delta 1-pyrroline-5- carboxylate synthase (P5CS) enzyme resulted in increasedproline levels to maintain osmotic homeostasis. The results demonstrates that the AKR1 protects proteins or enzymes that are involved in scavenging of cytotoxic compounds by detoxifying RCCs generated under salinity stress.

1. Introduction

Salinity stress is a major threat for agriculture that reduces plant productivity. More than 20% of cultivated land worldwide is affected by salt stress, therefore, it is necessary to identify and develop salinity tolerant varieties. When plants exposed to salinity, several multiple pathways and stress inducible gene expression results in improved adaptation. The induction of multiple stress-inducible genes encoding osmolytes, ion channels, detoxification enzymes confer salinity tolerance when overexpressed in sensitive plants (Veena and Sopory, 1999; Singla-Pareek et al., 2003: Sanan-Mishra et al., 2005). Physiologically exclusion, extrusion, compartmentalization by several Na^+-K^+ transporters are relevant mechanisms and can improve salinity tolerance (Gupta and Huang, 2014). Besides these mechanisms, plants tolerate saline conditions by a combination of several other mechanisms. Under salinity stress, reactive oxygen species (ROS) generated are detoxified either by non-enzymatic antioxidants like reduced glutathione (GSH), ascorbate (ASH), tocopherols and carotenoids etc. or by antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APx), glutathione peroxidise (GPx) and glutathione-S-transferase (GST) etc. (Xiong and Zhu, 2002; Kumar et al., 2003; Wang et al., 2003).

The ROS acts on proteins and lipids that generates highly toxic reactive carbonyl compounds like methylglyoxal, Acrolein, hydroxy nonenal (HNE), melandialdehyde etc. These compounds further acts on proteins to form aggregates or Advanced glycation end products (AGEs) or Advanced lipoxidation end products (ALEs) (Vistoli et al., 2013). The accumulation of carbohydrates like glucose, fructose, trehalose and other sugars has been demonstrated under salt stress (Parida et al., 2004). These carbohydrates mitigates ROS generated during saline stress by osmoprotection, carbon storage, and scavenging (Kerepesi and Galiba, 2000). However, in recent years it has been shown that changes in sugar metabolism is involved in cell damage due to sugar toxicity

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(Kawahito et al., 2009). Auto-oxidation of sugars generated during the photosynthetic calvin cycle and respiratory metabolism is inevitable and produces ROS and sugar derived reactive carbonyl compounds (RCCs) and damages cell metabolic activities (Takagi et al., 2014). It is also evident in microbes that the oxidative degradation of glucose in glycolysis generates by-products of RCCs (Kawahito et al., 2009). The enzymatic conversion of dihydroxyacetone phosphate catalysed by methyl glyoxal (MG) synthase produces MG (Richard, 1984). The RCCs like MG, glyoxal (GLY) and 3-deoxyglucosone (3-DG) formed by amodari re-arrangement of glucose inactivate protein function by modifying the amino acid residues. These RCCs has more resident time than the ROS, oxidize proteins and many proteins are carbonylated during oxidative stress that affect many cellular process (Brigitte et al., 2013).

Higher plants posses detoxifying mechanisms targeting sugar derived RCC MG (Negm, 1986 Mundree et al., 2000). The glyoxalase system (glyoxalase I and glyoxalase II) reduces MG into lactate using glutathione as electron donor (Thornalley, 1990). Overexpression of these two enzymes in tobacco showed enhanced MG detoxification under high concentrations of NaCl stress (Singla-Pareek et al., 2003; Yadav et al., 2005). In addition to glyoxalase pathway, aldo-ketoreductases (AKRs) have been shown to detoxify RCCs including MG. The expression of AKRs was induced by salt, osmotic and heat stresses and reduction of MG by NADPH dependent manner into acetol has been reported in microbes (Paulus et al., 1993; Gavidia et al., 2002). Similarly overexpression of *aldose reductases (ALR*) showed detoxification of reactive aldehydes including MG (Vander Jagt et al., 2001). The role of AKR4C10 and AKR4C11 in reducing sugar derived RCCs has been demonstrated in Arabidopsis in response to light and CO_2 (Saito et al., 2013). Recently, overexpression of *Arabidopsis thaliana* (AtAKR4C9) or alfalfa (*Medicago sativa)* (MsALR) genes in barley showed improved tolerance to salt, cadmium and reactive aldehydes (Csaba et al., 2016). Overexpression of *OsAKR1* in *E. coli* showed improved tolerance to ROS and methylglyoxal (Turoczy et al., 2011). In our recent study overexpression of *PsAKR*1 in tobacco detoxified the reactive carbonyl glyphosate and showed improved tolerance to herbicide (Vemanna et al., 2016). We demonstrate the potential of *PsAKR1*in detoxifying the salinity induced RCCs and improved protection of antioxidant, enzymes and osmolyte synthesizing enzymes resulting in improved tolerance to salinity in tobacco.

2. Material and methods

2.1. Plant material

Transgenic tobacco plants overexpressing *PsAKR1* gene under the control of a constitutive *ribulose 1,5-bis phosphate carboxylase* (*RBCS*) promoter were generated using pBINPLUS vector through Agrobacterium mediated transformation in our earlier studies. Three different homozygous lines of transgenic plants were identified and the expression levels of those lines were confirmed by RT-PCR using gene specific primers (Vemanna et al., 2016).

2.2. Imposition of stress

2.2.1. Seedling level

The germinated seeds of transgenic and wild type were placed on half MS agar medium supplemented with 150 and 300 mM NaCl. The seedling growth was observed 7 days after stress treatment and photographs were taken. Similarly the MG stress was also imposed by transferring germinated seedlings on half MS agar medium supplemented with 10 mM MG and growth and photographs were recorded after 10 days. In another experiment seedlings were raised in soilrite media for 15 days and irrigating with 150 and 300 mM NaCl imposed salinity stress. The seedling response was assessed after 8 days of stress period, seedling survival and photographs were recorded.

2.2.2. Excised leaf disc assays

Leaf discs were made from identical leaves of 3-month-old transgenic and wild type plants and placed on wet blotting paper in petri plates containing 150 mM, and 300 mM NaCl and incubated for 48 h in a temperature controlled light chamber at 28 °C and light intensity of 100 μm/s. After stress period leaf discs were transferred to water for 2 days of recovery and analysed for total chlorophyll content, MG levels and electrolyte leakage.

2.2.3. Hydroponic system

The plants were raised in half strength Hoagland media for 25 days and subsequently treated with 50 mM of NaCl for 8 days followed by 100 mM for 4 days and allowed to recover for 4 days. After recovery the biometric observations such as root length, fresh weight, Na^+ content in leaves and roots of transgenic and wild type plants were measured. The hydroponic solution containing Hoagland solution was aerated with a continuous stream of air.

