Journal of Plant Growth Regulation
https://doi.org/10.1007/s00344-020-10072-w

Enzymatic and Non-enzymatic Detoxification of Reactive Carbonyl Compounds Improves the Oxidative Stress Tolerance in Cucumber, Tobacco and Rice Seedlings

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Received: 15 June 2019 / Accepted: 14 January 2020

Abstract

Detoxification of reactive carbonyl compounds (RCC) is crucial to sustain cellular activity to improve plant growth and development. Seedling growth is highly affected by accumulation of RCC under stress. We report non-enzymatic, enzymatic mechanisms of detoxification of RCC in the cucumber, tobacco and rice seedling systems exposed to glucose, NaCl, methyl viologen (MV) induced oxidative stress. The cucumber seedlings exposed to carbonyl stress had higher levels of malondialdehyde (MDA), protein carbonyls (PCs) and advanced glycation end-product *N*-carboxymethyl-lysine (AGE-CML) that negatively affected the seedling growth. The overexpression of enzyme encoding aldo-keto reductase-1 (*AKR1*) in tobacco and rice showed detoxification of RCC, MDA and methylglyoxal (MG) with improved seedling growth under glucose, NaCl and MV-induced oxidative stress. Further, small molecules like acetylsalicylic acid (ASA), aminoguanidine (AG), carnosine (Car), curcumin (Cur) and pyridoxamine (PM) showed detoxification of RCC non-enzymatically and rescued the cucumber seedling growth from glucose, NaCl and MV-stress. In autotrophically grown rice seedlings these molecules substantially improved seedling growth under MV-induced oxidative stress. Seedlings treated with the small molecules sustained higher guaiacol peroxidase (GPX) enzyme activity signifying the role of small molecules in reducing carbonyl stress-induced protein inactivation and AGE-CML protein modifications. The results showed that besides enzymatic detoxification of RCC, the small molecules also could reduce cytotoxic effect of RCC under stress. The study demonstrates that small molecules are attractive compounds to improve the seedling growth under stress conditions.

Keywords Small molecules · Reactive carbonyl scavengers · Seed germination · Plant growth · Oxidative stress

Introduction

Plants being sessile are exposed to diverse stresses that limit the growth, development and affect potential yields. Oxidative stress affects the plant growth severely is ubiquitous to diverse abiotic factors. The reactive oxygen species (ROS) generated under abiotic stresses when light intensity is excess than the required for CO_2 assimilation (Dat et al. 2000). ROS reacts with proteins, lipids and other secondary molecules and generates highly oxidative and electrophilic compounds called reactive carbonyl compounds (RCC). These reactive carbonyls diffuse throughout the chloroplasts and form adducts with stoma and thylakoid proteins (Yamauchi et al. 2011). These RCC are deleterious to the cell, are more stable than ROS and have longer half-life ranging from minutes to hours (Pamlona 2011). RCC are mainly produced by auto-oxidation or enzymatic cleavage

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00344-020-10072-w) contains supplementary material, which is available to authorized users.

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Published online: 27 January 2020

of lipids by lipid peroxidation mediated processes and generates RCC such as α-β unsaturated aldehydes like acrolein, crotonaldehyde, hydroxynonenol (HNE) and keto-aldehydes like 4-oxo-trans-2-nonenal (ONE) (Uchida et al. 1998; Uchida 2000; Takabe et al. 2001). Also the non-enzymatic process of RCC production through glucose reacting with proteins called glycation produces RCC such as glyoxal, methylglyoxal (MG), glucosone, 3-deoxyglucosone and acrolein (Uchida et al. 1998). Many of these molecules are cytotoxic and lead to carbonyl stress. RCC generated due to lipoxidation and glycoxidation reaction eventually lead to the formation of advanced glycation/advanced lipoxidation end products (AGEs/ALEs). RCC mainly damage and degrade proteins, forms adducts and also cause phospholipid damage that leads to activation or alteration in signaling pathways. The RCC bring in a functional change in protein with altered properties such as charge, hydrophobicity, elasticity, solubility, the formation of cross-links and aggregates (Pamplona 2011). The accumulation of MDA, HNE, etc. under stress affected many photosynthetic and Calvin cycle enzymes that affect growth in tobacco (Mano 2012). The rice genotypes exposed to oxidative stress have accumulated higher levels of RCC and caused loss of seed vigor (Nisarga et al. 2017). To improve the cellular tolerance of the plants under stress, it is crucial to have broad-spectrum mechanisms.

The broad-spectrum aldo-keto reductases (AKRs) had shown the potentiality to detoxify RCC. Overexpression of *PsAKR1* detoxifies malondialdehyde (MDA), methylglyoxal (MG) under salinity and improved seed viability in rice and tobacco (Vemanna et al. 2017; Nisarga et al. 2017). Similarly, the overexpression of *AKR4C9* from *Arabidopsis* also improved tolerance to oxidative stress linked to the detoxification of reactive Aldehydes (Simpson et al. 2009). Overexpression of *MsALR* and AKR in tobacco have been shown to detoxify cytotoxic RCC and increase tolerance to methyl viologen (MV), heavy metal, UV-B irradiation and also drought and salinity (Oberschall et al. 2000; Hideg et al. 2003; Hegedusab et al. 2004). A similar response was also seen in tobacco transgenics expressing *ZmALDH22A1* to diverse abiotic stresses like NaCl, mannitol and even ABA (Huang et al. 2008). Another group of RCC detoxifying enzymes are aldehyde dehydrogenases (ALDH), overexpression of *ZmALDH22A* in tobacco and *ALDH3I1*, *ALDH3H1* and *ALDH7B4* in *Arabidopsis* improved tolerance to diverse abiotic stresses by substantially reducing the lipid peroxidation process (Kotchoni et al. 2006).