2.2.4. Whole plant stress

The salinity stress was imposed to soil grown plants in pots by a method developed at the Central Soil Science Research Institute (CSSRI) Karnal, India. The plants were raised in soil with farmyard manure (80:20%) and grown till 45th day under normal conditions. Subsequently the soil was irrigated with known concentrations of mixture of salts of NaCl (0.5 M), $CaCl_2$ (0.150 M) $MgCl_2$ (0.08 M) and Na_2SO_4 (0.1 M). The method involves equilibrating the soil with a mixture of salts to simulate natural field salinity stress condition. Pots were irrigated with known volume of salts to maintain salinity stress of 8 d S/m (Smitharani et al., 2014). Subsequently irrigation was controlled not to allow any seepage of salts. One set of plants was harvested at 60^{th} day to measure root growth, MG and total dry matter. Another set of survived plants were continued to grow till the seed set and harvest.

2.3. Quantification of H_2O_2 levels

The xylenol orange reagent was prepared in 50 ml of distilled water containing 50 mM ferrous ammonium sulphate in 1 ml of 2.5 M H_2SO_4 and 62.5 μl of 125 μM xylenol orange along with 0.9019 g sorbitol. The leaf discs treated with NaCl was extracted in phosphate buffer of pH 7.5 and 25 μl supernatant was mixed with 275 μl of xylenol orange reagent. The reaction mix was incubated for 30 min at room temperature and absorbance was measured at 560 nm against blank (xylenol orange reagent) (Gay et al., 1999).

2.4. Total chlorophyll content

Chlorophyll was extracted from 200 mg of leaf tissue in 10 ml of acetone: DMSO (1:1) and the supernatant were made up to final volume of 25 ml. The absorbance was recorded at 663 and 645 nm using UV–visible spectrophotometer (UV 2450, Shimadzu Corporation, Kyoto, Japan). Total chlorophyll was estimated by using the following formula and expressed as mg/g FW (Babitha et al., 2015a).

$$CHLa = (12.7(A663) - 2.69(A645)^*V)/(W^*1000)$$

$$CHLb = (22.9(A6.45) - 4.68(A663)V)/(W^*1000)$$

$$TCHL\ (mg/g.\ FW) = CHLa + CHLb$$

where, CHL = chlorophyll, V = volume, W = weight and

TCHL = Total chlorophyll.

2.5. Electrolyte leakage

The leaf tissues after NaCl treatments taken and immersed in a known volume of water and initial electrical conductivity (EC) was measured by conductivity meter (Elico, CM180, Hyderabad, India). Subsequently leaf tissues were boiled at 100° C for 20 min and final electrical conductivity was measured (Babitha et al., 2015b). The electrolyte leakage was calculated using the following formula:

$$EC\ (\%) = (IEC/FEC) \times 100$$

where, IEC = initial EC, FEC = final EC and expressed in micro siemens (μS).

2.6. Evans blue assay

The roots samples from hydroponically grown NaCl treated plants were immersed in Evans blue solution (2 μm Evans blue dye dissolved in 0.1% CaCl2, pH-5.5) (Sigma, Bangalore, India) for 30 min. The stained roots were washed with 0.1% CaCl2 (El-Sayed et al., 1995) and observed under microscope and images were taken under bright field microscope. Further to quantify the Evans blue taken up by the roots, 100 mg washed roots were ground with 500 μl of 1% SDS. The extract was centrifuged at 8000 g for 10 min and the supernatant was collected and OD at 600 nm was quantified using spectrophotometer.

2.7. Schiff's test

The roots from hydroponically grown NaCl treated plants were excised and immediately soaked in Schiff's reagent (Himedia, Bangalore, India) for about 30 min. The roots were washed and root tips were observed for pink colouration under bright field microscope as a measure of reactive aldehydes.

2.8. Quantification of Na^+content in root and shoot

The sodium content in root and shoot samples (200 mg of powdered sample) were quantified after digesting with di-acid mixture (5 ml) (10: 4: nitric acid: per chloric acid) using Inductively Coupled Plasma Optical Emission Spectrometry (ICPOES, Thermo Fisher). (Mohammed et al., 2010).

2.9. Quantification of MDA levels

The leaf tissue was homogenized in 5 ml of 10% (W/V) trichloroacetic acid (HiMedia, Nasik, Maharashtra) and 0.25% of thiobarbituric acid and centrifuged at 12,000 rpm for 15 min at room temperature. The supernatant was mixed with an equal amount of thiobarbituric acid [0.5% in 20% (W/V) trichloroacetic acid] (Sigma aldrich, Bangalore, India). The mixture was boiled for 25 min at 100° C. Absorbance of the supernatant was measured at 532 nm and 600 nm and corrected for nonspecific turbidity by subtracting the absorbance at A600 (Loreto and Velikova, 2001). The standard MDA (Sigma Aldrich, Bangalore, India) was used to develop the standard graph.

2.10. Methylglyoxal levels

MG was quantified in the seedlings and leaf tissues exposed to salinity stress according to Yadav et al. (2005). The plant tissue (100 mg) was ground in a 2 ml of distilled water and centrifuged at 8000 g for 10 min at 4° C and supernatant was collected. To quantify the MG content, 250 μl of 7.2 mM 1,2- diamino benzene (1,2-phenylenediamine), 100 μl of 5 M per chloric acid and 650 μl of the neutralized supernatant treated with activated charcoal were mixed together. The absorbance was read at 336 nm using spectrophotometer (Spectra max plus-384, Spence Biotech pvt ltd, Bangalore).

2.11. Estimation of proline

The proline content was estimated by the method described by Bates et al. (1973). A 5% homogenate from 100 mg lyophilized leaf sample was prepared with 3% aq. Sulfosalicylic acid using mortar and pestle and centrifuged at 12,000 rpm at 4° C for 15 min. The supernatant (2 ml) was collected and 2 ml of glacial acetic acid and 2 ml of acid ninhydrin reagent was added. The reaction mixture was incubated in a boiling water bath for 60 min and then cooled on ice. After cooling, 4 ml of toluene was added and incubated at room temperature for 30 min. Tubes were then shaken for 15 s and allowed to stand for 10 min for phase separation. The upper phase was separated and absorbance was measured at 520 nm using spectrophotometer using toluene as blank. Concentration of proline was calculated using standard curve from proline (Himedia, Bangalore, India) and expressed as μmoles/g DW (Vemanna et al., 2015).

2.12. Estimation of reduced glutathione (GSH)

A 5% homogenate from 100 mg lyophilized leaf was prepared in 6% (w/v) TCA. The homogenate was centrifuged at 12,000 rpm for 5 min at 4 °C. Supernatant was made up to 2 ml and used for the estimation of reduced glutathione. An aliquote of 0.1 ml of sample was made upto 1.0 ml with sodium phosphate buffer (pH 8.0) followed by addition of 2 ml of freshly prepared 5,5'- dithiobis (2-nitrobenzoic acid) [DTNB] reagent. After 10 min, the absorbance was recorded at 412 nm and the concentration of total glutathione was calculated using standard curve for GSH (Sigma Aldrich, Bangalore, India). The concentrations of reduced and oxidized glutathione are expressed as nmoles/g (Griffith, 1980).

2.13. Measurement of enzyme activities

To measure enzymatic activities leaf tissues was taken from the 300 mM NaCl treated to 25 day-old-plants. After 15 days of treatment from the 100 mg of lyophilized leaf samples total protien was extracted in 1.5 ml ice cold 0.1 M potassium phosphate buffer (pH 7.5) containing 1.0 mM EDTA, 0.1 mM Triton X-100 and 2% Polyvinyl pyrrolidone (PVP) using pre-chilled mortar and pestle. The homogenate was transferred to microfuge tube and centrifuged at 12,000 rpm for 20 min at 4 °C. The supernatant was used for measurement of the enzyme activity. The protein content of the enzyme extract was determined by bradford reagent (Broadford, 1976) and the specific activity is expressed as units/mg protein.

2.13.1. Ascorbate peroxidase activity

The reaction mixture contained 50 mM phosphate buffer (pH 7), 1 mM ascorbic acid, 1% triton X-100, 2.5 mM H_2O_2 and 100 μl of the enzyme source in a final volume of 3.0 ml. The reaction was initiated by addition of enzyme source and the decrease in absorbance was measured at 290 nm. The enzyme activity was calculated using the molar extinction co-efficient of ascorbate ($\epsilon = 2.8\ mM^{-1}\ cm^{-1}$, at 290 nm). The specific activity is expressed as units/mg protein (Nakano and Asada, 1981).

2.13.2. Glutathione reductase activity

The reaction mixture containing 0.1 M Tris buffer (pH 7.8), 2 mM

EDTA, 50 μM NADPH, 0.5 mM GSSG and 0.3 ml of enzyme source in reaction volume of 3.0 ml. The reaction initiated by addition of NADPH and the decrease in absorbance was measured at 340 nm. The enzyme activity was calculated using the molar extinction coefficient of NADPH (€ = 6.022 $mmol^{-1}$ cm^{-1}, at 340 nm) (Foyer and Halliwell, 1976).

2.13.3. Pyrroline-5-carboxylate synthetase activity

The reaction mixture containing 0.2 ml of 0.1 M Tris-HCl buffer (pH 7.2), 25 mM $MgCl_2$, 75 mM sodium glutamate and 5 mM ATP in the final volume of 3.0 ml. reaction was initiated by addition of 120 μl of 10 mM NADH and the activity was measured as the rate of decrease in absorbance at 340 nm. The enzyme activity was calculated using the molar extinction coefficient of NADH, 6.22 $mmol^{-1}$ cm^{-1} at 340 nm (Garcia-Rios et al., 1997).

2.14. Statistical analysis

The data obtained in different experimental results was analysed using two-way analysis of variance (ANOVA) as per the procedure given by Fischer (1960). Data points with different lowercase letters indicate significant differences ($P < 0.05$) between transgenic lines and wild type as determined by Tukey's HSD test.

3. Results

3.1. AKR1 overexpressing transgenic tobacco plants showed higher survival and tolerance to salinity stress

The tobacco transgenic lines expressing *AKR1* (Fig. 1a) from our earlier studies were used and three homozygous lines that showed relatively higher levels of mRNA expression levels (Fig. 1b) were characterized for salinity stress tolerance. The seedlings were exposed to 150 mM and 300 mM of NaCl under two different conditions. In one set of experiment, seedlings growth response was observed on half MS media supplemented with NaCl and other set seedling growth was assessed in soilrite in small pots irrigated with NaCl. Seedling survival and growth in transgenics was less affected on MS agar media supplemented with NaCl compared to wild type. In soilrite, the survival and growth of the seedlings was affected under stress both in wild type and transgenics in a dose dependent manner. However, the *AKR1* expressing seedlings showed enhanced tolerance at both the salinity concentrations (Fig. 1c and d). More distinct differences were observed between transgenic and wild type seedlings when exposed to 300 mM NaCl. The transgenic seedlings showed 95% of survival on 150 mM NaCl and wildtype seedlings showed 60% survival. At 300 mM NaCl the wildtype seedlings showed chlorosis and stunted phenotype and less survival. Further, whereas the transgenics continued to grow and showed 75% survival (Fig. 1e).

Salinity stress response was also studied in excised lead discs. Leaf discs from 50-day-old transgenic and wild type tobacco plants were floated on 150 and 300 mM NaCl for 48 h and transferred to water for 2 days. The NaCl induced H_2O_2 in transgenic plants was significantly low compared to wild type plants (Fig. 2a and b). Similarly lipid peroxidation was significantly lower in transgenic plants compared to wild type plants as indicated by reactive carbonyl MDA levels (Fig. 2c). Even accumulation of MG was low in transgenic plants (Fig. 2d). Salinity-induced degradation of chlorophyll was less in *AKR1* overexpressing lines compared to wild