Besides the enzymatic scavenging options, non-enzymatic RCC scavenging mechanisms are also effective and several natural molecules including antioxidants, polyamines, phenols, ascorbic acid, glutathione, α-tocopherol and several synthetic molecules such as metformin, pentoxyfylline, aminosalicylic acid have also been demonstrated in vitro and in vivo studies to prevent the formation of AGEs and ALEs (Rahbar et al. 2000; Chinchansure et al. 2015; Younus and Anwar 2016; Abbas et al. 2016). In addition, synthetic molecules such as acetylsalicylic acid (ASA), aminoguanidine (AG) and natural compounds such as carnosine (Car), curcumin (Cur) and pyridoxamine (PM) have been shown to detoxify RCC in animal studies (Kim et al. 2011; Boldyrev et al. 2013; Sadowska-Bartosz and Bartosz 2015; Younus and Anwar 2016). Synthetic and natural molecules can inhibit glycation process by interfering with the attachment of reducing sugars with amino groups of proteins, by inhibiting the late stage of glycation or by preventing Amadori product formation (Younus and Anwar 2016). ASA can block the attachment between reducing sugars and amino groups by acetylating free amino groups of a protein at the early stage of glycation process (Peng et al. 2008). AG inhibits the RCC and glycation products to prevent protein–protein and protein-lipid cross-linking under glucose and lipid peroxidation triggered in the diabetic milieu (Yavuz et al. 2002). Car, a dipeptide composed of beta-alanyl-L-histidine reacts with $O_2^{\cdot-}$ and $OH^{\cdot-}$ radicals and forms adduct with HNE and other RCC to prevent the glycation process (Rashid et al. 2007; Boldyrev et al. 2013). Cur acts as trappers for RCC compounds by reacting with methylgloxal (MG), 3-deoxyglucosone (3-DG), MDA and HNE and prevents association with proteins (Hu et al. 2016). Cur also suppresses the activity of the receptor for the advanced glycation end product (RAGE) to eliminate the effects of AGEs (Wolffenbuttel et al. 1998; Du et al. 2012; Larasati et al. 2018). PM is the B6 vitamin is a potent post-amadori AGE inhibitor. PM has multiple mechanism of action by blocking oxidation of the amadori intermediate, trapping of reactive carbonyl and dicarbonyl compounds derived from the amadori compounds (Voziyan and Hudson 2005). However, a substantial knowledge gap exists in using these small molecules in plants, because their mechanism in vivo has not been elucidated (Hong et al. 2016).

Germinating seedlings often experience moisture stress under receding soil moisture conditions in the field. Hence, improving seedling tolerance under stress has relevance in establishing proper crop stand. Under stress, oxidative stress-induced RCC affect seedling growth and survival. In an earlier study, we demonstrated that seedling vigor is highly affected by accumulation of RCC during seed storage in rice and tobacco (Nisarga et al. 2017). Therefore the major emphasis in the study was to quantify the oxidative stress-induced RCC and assess the importance of enzymatic and non-enzymatic detoxifying mechanisms to improve carbonyl stress tolerance. In this study, we show that transgenic plants overexpressing AKR1 showed RCC scavenging ability. Further, we demonstrate that novel natural and synthetic small molecules like ASA, AG, Car, Cur and PM showed improved seedling growth with a reduction in RCC, PCs and maintenance of enzymatic functions under stress conditions.

These molecules could be exploited in early seed germination to improve growth and productivity of crops.

Materials and Methods

Stress Imposition to Seedlings

To create carbonyl stress, the cucumber [cv. Guntur Local (Udayakumar et al. 1976)] seeds were surface sterilized with 0.4% sodium hypochloride (HiMedia, Nasik, Maharashtra) for 10 min then rinsed thrice with autoclaved sterilized distilled water and placed on a wet blotting paper in petri dishes (90 mm × 13 mm) for 24 h at 28 °C and 75% relative humidity in a dark growth chamber. Uniformly germinated seedlings were used for further experiments. Carbonyl stress was imposed by transferring the seedlings to different concentrations of glucose (2–8%) (HiMedia, Nasik, Maharashtra) NaCl (50–200 mM) (Sigma-Aldrich, Bangalore, India), MV (2–25 µM) (Sigma-Aldrich, Bangalore, India). Further cucumber seedlings were allowed 48 h and seedling growth was recorded. Further, based on initial experiments optimum concentration of 4% glucose, 150 mM NaCl and 20 µM MV was used to assess the seedling growth and RCC estimation. Minimum of 25 seedlings were treated with each stress inducers.

Rice Seedlings

Rice (Accession AC39020) seeds were germinated in sterile sand trays in greenhouse conditions with ¼th strength Hoagland nutrient solution (Hoagland 1950). 7-day-old rice seedlings of uniform size with intact root system were pretreated with different small molecules for 12 h. Subsequently, the seedlings were transferred to 5 µM MV and exposed to high light (600 µmol/m^2/s light intensity) for 16 h. Later, the seedlings were transferred to ¼th strength Hoagland solution for 48 h for recovery. The seedling growth was measured and leaves were used to check membrane integrity using Evan's blue assay (Preethi et al. 2017).

Tobacco and Rice Transgenic Plants

Tobacco (variety KST-19) transgenic lines expressing *PsAKR1*, *OsAKR1* and *OsALR1* genes were developed previously in our laboratory were used in this study. All the three AKR genes were constitutively expressed under ribulose 1,5-bisphosphate carboxylase (RBCS) promoter and terminator. Three independent transgenic lines from each construct were used for all the experiments. Rice transgenics expressing *PsAKR1* were developed using modified *in planta Agrobacterium*-mediated transformation method (Vemanna et al. 2016). The PCR positive transgenic lines with stable integration of *PsAKR1* were used in the present study.

Response of Yeast mutants: The mutants and the wild type strains were grown on YPD media (Vemanna et al. 2016) (HiMedia, Nasik, Maharashtra) with 3% glucose, 100 mM NaCl and 10 µM MV in Yeast extract-peptone-dextrose (YPD) medium and the phenotypes were observed after 72 h.

Crude Protein Bioassay with Cucumber Seedlings

To assess the seedling rescue by AKR protein from carbonyl stress by scavenging RCC, a cucumber seedling assay was designed. The bacterial cells expressing *PsAKR1*, *OsAKR1* and *OsALR1* under the bacterial expression system pET32a developed earlier in our laboratory (Vemanna et al. 2016) were used to generate surrogate AKR proteins. The efficacy of these AKR bacterial proteins against glucose was studied using cucumber seedling bioassay. Known amount of bacterial crude protein from *PsAKR1*, *OsAKR1* and *OsALR1* expressed in the bacteria was infiltrated into the cucumber seedlings under mild vacuum as described by (Zhang et al. 2017). Subsequently, these cucumber seedlings were incubated in 4 and 5% glucose for 48 h. Following which, the seedlings were transferred to petri dishes with water and recovery growth was recorded after 48 h.

Treatment of RCC Scavengers/Inhibitors

The effect of different small molecules [50 µM ASA, 10 µM AG, 10 µM Car, 1 µM Cur and 10 µM PM, (Sigma-Aldrich, Bangalore, India)] were assessed in the cucumber seedling bioassay system. Ten uniformly germinated 2-day-old seedlings were transferred to 4% glucose, 150 mM NaCl and 20 µM MV along with different small molecules. All the petri dishes were covered, sealed with parafilm, and incubated in a dark growth chamber at 28 °C with 75% relative humidity. After 48 h, seedling growth was measured and plant sample was frozen in liquid nitrogen and stored in − 80 °C deep freezer and subsequently MDA and PCs contents were estimated.

MDA Estimation

About 0.5 g of cucumber seedlings were homogenized in 5 mL of 10% (W/V) trichloroacetic acid (HiMedia, Nasik, Maharashtra) and 0.25% of thiobarbutiric acid. The homogenate was centrifuged at 16,000×*g* for 15 min at room temperature. The supernatant was mixed with an equal amount of thiobarbutiric acid [0.5% in 20% (W/V) trichloroacetic acid] (Sigma-Aldrich, Bangalore, India) and the mixture was boiled for 25 min at 100 °C followed by centrifugation for 5 min at 6300×*g* to clarify the solution. Absorbance of

the supernatant was measured at 532 nm and 600 nm and corrected for nonspecific turbidity by subtracting the absorbance at 600 nm. The standard MDA (Sigma-Aldrich, Bangalore, India) was used to develop the standard graph (Nisarga et al. 2017).

Methylglyoxal Estimation

The quantification of MG was performed according to (Yadav et al. 2005) with slight modifications. 300 mg of seedlings were ground using motor and pestle and extracted in 3 mL of 0.5 M perchloric acid (Sigma-Aldrich, Bangalore, India). The extract was incubated for 20 min on ice and centrifuged at 4 °C at 12,000×*g* for 10 min. The color pigments in the supernatant were decolorized by adding activated charcoal (10 mg/ml), kept for 20 min at room temperature, and centrifuged at 12,000×*g* for 15 min. The collected supernatant was neutralized by incubating for 15 min using a saturated solution of potassium carbonate and centrifuged at 12,000×*g* for 15 min. MG estimations were performed using neutralized supernatant. MG quantification was performed by adding the solutions in the following order 250 µL of 7.2 mM 1,2-diaminobenzene, 100 µL of 5.0 M perchloric acid, and 650 µL of the neutralized supernatant with a total volume of 1.0 ml. The absorbance of the derivatized MG was read at 336 nm using a spectrophotometer (Spectra max plus-384, Spinco Biotech Pvt Ltd, Bangalore).

Protein Carbonyls (PCs) Estimation

The frozen sample (100 mg) was ground in 2 mL of 50 mM phosphate buffer (pH 7.4). The homogenate was vortexed and centrifuged at 10,000×*g* for 30 min at 4 °C. The supernatant was subjected to another round of centrifugation at 10,000×*g* for 30 min. The supernatant was treated with 1 mL of 10% TCA and incubated on ice for 10 min for the proteins to precipitate. Centrifuged at 10,000×*g* for 30 min. 100 µL of 1.0 N NaOH + 900 µL of phosphate buffer saline (PBS) (pH 7.4) was added to the pellet and incubated at 4 °C for 20 min. Total protein concentration was estimated using Lowry's method (Lowry et al. 1951). Further 500 µL of DNPH was added to 1 mg of protein sample and incubated at room temperature for 10 min and then 250 µL of 6.0 M NaOH was added and incubated at room temperature for 10 min. Absorbance was read at 450 nm (Mesquita 2014).

Western Blotting

To assess the carboxymethyl lysine-mediated protein modifications in stress seedlings, western blotting was performed. Ten (10) µg of total protein from seedlings treated with glucose and small molecules were separated on an SDS-PAGE gel followed by semi-dry transfer of the proteins on to a polyvinylidene difluoride (PVDF) membrane. To check the equal transfer of proteins from the gel, the membrane was stained with Ponceau S and anti-carboxymethyl Lysine (CML) antibody (Abcam, UK) was used to quantify the CML levels (Banarjee et al. 2018). Chemiluminescent detection was performed using ClarityTM Western Chemiluminescent Substrate (Bio-Rad, USA) on a Syngene Dyversity Gel Documentation system. The AGE–CML modified proteins were quantified using the Image J tool.

In-Gel Guaiacol Peroxidase (EC 1.11.1.7) Assay

The frozen seedlings (100 mg) were homogenized in 50 mM Tris–HCl (pH 7.5) buffer containing 40 mM phenylmethylsulfonyl fluoride (PMSF), and 2% (w/v) polyvinylpolypyrrolidone (PVPP). The extract was centrifuged at 15,000×*g* for 20 min at 4 °C and the resultant supernatant was used for GPX enzyme assay. The amount of protein was calculated according to Lowry et al. (1951). Native polyacrylamide gel electrophoresis (Native-PAGE) was performed on a 10% gel according to Laemmli (1970). The activity of GPX isoforms was visualized according to the staining procedure of Birecka (1978) and the gels were captured in a gel documentation system (Bio-Rad).

Statistical Analysis

The data obtained in different experimental results were analyzed a simple variance (ANOVA) as per the procedure is given by Fischer (1960). The differences between the means were compared by Student's *t* test at $P < 0.05$.

Results

Reactive Carbonyl Compounds Affect Seedling Survival and Growth Under Stress Conditions

The effect of carbonyl stress induced by glucose, NaCl and MV was assessed in cucumber seedlings. A concentration dependant decrease in growth was observed in all the stresses (Fig. S1). In subsequent experiments besides the growth response, accumulation of RCC like MDA and PCs in seedlings exposed to 4% glucose, 150 mM NaCl and 20 µM MV was examined. Significant reduction in shoot and root growth was observed in all the stresses (Fig. 1a). An increase in MDA content was observed in glucose (2.4-fold), NaCl (2.3-fold) and MV-induced stress (2.1-fold) when compared to control seedlings (Fig. 1b). The PCs content was 3.4, 3.6 and fourfold in glucose, NaCl and MV-stress, respectively (Fig. 1c). The results clearly suggest that the RCC and protein carbonyls accumulate and reduce seedling growth under stress conditions.