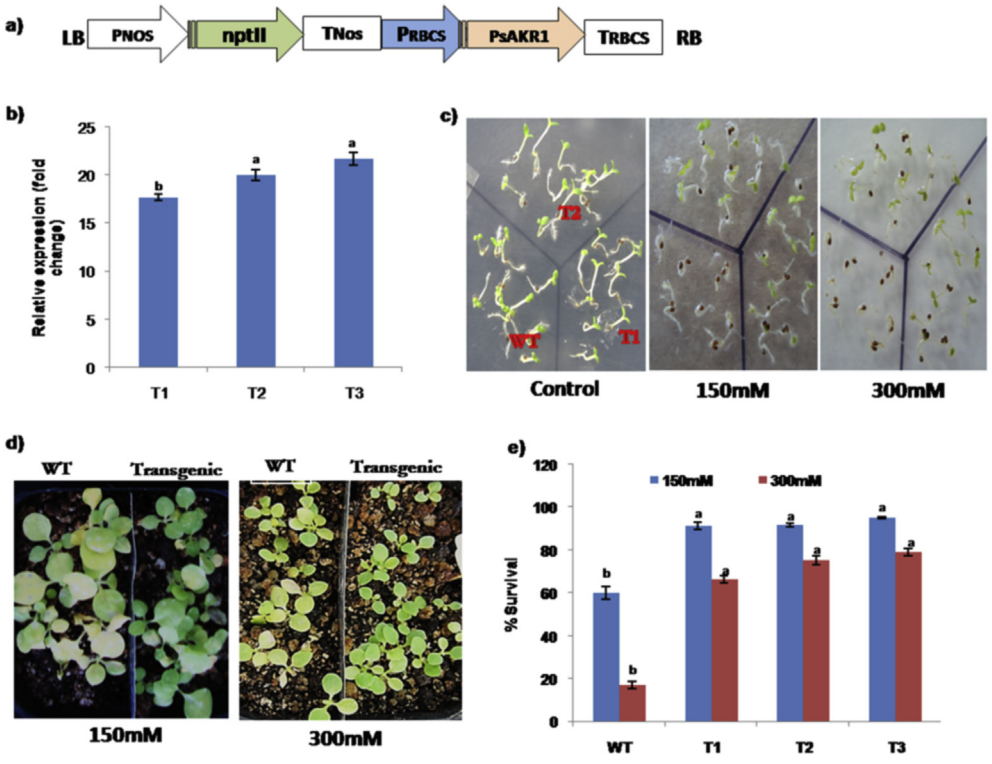

Fig. 1. Transgenic AKR1 expressing tobacco seedlings showed tolerance to NaCl stress, a) Schematic representation of gene construct with *PsAKR1*, b) Relative expression levels of AKR1 transcripts in transgenics, c) Representative photograph of transgenic and wild type seedlings on MS agar media supplemented with 150 mM and 300 mM NaCl stress, d) Response of transgenic and wild type seedlings grown on soilrite irrigated with 150 and 300 mM NaCl, photographs were taken after 8 days of NaCl application, e) Survival percentage of transgenics and wild type seedling, Error bars indicate mean values from three biological replicates that are having 25 seeds each. Different letters above the error bar indicate differences in significance. Two way ANOVA with $P < 0.05$ with Tukey's honest significant differences (HSD) means separation test ($a = 0.05$) among transgenic and wild type were conducted.

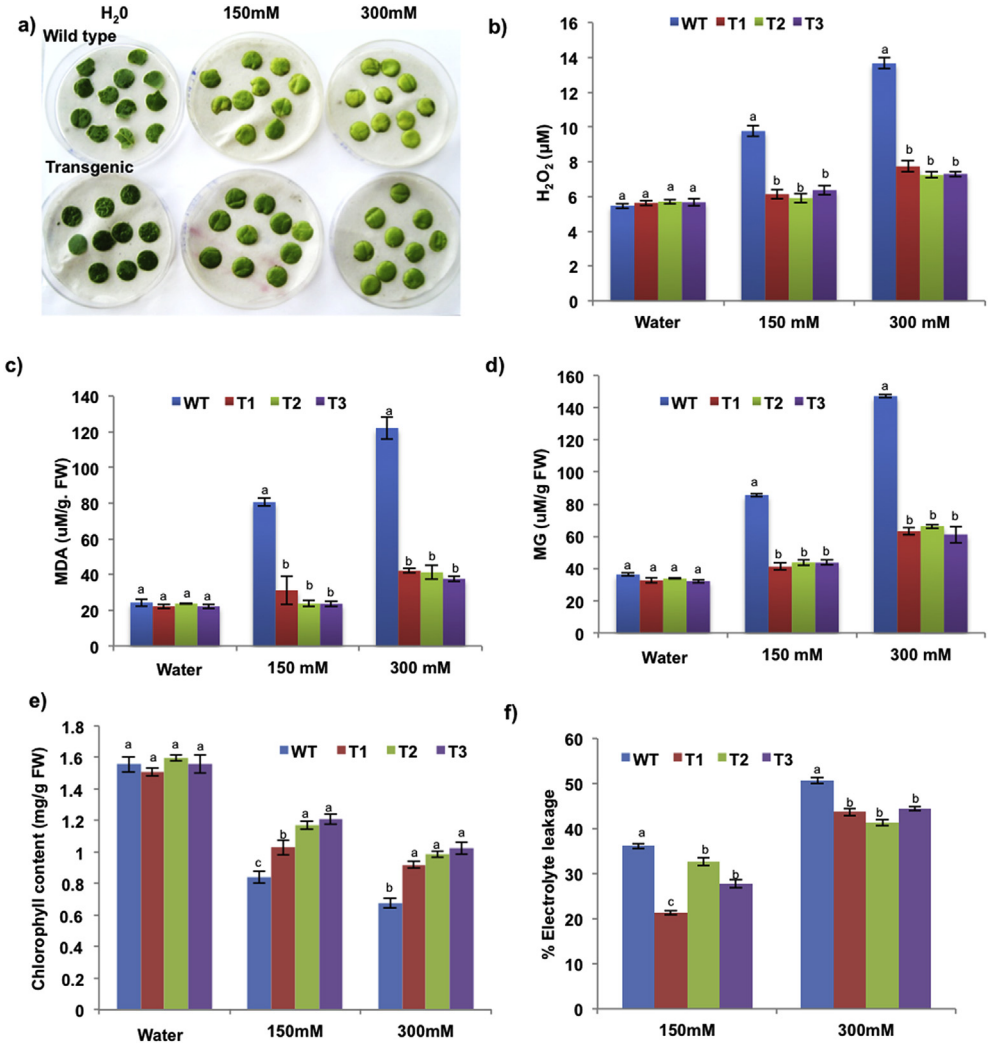

Fig. 2. Transgenic plants showed improved tolerance to NaCl stress in excised lead disc assay. a) Photograph showing the extent chlorophyll content in transgenic and wild type leaf discs treated with 150 and 300 mM of NaCl after 4days of stress. At the end of stress different biochemical parameters b) H_2O_2 c) Malondialdehyde d) methyl glyoxal (MG), e) Total chlorophyll content and f) electrolyte leakage was determined in NaCl treated leaf discs. WT-wild type, T1-T3- transgenic plants. Error bar indicate mean values from three biological replicates that are having 10 leaf discs. Different letters above the error bar indicate differences in significance. Two way ANOVA with $P < 0.05$ with Tukey's honest significant differences (HSD) means separation test ($a = 0.05$) among transgenic and wild type were conducted.

type plants (Fig. 2e). The membrane stability in wild type plants was highly affected as evidenced by higher electrolyte leakage (Fig. 2f).