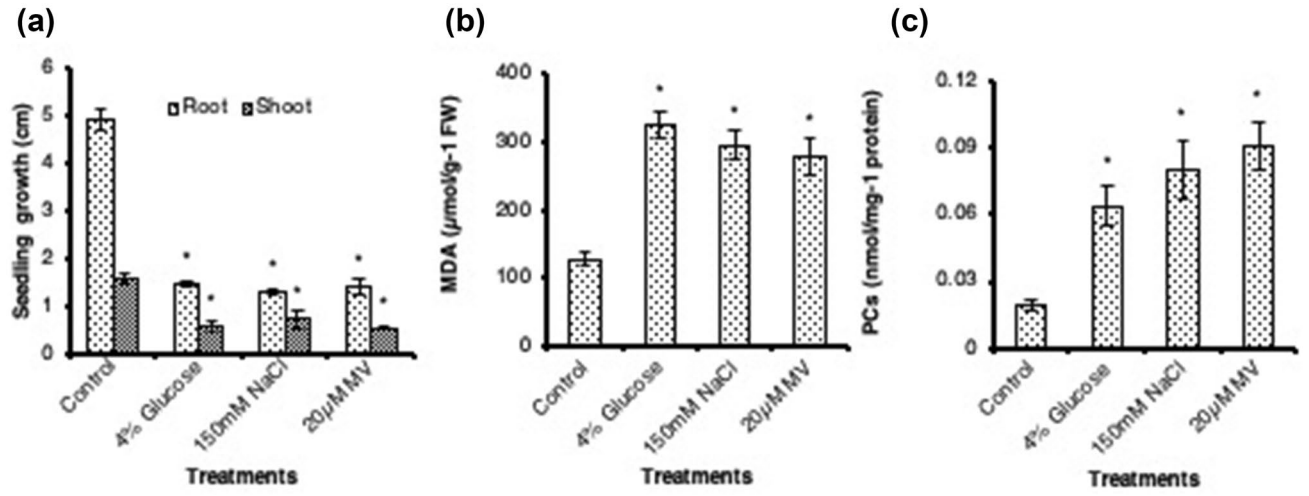

Fig. 1 Response of pre-germinated cucumber seedlings to 4% glucose, 150 mM NaCl and 20 µM MV-induced oxidative stress. **a** seedling root and shoot growth, **b** malondialdehyde (MDA) and **c** protein carbonyls (PCs) content. The seedlings treated with distilled water were maintained as control. The growth measurement, MDA and PCs contents were quantified after 2-days of exposure to stress. Error bars indicate the data from 5 biological replications in each treatment. Statistically significant differences between control and stress treatments were analyzed by Student's *t* test (*$p \leq 0.05$)

Aldo-Keto Reductases (AKRs) Rescue Tobacco and Rice Seedlings from Glucose, NaCl, and MV-Induced Carbonyl Stress

Several enzymes like AKRs, aldehyde reductases (ALR), Aldose reductases (ADR) and glyoxalases have been shown to detoxify RCC (Hegedusab et al. 2004; Kotchoni et al. 2006; Huang et al. 2008; Nisarga et al. 2017). Our earlier studies using tobacco transgenics overexpressing AKRs showed improved NaCl stress tolerance (Vemanna et al. 2017). The detoxification of glycation-induced carbonyls under glucose stress and lipoxidation induced carbonylation under MV and NaCl stress by AKRs was assessed in AKR overexpressing tobacco plants which were previously developed by our group. The transgenic seedlings overexpressing either *OsAKR1*, *PsAKR1* or *OsALR1* showed improved growth under all these stresses when compared to wild type seedlings (Figs. 2a and S2a). The MG and MDA levels were significantly less in AKR transgenics compared to wild type (Fig. 2b, c). The results indicate that improved detoxification of cytotoxic compounds MG and MDA by AKR enzymes rescue tobacco seedlings and enhance stress tolerance. To further validate the observed response of tobacco seedlings, carbonyl stress response was assessed in *PsAKR1* rice transgenics exposed to glucose, NaCl, and MV-induced stress (Fig. 3a). The *PsAKR1* rice transgenics have maintained higher shoot and root length compared to wild type (Fig. 3b).

AKR Proteins Detoxify RCC and Rescued Cucumber Seedlings from Glucose, NaCl and MV-Induced Stress

The relevance of AKR proteins in scavenging the reactive carbonyl compounds was studied by an indirect assay using cucumber seedlings. All the three AKR genes were cloned into the pET expression vector and subsequently overexpressed in *Escherichia coli* and surrogate AKR proteins were infiltrated into the cucumber seedlings. Subsequently, the seedlings were incubated in 4 and 5% glucose for 48 h and transferred to the water for 48 h and recovery growth was measured. The cucumber seedlings treated with *PsAKR1*, *OsAKR1* and *OsALR1* crude proteins showed significantly higher recovery in growth on 4 and 5% of glucose compared to vector control (pET32a) (Fig. S3). The results reveal that ectopically supplied AKR proteins could rescue cucumber seedlings from the glucose-induced stress.

The Yeast AKR Family Mutants are Hyper-sensitive to RCC Induced by Glucose, NaCl and MV-Induced Stress

The relevance of AKRs in detoxification of RCC has also been analyzed in yeast (*Saccharomyces cerevisiae*) mutants *GCY1* (Acc. No. SGD: S000005646-YOR120W), *YPR1* (Acc. No. SGD: S000002776-YPR368W) and *ARA1* (Acc. No. SGD000000353-YBR149W) which belongs to AKR group of enzymes. The wild type and the Yeast mutants were

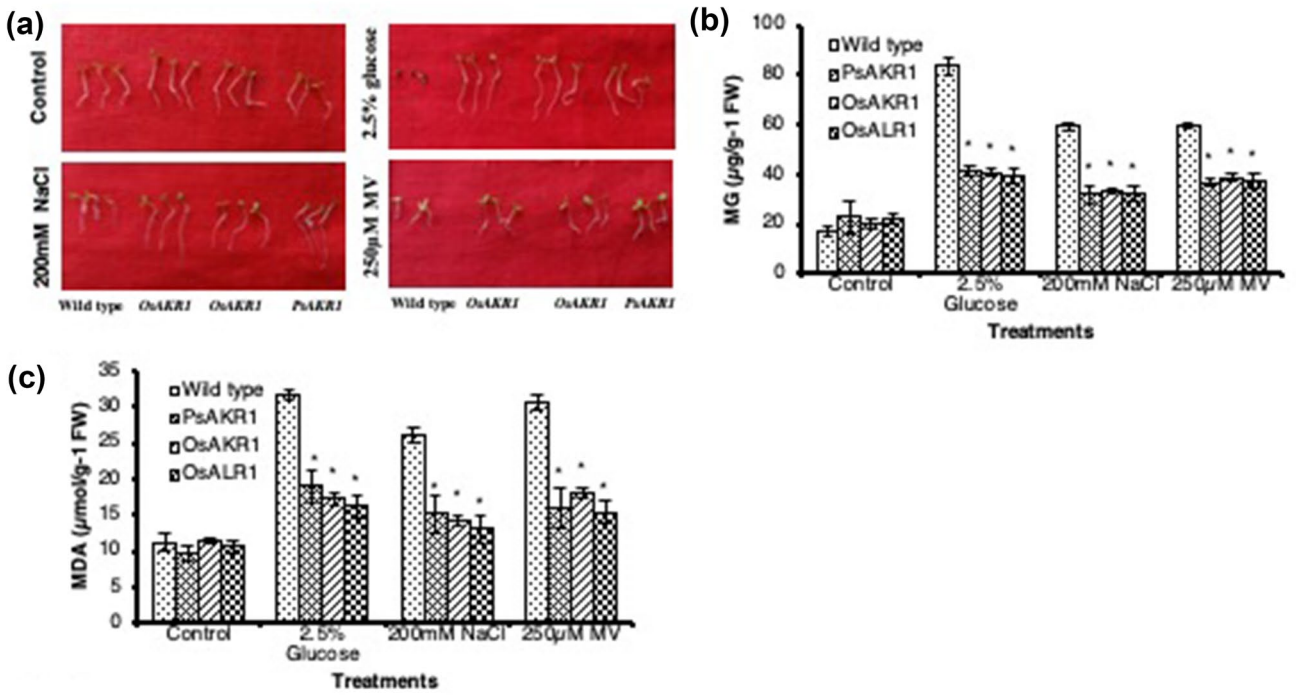

Fig. 2 Response of *AKR1* overexpressing tobacco transgenics to carbonyl stress induced by 2.5% glucose, 200 mM NaCl and 250 µM methyl viologen. The tobacco seedlings were exposed to stress for 5-days and subsequently the root and shoot growth was recorded. **a** photographs showing the growth response of tobacco transgenics overexpressing *OsAKR1*, *PsAKR1* and *OsALR1* genes under glucose, NaCl and MV-induced stress. **b** levels of methylglyoxal (MG), and **c** malondialdehyde (MDA) in wild type and transgenics under stress conditions. Bar graphs representing the figures and indicate the data from 3 biological replicates. Statistically significant differences between wild type and transgenics were analyzed by Student's *t* test (*$p \leq 0.05$)

exposed to glucose, NaCl and MV-induced stress in Yeast extract-peptone-dextrose agar (YPDA) media. The AKR mutants showed hyper-sensitive phenotype with delayed growth rates in all stresses indicating sensitivity to carbonyl stress (Fig. S2b). The results clearly demonstrate that AKRs are potential enzymes that can mitigate the carbonyl stress.

Small Molecules Detoxify RCC Generated Under Stress and Restored Seedling Growth

Besides the enzymatic scavenging of cytotoxic compounds, several small molecules and metabolites are known to detoxify the ROS and even RCC (Uchida et al. 1998). Small molecules such as ASA, AG, Car, Cur and PM have been shown to detoxify RCC in several studies including cell culture studies (Younus and Anwar 2016). We assessed the effect of these small molecules to rescue the cucumber seedlings from carbonyl stress induced by glucose, NaCl and MV. The glucose, NaCl and MV-stress response of the cucumber seedlings were assessed with different concentrations of small molecules. At 50 µM ASA, 10 µM AG, 10 µM Car, 1 µM Cur and 10 µM PM, the maximum recovery of the growth was observed under glucose-induced stress (Fig. S4). Similar responses were found in NaCl and MV-induced stress. The extent of root and shoot growth recovery in all the small molecules treatment are significantly higher compared to the seedlings exposed to glucose, NaCl and MV-induced stress (Fig. 4a, b). However, under glucose stress, the recovery response of the seedlings was much higher as compared to NaCl and MV-induced stress.

The bio-efficacy of small molecules in scavenging RCC was tested by assessing the recovery response of cucumber seedlings to glucose, NaCl and MV-induced stresses and by quantifying the MDA and PCs levels. In the presence of the small molecules, the cucumber seedlings exposed to carbonyl stress showed reduced MDA and PCs content (Fig. 5a, b). In glucose, a significant increase in MDA and PCs content was observed compared to control conditions. However, the stressed seedlings supplemented with small molecules the MDA and PCs levels was significantly less as compared to stress alone (Fig. 5a, b). A similar trend was observed in seedlings exposed to NaCl and MV-induced stress.

In all stresses there is a significant decrease in growth compared to control and it is also related to a substantial increase in MDA and PCs levels. In stressed seedlings treated with small molecules, the extent of growth reduction was relatively less and was associated with reduced

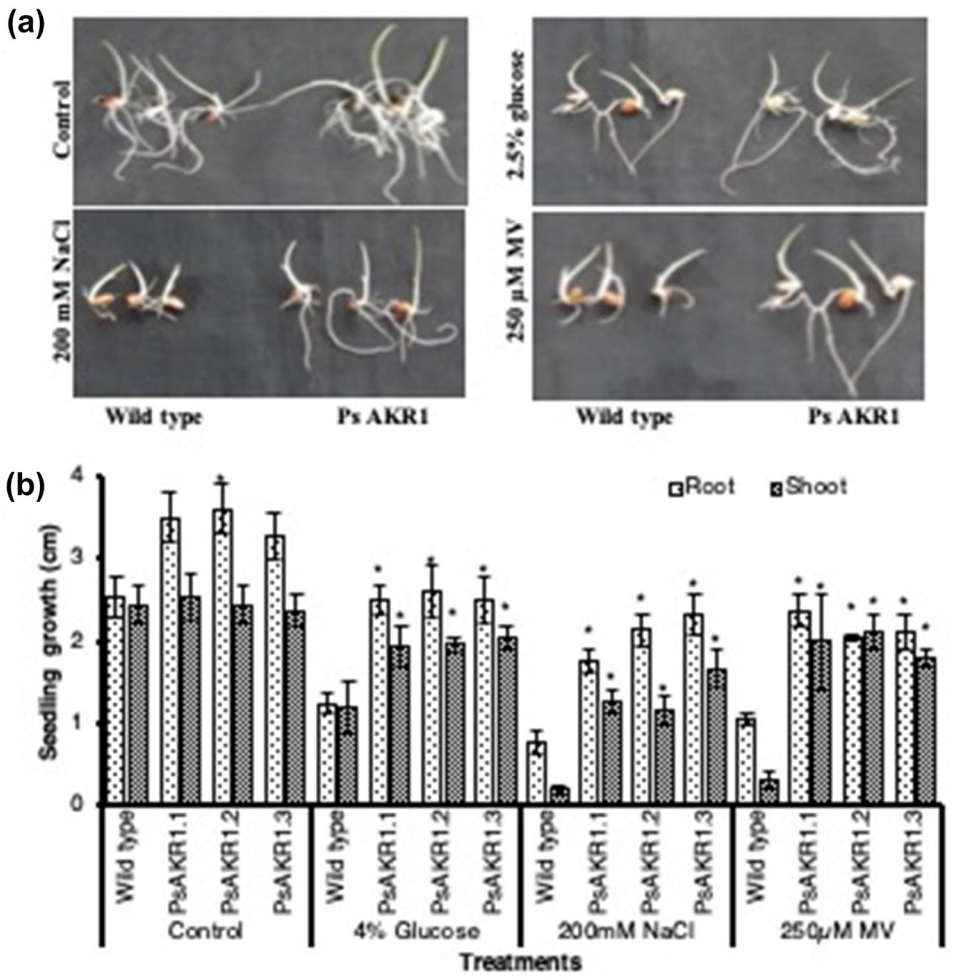

Fig. 3 Response of *PsAKR1* overexpressing rice transgenics to carbonyl stress induced by 2.5% glucose, 200 mM NaCl and 250 µM methyl viologen. Pre-germinated AKR1 transgenic seedlings were transferred to different stress conditions and seedling growth was recorded after 5-days. **a** photographs show the growth response of wild type and transgenic seedlings under control, 2.5% glucose, 200 mM NaCl and 250 µM methyl viologen. **b** bar graphs representing the figures and indicate the data from 3 biological replicates. Statistically significant differences between wild type and transgenics were analyzed by Student's *t* test (*$p \leq 0.05$)