3.2. Overexpression of PsAKR1 rescued tobacco seedlings from methylglyoxal effect

In salinity stress, accumulation of methylglyoxal was less in *AKR1* expressing transgenics (Fig. 2d), therefore to assess whether overexpression of *AKR1* in tobacco rescues the seedlings from MG induced effect, both the transgenic and wild type seedlings were germinated on 10 mM MG. MG did not affect the growth of transgenic seedlings (Fig. 3a) and showed 100% survival, whereas only 10% of seedlings survived in wild type (Fig. 3b). The transgenic leaf discs floated on 10 mM MG also showed 3-fold lower MG levels than the wild type plants (Fig. 3c and d). Further the H_2O_2 levels was also significantly less in transgenics compared to wild type plants (Fig. 3e). Even the chlorophyll content was significantly higher in transgenic plants (Fig. 3f).

3.3. AKR1 expression in transgenic plants detoxify reactive carbonyl compounds under NaCl stress

The transgenic and wild type plants grown hydroponically were less affected by salinity stress than wild type plants (Fig. 4a and Supplementary Fig. S1). The transgenic plants maintained higher root growth compared to wild type plants under NaCl stress (Fig. 4b). The effect of NaCl in generation of reactive aldehydes in roots was quantified by Schiff's reagent staining. The transgenic roots showed very less staining of schiff's reagent whereas wild type showed dark staining indicating higher levels of RCCs (Fig. 4c). Root plasma membrane stability as assessed by Evan's blue staining was very less in transgenics compared to wild type (Fig. 4 d & e). The tolerance was also reflected with higher fresh weight in transgenic plants compared to wild type plants at the end of stress period (Fig. 4f). The Na^+ concentration was less in transgenics compared to wild type plants in both roots and leaves (Fig. 4g).

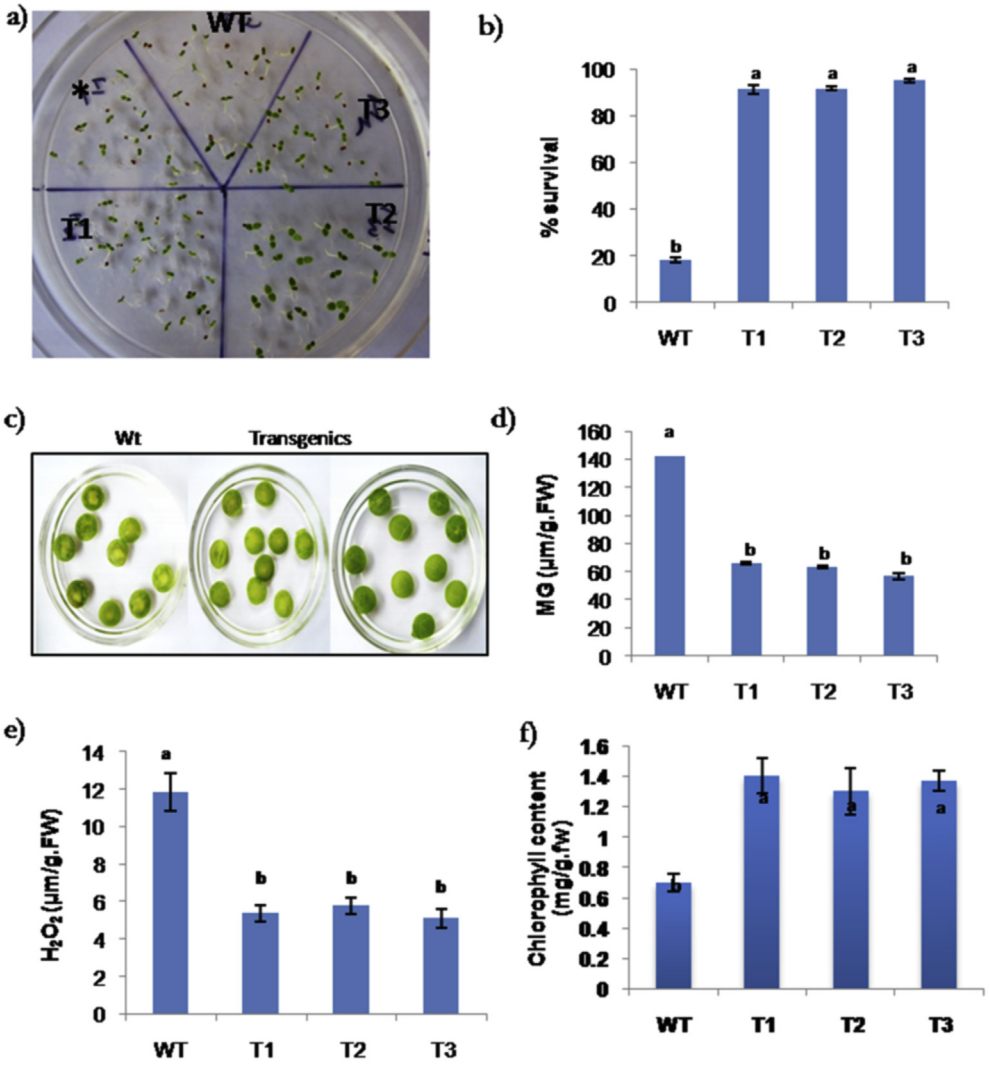

Fig. 3. Effect of methylglyoxal (MG) on *AKR1* expressing tobacco transgenic seedlings. a) Response of wild type and transgenic seedlings to 10 mM MG, b) Seedling survival percentage on MG treatment, n = 50, c) Leaf disc assay on 10 mM MG, Photographs taken after 3 days of recovery response and levels of d) MG, e) H_2O_2 and f) Chlorophyll content was determined. WT-wild type, T1-T3- transgenic plants. N = 5. Experiments were repeated 3 times with minimum of 3 biological replicates from each transgenic lines. Two way ANOVA with $P < 0.05$ with Tukey's honest significant differences (HSD) means separation test (a = 0.05) among transgenic and wild type were conducted. Different letters above the error bar indicate differences in significance.

3.4. AKR1 protects antioxidant enzymes and improve proline levels under salinity stress

To assess the effect of salinity on *AKR1* overexpressing plants at whole plant level, the plants were irrigated with a mixture of salts [NaCl (0.5 M), $CaCl_2$ (0.150 M) $MgCl_2$ (0.08 M) and Na_2SO_4 (0.1 M)] to simulate natural field salinity stress condition. Salt stress of 8 d S/m was imposed when the plants were 45-day-old. The wild type plants showed significant growth retardation, in contrast transgenic plants were less affected with better growth (Fig. 5a). At 60 days, transgenic plants showed 90% of survival and wild type plants showed less than 35% survival under NaCl stress. Amongst the wild type plants some of them showed complete senescence and few plants showed less senescence and completed life cycle. The biometric data taken in some of the selected wild type plants showed reduced root growth, increased MG and reduced biomass under stress. Transgenic plants maintained higher root growth, root density, root weight and total dry matter (TDM) compared to wild type plants (Fig. 5b and c), even the MG levels was lower than the wild type plants (Fig. 5d and e). Further in another set of plants that are allowed till the seed set at harvest, few critical growth parameters such as plant height, time taken for flowering, seed weight and root growth was assessed in both wild type and transgenic plants till harvest. Only 15% of wild type plants survived and in these plants growth of wild type plants was severely affected by continuous exposure to salinity stress whereas transgenic plants were less affected (Table 1). Due to stress, wild type plants showed early flowering, however transgenics showed less advance in flowering compared to non stress control plants (Table 1).