levels of MDA and PCs. However, the effectiveness of these molecules varied in different stresses in recovery response. The recovery growth is substantially high in seedlings treated with small molecules under glucose stress compared to other stresses. The PCs levels were also much less in glucose stress in seedlings treated with small molecules. Even within the same stress, the effectiveness of molecules differed to a certain degree and it was related to the recovery growth and accumulation of MDA and PCs. The seedlings treated with ASA showed reduced MDA and PCs levels with higher growth whereas Cur-treated seedlings had higher MDA and less growth compared to other molecules. In all stresses, the seedlings treated with ASA and Car showed lower MDA and PCs levels. The results clearly indicate that one of the reasons for the reduction in growth was due to the accumulation of RCC like MDA and even PCs. A significantly negative relation was observed between MDA levels and growth (Fig. 5c) and between PCs and growth (Fig. 5d). A positive relation was observed between PCs levels and MDA levels under stress conditions (Fig. 5e).

Small Molecules Rescue the Recovery Growth of Rice Seedlings Under MV-Induced Oxidative Stress

The effect of these small molecules on MV-induced oxidative stress was assessed in autotrophically grown rice seedlings under high light conditions. The 7-day-old rice seedlings were incubated in different concentrations of small molecules for 12 h and subsequently exposed to MV-stress under high light (600 µmole/m^{-2}/s^{-1}) for 16 h. The recovery in shoot growth and the extent of membrane damage was assessed after 48 h. A significant reduction in growth was seen in seedlings treated with MV alone (Fig. 6a). With small molecules, stressed rice seedlings showed significantly higher recovery growth and fresh weight compared to MV treated seedlings (Fig. 6c, d). The carbonyl stress response was also evident as seen in membrane integrity differences. The significantly higher Evan's blue staining was seen in MV treated seedlings compared to control and small molecules treatment (Fig. 6b). These results clearly confirm that the stress-induced RCC are detoxified by small molecules even in autotrophically grown rice seedlings.

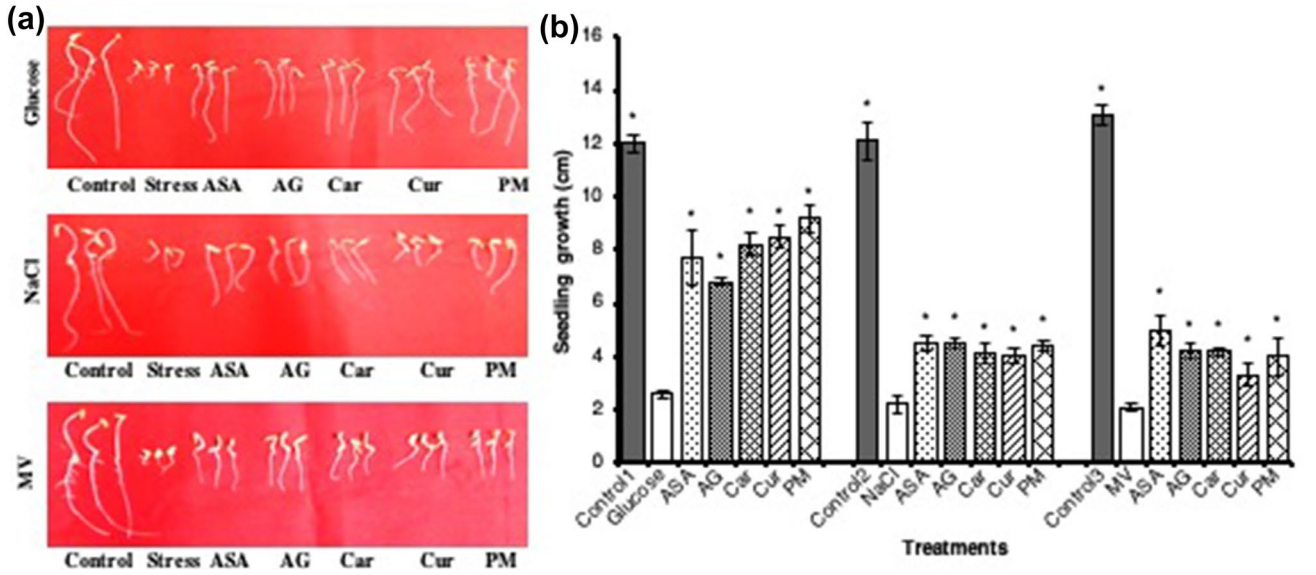

Fig. 4 The growth response of pre-germinated cucumber seedlings to different small molecules subjected to carbonyl stress induced by 4% glucose, 150 mM NaCl and 20 µM methyl viologen in comparison with control (distilled water). The seedling growth was measured after 2-days of exposure to stress. **a** the photographs showing the growth response of cucumber seedlings with different small molecules exposed to 4% glucose, 50 mM NaCl and 20 µM methyl viologen. **b** bar graph representing the seedling growth in terms of root and shoot growth (cm). Error bars indicate the data from 15 biological replicates. Statistically significant differences between small molecules treatments and stress alone were analyzed by Student's *t* test (*$p \leq 0.05$). (Control = water; ASA = 50 µM acetylsalicylic acid; AG = 10 µM aminoguanidine; Car = 10 µM carnosine; Cur = 1 µM curcumin; PM = 10 µM pyridoxamine)

Small Molecule Could Reduce the Ages Accumulation Under Stress

Advanced glycation end products (AGEs) are known to be produced by non-enzymatic glycation between proteins and D-glucose. The non-enzymatic Schiff base reaction of glucose with proteins produces N-epsilon-carboxymethyllysine (CML or Carboxymethyl Lysine) by an amadori rearrangement. Significantly higher levels of CML modified proteins accumulated over time due to glucose and NaCl induced stress conditions when compared to control conditions in cucumber seedlings (Fig. 7). The accumulation of these CML modified proteins were significantly reduced when seedlings are treated with small molecules ASA, AG, PM, Car under glucose-induced stress. However, the Cur-treated seedlings did not show any reduction in CML which is on par with the glucose treated seedlings (Fig. 7c). A similar reduction in CML proteins was observed in NaCl treatment; however, in the case of Car treatment, the seedlings showed significantly higher levels of CML modified proteins compared to NaCl stress (Fig. 7f). Overall the small molecules could reduce the effect of RCC on proteins by inhibiting the formation of CML.

Small Molecules Could Protect Antioxidant Enzyme Guaiacol Peroxidase (EC 1.11.1.7) from RCC Damage

One of the primary effects of RCC is protein carbonylation, which alters the function of the target proteins or enzymes, thus affecting cell metabolism. To study the effectiveness of small molecules in protecting guaicol peroxidase (GPX) the activity of this enzyme was assessed in cucumber seedlings exposed to glucose and NaCl stress in the presence of the small molecules. The in-gel assays with guaiacol as substrate showed a significant reduction in GPX levels in glucose and NaCl treated seedlings (Fig. 8). The GPX activity on the gels revealed three isoforms of peroxidase designated GPX I, II, and III. With small molecule treatment, the enzyme activity was significantly higher as observed in higher levels of isoform activities (Fig. 8). The result shows that the small molecules which detoxify the RCC may sustain the enzymatic activity under RCC induced stress conditions.

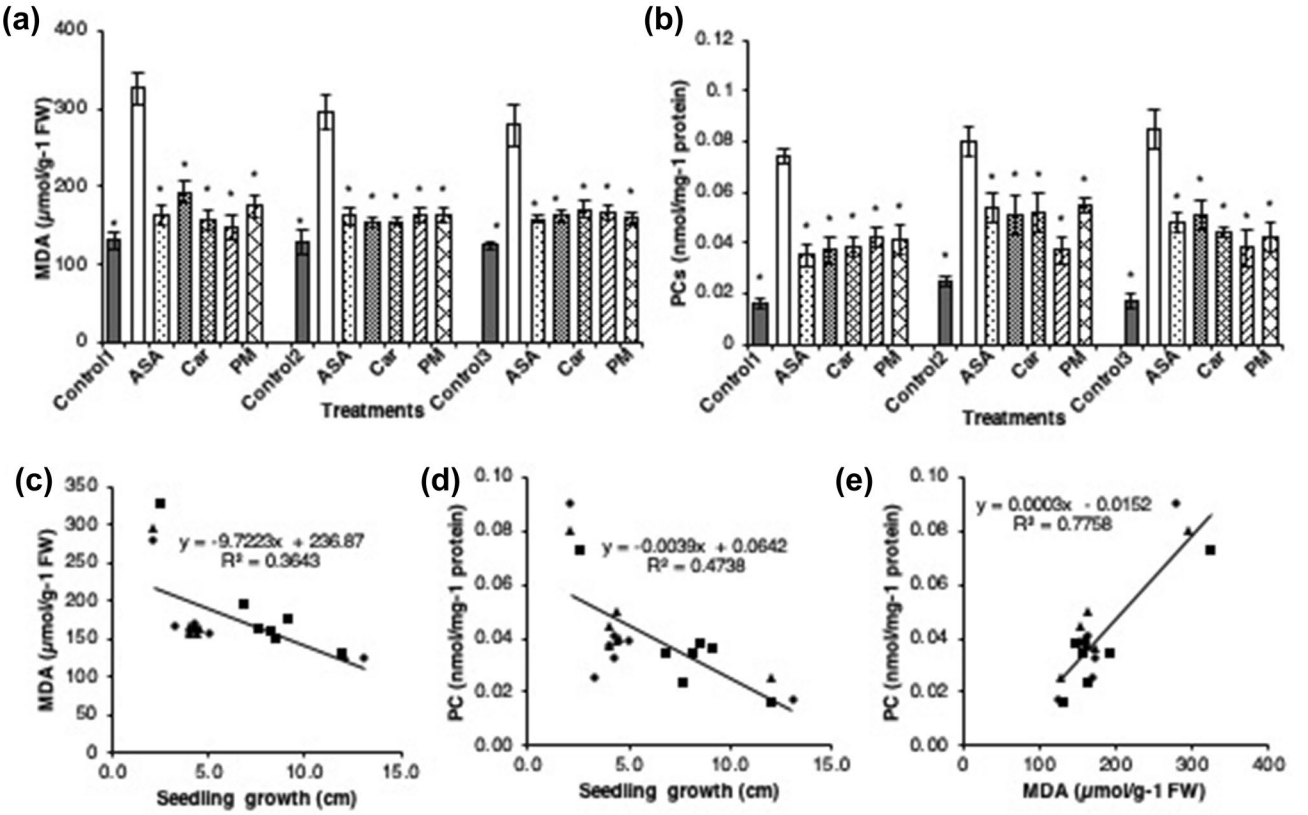

Fig. 5 Effect of malondialdehyde (MDA) and protein carbonyls (PCs) content in pre-germinated cucumber seedlings exposed to different small molecules with 4% glucose, 150 mM NaCl and 20 µM methyl viologen in comparison with control (distilled water). **a** MDA content **b** PCs content. Error bars indicate the data from 5 biological replicates. Statistically significant differences between small molecules treatments and stress alone were analyzed by Student's *t* test (*$p \leq 0.05$). **c** Inverse correlation between MDA and seedling growth and **d** PCs and seedling growth; **e** positive correlation between PCs and MDA under stress with small molecule treatment. (ASA = 50 acetylsalicylic acid; AG = aminoguanidine; Car = carnosine; Cur = curcumin; PM = pyridoxamine)

Discussion

Reactive oxygen species (ROS) are ubiquitous under stress and induce reactive carbonyl compounds (RCC) that further react with proteins to form protein aggregates resulting in dysfunction of cellular metabolic activities. In this study, we have used glucose, NaCl and MV to induce carbonyl stress. The effects of MV and salinity-induced ROS on lipids causing lipid peroxidation and glucose-induced carbonyl stress generating MG and glyoxal are well documented in different crops (Singla-Pareek et al. 2003; Yadav et al. 2005; Kotchoni et al. 2006). In all these stresses a substantial reduction in seedling growth was observed and is associated with an increase in MDA, MG, AGE-CML, PCs and other cytotoxic compounds that damage the cellular metabolism. Despite being damaging agents, the role of a few RCC has been shown in cellular signaling mechanisms in plants (Thornalley 1990). However, to avoid cellular damage by these RCC, the levels should be maintained in a certain range. Plants have evolved the mechanisms to detoxify and repair the damage caused by these reactive molecules (Hudig et al. 2018). Scavenging systems involving single- or multiple-step mechanisms by enzymatic and non-enzymatic reactions are highly effective in maintaining the equilibrium.