3.5. AKR1 transgenics showed improved activity of metabolic enzymes under soil salinity conditions

Under stress conditions formation of protien carbonyls is predominantly due to RCCs. Therefore, RCCs may affect the function of many enzymes. In view of this the effect of salinity stress on the enzymatic activity of few selected enzymes was studied. The 25-day-old *AKR1* overexpressing transgenic and wild type plants were exposed to 300 mM NaCl. From these plants relative activity of a few important enzymes that are known to upregulate under stress

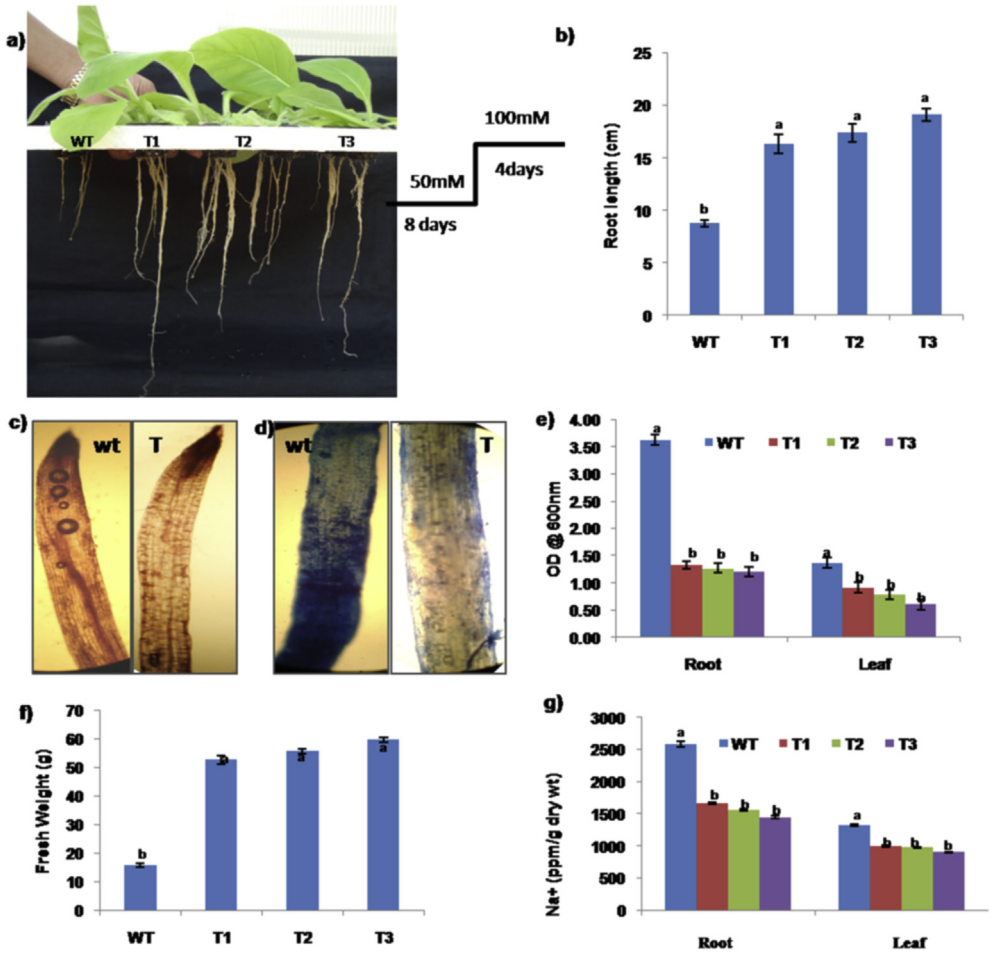

Fig. 4. Transgenic plants expressing *AKR1* detoxify reactive carbonyl compounds under NaCl stress. Transgenic and wild type plants were grown hydroponically with NaCl containing Hoagland solution. a) Phenotypic response b) Root length, c) RCCs detection using Schiff's reagent, d) Evans blue stain taken up by the roots exposed to NaCl stress, e) Levels of evans blue stain in roots and leaf tissue treated with NaCl, f) Fresh weight of seedlings, g) Na^+ content in leaf and roots. n = 10, experiments was repeated at least 3 times and the values are mean of all the three times. Two way ANOVA with $P < 0.05$ with Tukey's honest significant differences (HSD) means separation test (a = 0.05) among transgenic and wild type were conducted.

such as glutathione reductase (GR), ascorbate peroxidase (APX) and delta 1-pyrroline-5- carboxylate synthase (P5CS) were studied. The transgenic plants had higher levels of glutathione under stress (Fig. 6a). The higher levels of GSH in transgenic plants could be due to higher activity of GR enzyme (Fig. 6b). The transgenic plants also showed relatively higher APX enzyme activity at 300 mM NaCl stress (Fig. 6c). The activity of these enzymes was less in wild type plants compared to transgenic plants. The proline levels in transgenic plants was significantly higher compared to wild type (Fig. 6d) under saline stress. This increase in proline is due to the increased activity of delta 1-pyrroline-5- carboxylate synthase (P5CS) that is involved in biosynthesis of proline (Fig. 6e). In *AKR1* overexpressing plants these enzyme activity was higher and not affected and hence better scavenging of ROS and RCCs that are generated under salinity stress.

4. Discussion

Several cytotoxic compounds including ROS and RCCs generated under salinity stress are potentially harmful for cell integrity (Asada, 1999). Under NaCl stress the generation of RCCs like MG, MDA, HNE etc. Have been showed in different crops (Yadav et al., 2005; Mano, 2012). The Methylglyoxal (MG), glyoxal (GLY), 3-deoxyglucosone (3-DG), MDA, HNE, and acrolein are formed by Schiff's bases, amodari re-arrangement of glucose and lipid peroxidation end products. These molecules react with several proteins that are involved in cellular metabolic processes by modifying the side chains of aminoacids by forming protein aggregates (Negm, 1986; Mundree et al., 2000). The glyoxalase I and glyoxalase II reduces MG into lactate using glutathione as electron donor and overexpression of these two enzymes in tobacco showed enhanced tolerance to salinity and MG (Singla-Pareek et al., 2003; Yadav et al., 2005). The other group of enzymes aldo-keto reductases have shown to detoxify broad substrate RCCs. Overexpression of *Aldo-keto reductase (OsAKR1)* from rice, *MsALR1* in tobacco showed tolerance by reducing 4-HNE and MG levels (Turcozy et al., 2011; Hegedüs et al., 2004; Oberschall et al., 2000; Hideg et al., 2003). Role of AKR4C10 and AKR4C11 in reducing sugar derived RCCs have been demonstrated in Arabidopsis in response to light and CO_2 (Saito et al., 2013). Our previous studies have shown that overexpression of *PsAKR1, OsAKR1* in tobacco showed improved tolerance to carbonyl molecule glyphosate (Vemanna et al., 2016). In this present study, higher levels of RCCs were detected in wild type tobacco plants under NaCl stress and overexpression of *PsAKR1* in transgenic plants showed reduced RCCs such as MG, MDA levels and improved tolerance to salinity stress. The *PsAKR1* overexpressing tobacco plants showed reduced accumulation of ROS with reduced electrolyte leakage and higher chlorophyll content

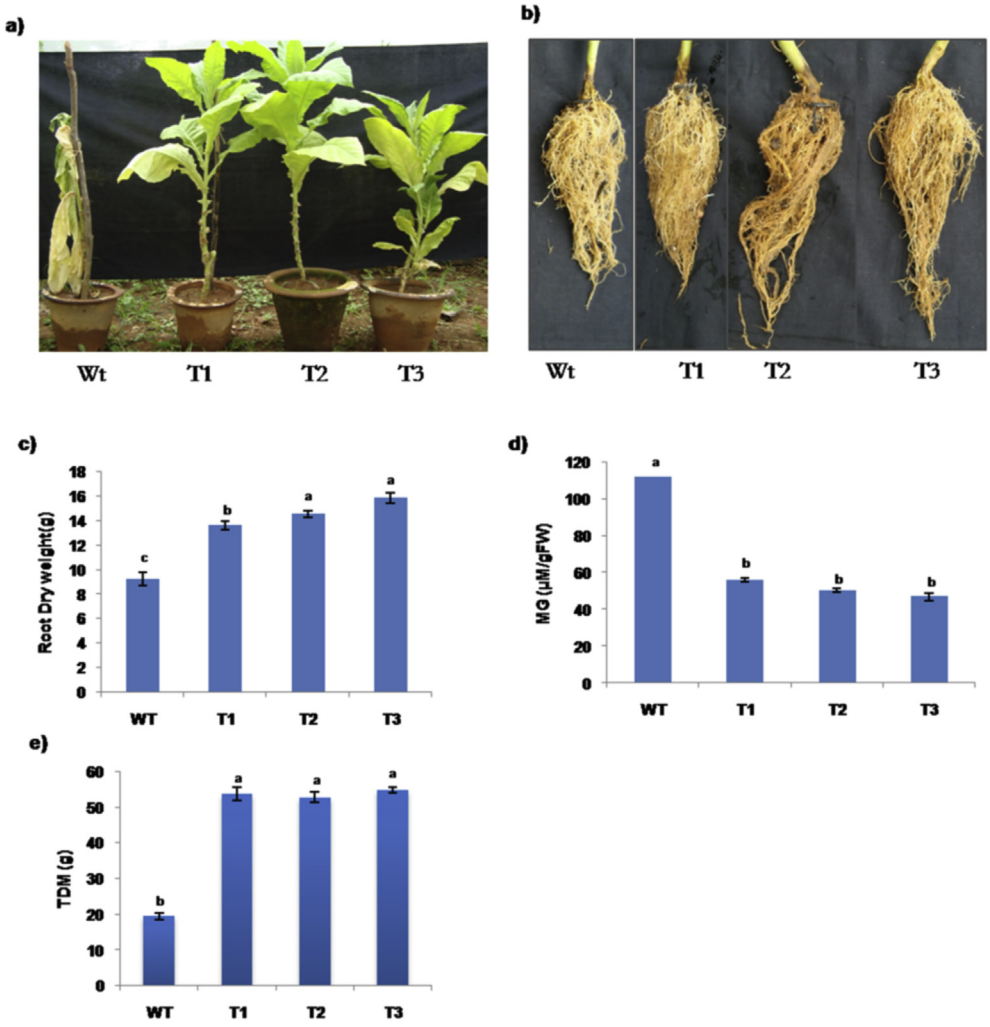

Fig. 5. Response of tobacco transgenic and wild type plants to salinity stress at whole plant level. 45 days-old transgenic and wild type plants exposed to salinity stress of 8 dS/m, one set of plants were assessed at 60th day for a) Phenotypic response, b) Variation in root growth, c) Root dry weight, d) Methylglyoxal levels and e) Total dry matter (biomass). The plants were harvested and both root and shoot was dried in oven and total biomass was measured. n = 5, minimum 5 plants were used for root growth studies from each line. Two way ANOVA with $P < 0.05$ with Tukey's honest significant differences (HSD) means separation test ($a = 0.05$) among transgenic and wild type were conducted.

Table 1
Comparison of various growth parameters of the wild type and AKR1 expressing transgenic tobacco plants grown salinity stress.

Parameter	Non-treated control plants				Salinity stress			
	WT	T1	T2	T3	[a]WT	T1	T2	T3
Plant Height (cm)	115 ± 5	124 ± 4	120 ± 3	130 ± 5	65 + 10	105 ± 2	108 ± 3	98 ± 2
Flowering time (Days)	132 ± 2	138 ± 2	132 ± 3	134 ± 2	105 + 4	120 ± 2	121 ± 2	118 ± 1
Seed weight per pod (mg)	128 ± 4	140 ± 2	134 ± 3	136 ± 3	68 + 6	115 ± 1	113 ± 1	118 ± 1
Root growth Inhibition (%)	0	0	0	0	60	15	20	18

Experiments were repeated at least three times, and the values represent means of different samples. In all cases, *n=5*.
[a] Only 15% of wild type plants survived and the biometric observations were recorded from those survived seed set and harvested plants.

under salinity stress. The improved tolerance to MG signifies the tolerance is due to detoxification of RCCs by *AKR1* overexpression. Similarly overexpression of AtAKR4C9 and MsALR in barley showed reduced RCCs (Csaba et al., 2016).

The RCCs are known to affect several metabolic processes predominantly forming adducts with protiens or by formation of protien carbonyls and hence affect function of many enzymes. Acrolein an RCC significantly reduced the activity of the calvin cycle enzymes glyceraldehyde phosphate dehydrogenase (GAPDH) Fbpase, Aldolase, phosphoribulo kinase (PRK) etc was significantly reduced and also showed reduced glutathione (Mano et al., 2009; Yin et al., 2010). We provide experimental evidences that in the AKR expressing transgenic lines, the activity of few stress induced enzymes in transgenics is higher compared to wild type. The glutathione reductase enzyme activity is significantly increased in transgenic plants that resulted in increased accumulation of glutathione levels. The higher levels of antioxidant glutathione in *PsAKR1* expressing transgenics may protect cellular metabolic process against a range of cytotoxic compounds including heavy metals (Dixon et al., 1998). Though the glutathione levels increases

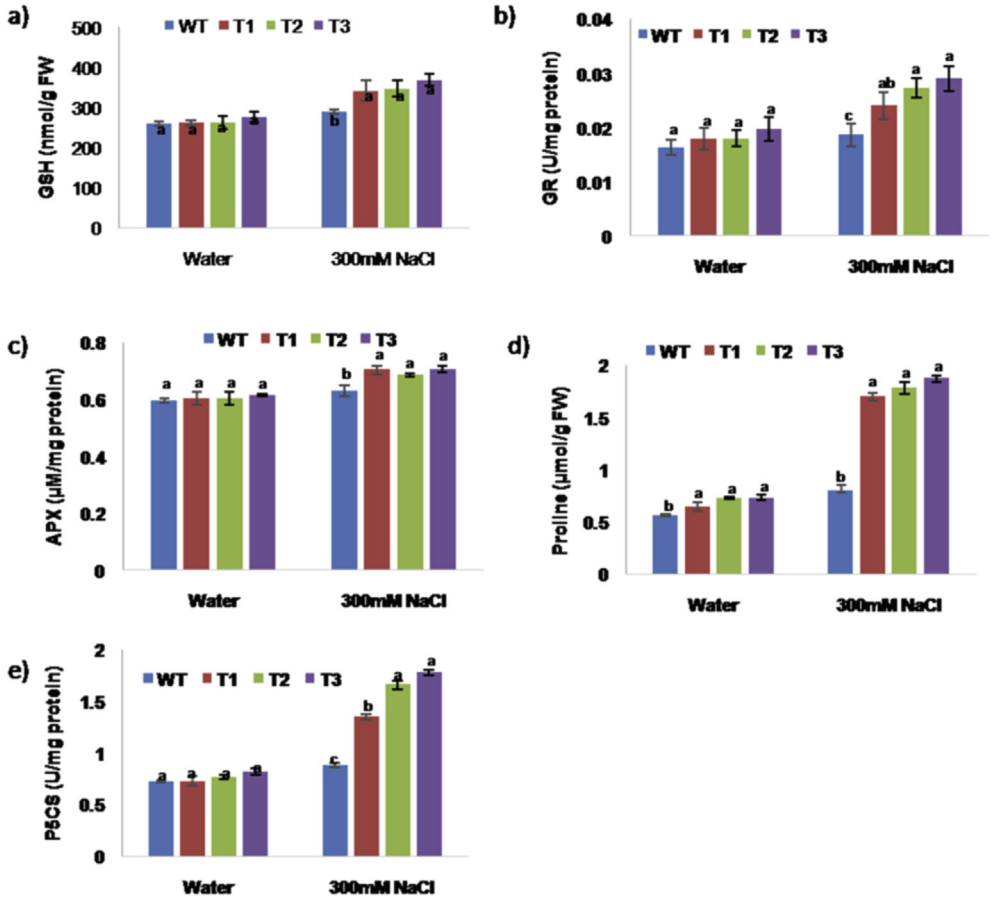

Fig. 6. AKR1 protects enzymes and maintain higher activity under NaCl stress. The 25 day-old transgenic and wild type plants exposed to NaCl (300 mM) stress and after 15 days the enzyme activity was measured from leaf tissues. a) Glutathione levels, b) Glutathione reductase enzyme activity, c) Ascorbate peroxidase activity, d) Proline levels and e) delta 1-pyrroline-5- carboxylate synthase (P5CS) enzyme activity in NaCl treated and water control tissues. n = 3 all the measurements were done atleast 3 times with 3 biological replications. Two way ANOVA with $P < 0.05$ with Tukey's honest significant differences (HSD) means separation test ($a = 0.05$) among transgenic and wild type were conducted.

under stress (Celik and Atak, 2012), the marginal increase in glutathione in wild type plants could be attributed to RCCs associated inactivation of glutathione reductase. Carbonylation of several enzymes like GST and SOD enzymes as well as other calvin cycle associated proteins have been reported in apoptotic response to oxidative stress (Magi et al., 2004).

The AKR1 expressing transgenic plants also showed increased activity of APX under stress compared to wild type plants suggesting less affected due to protien carbonylation by salinity stress. The protein carbonylation of antioxidant enzymes superoxide dismutase (MnSOD), Peroxiredoxin-1,-3, and -5, Glutathione peroxidase 1, Glutathione S-transferase M1/A4, Aldehyde dehydrogenase under oxidative stress has been reported (Brigitte et al., 2013). The *AKR1* expressing transgenics also showed significantly reduced levels of H_2O_2. The reduced levels of H_2O_2 levels could be due to higher activity of APX, glutathione and other enzymes that may be protected from RCC modifications on proteins. The *AKR1* expressing tobacco transgenics maintained higher levels of proline under saline conditions that suggests improved osmotic homeostasis because of higher activity of biosynthetic enzyme P5CS. Although wild type plants showed slightly increased levels of proline but the transgenic plants had significantly higher levels of proline (Fig. 6d) that can play role as osmolyte to protect from oxidative damage. The increased accumulation of proline was also evident is Turkish tobacco plants exposed to higher NaCl levels (Celik and Atak, 2012). The accumulation of proline is one of the adaptive strategy in plants and many studies demonstrat the role of proline in improving stress adaptation in crop plants (Hare et al., 1999; Savouré et al., 1997).

Both wild type and transgenic plants accumulated higher concentrations of Na^+ in roots compared to shoot. However, in wild type plants Na^+ accumulation was higher in both in root and shoot compared to transgenic plants. Similar observations were reported in transgenic tobacco plants expressing glyoxalase I and II genes (Singla-Pareek et al., 2003). Our results demonstrates that the transgenic plants also showed tolerance by protecting the cellular enzymes and also Na+ homeostasis. However, the role of AKR1 in regulating the Na^+ uptake is not clear. The increased enzymatic activities in AKR1 transgenics can protect the functional proteins and plasma membrane from ROS and RCCs and subsequently reduces the protein carbonylation and formation of ALEs and AGEs resulting in improved tolerance to salinity stress.

Author contributions

R.S·V and U.K.M concaved the project and R.S·V designed and executed the experiments. B.K.C did hydroponic experiments. J.K.S and A.R.V estimated antioxidant and scavenging enzyme activity. R.S.V and B·K·C analysed the data and R.S·V, S·S·K and U.K.M wrote manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2017.02.012.

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