A few groups of enzymatic systems have been identified that showed detoxification of a specific or broad spectrum of RCC under stress (Yadav et al. 2005; Vemanna et al. 2016, 2017). The reactive MG scavenged by the glyoxalase system has been shown to convert MG to lactate in the presence of glutathione in tobacco under salinity and drought stress (Yadav et al. 2005; Singla-Pareek et al. 2003). The other groups of enzymes, the AKRs, have broad substrate specificity to detoxify RCC (Yadav et al. 2005; Vemanna et al. 2016, 2017). Our study demonstrates reduced MDA and MG levels under glucose, NaCl and MV-stress in tobacco transgenics overexpressing *OsAKR1*, *PsAKR1* and *OsALR1*. Even the rice transgenics overexpressing *PsAKR1* also showed improved seedling growth under these stress conditions. The relevance of AKRs has also been assessed in yeast AKR mutants. The tobacco plants overexpressing

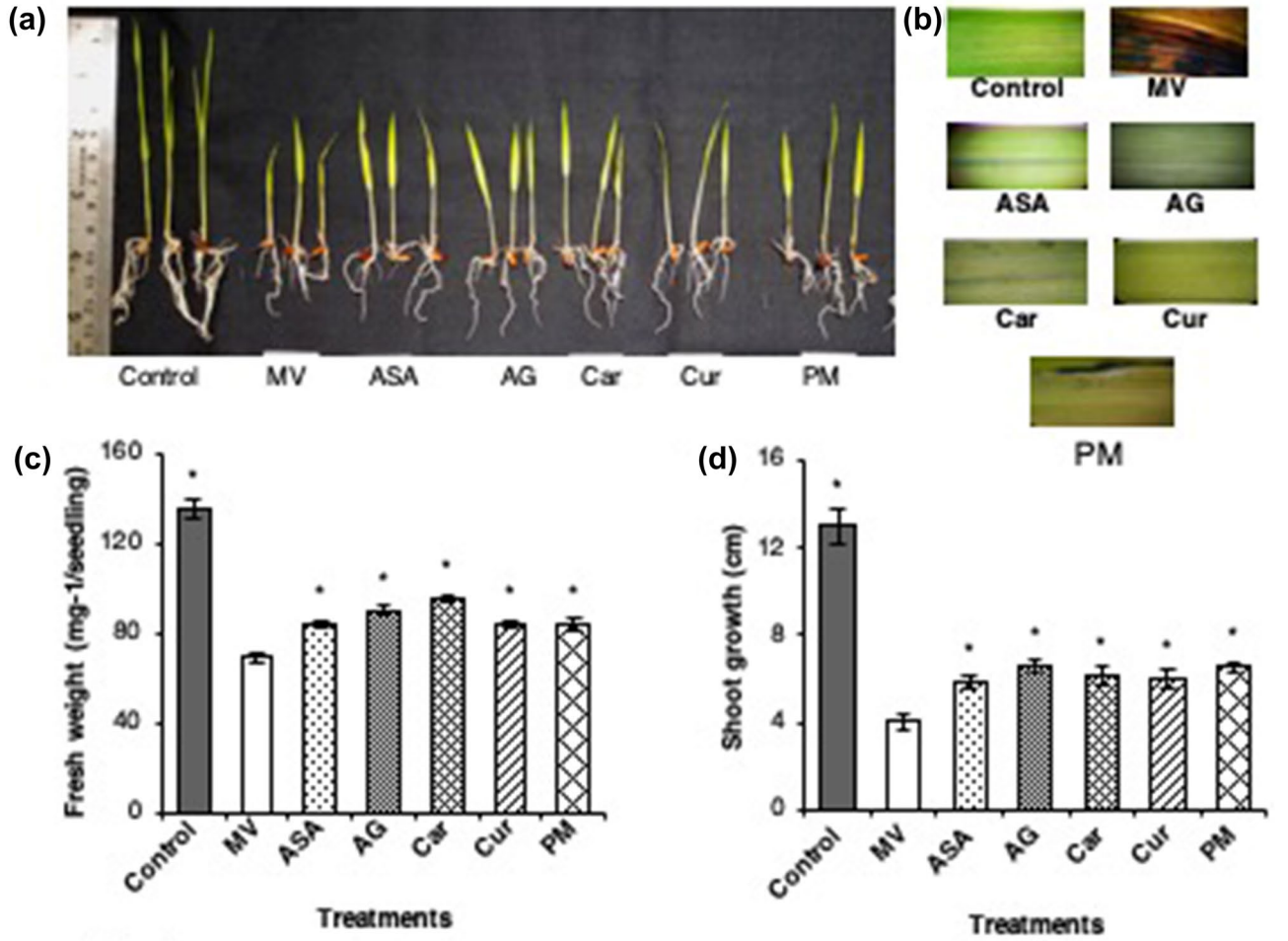

Fig. 6 Response of autotrophically grown rice seedlings to MV-induced oxidative stress and small molecules exposed to high light conditions (600 µmole/m^{-2}/s^{-1}) in comparison with control (distilled water). **a** phenotypic response of seedlings to small molecules under MV-stress, **b** membrane integrity by Evans blue assay, **c** bar graphs representing Fig. 6a with the seedling growth (cm), **d** seedlings fresh weight (mg). Error bars indicate the data from five biological replicates. Statistically significant differences between small molecules treatments and MV-stress alone were analyzed by Student's *t* test (*$p \leq 0.05$). (ASA = 50 µM acetylsalicylic acid; AG = 10 µM aminoguanidine; Car = 10 µM carnosine; Cur = 1 µM curcumin; PM = 10 µM pyridoxamine)

PsAKR1, *OsAKR1*, *OsALR1* also showed improved tolerance to glyphosate and NaCl induced stress (Vemanna et al. 2016, 2017). Several other studies have also shown that the overexpression of *OsAKR1*, *OsALR1* in tobacco reduced the levels of 4-HNE, methylglyoxal and improved tolerance under NaCl stress (Oberschall et al. 2000; Hideg et al. 2003; Hegedusab et al. 2004; Turoczy et al. 2011). The role of *AKR4C10* and *AKR4C11* in reducing sugar derived RCC have been demonstrated in *Arabidopsis* in response to light and CO_2 (Saito et al. 2013). These studies clearly demonstrate that AKRs are potential scavenging enzymes of RCC generated under stress.

Detoxification of RCC by several natural and synthetic molecules such as flavonoids, phenol derivatives, imidazole, thiazolidine, and sulfonates has been shown (Hu et al. 2016; Younus and Anwar 2016). In addition, several molecules such as ASA, AG, Car, Cur and PM have been shown to detoxify RCC in cell culture studies (Yavuz et al. 2002; Sadowska-Bartosz and Bartosz 2015). Several antiglycating compounds from plants and synthetic origin such as ASA, AG, Car, Cur and PM have been shown to inhibit the glycation compounds either by blocking of sugar attachment to proteins attenuating glycoxidation, trapping or scavenging glycation intermediates, dicarbonyls and breakage of AGEs (Younus and Anwar 2016; Sun et al. 2018). The reduction of RCC and higher recovery in seedling growth in cucumber or rice when exposed to glucose, NaCl and MV-stress could be attributed to the effect of small molecules because they react with broad-spectrum RCC and convert them into harmless molecules. The glycation-induced RCC react with proteins to form AGE (CML, N-(Carboxymethyl)lysine). The significant reduction in levels of AGE-CML in ASA, AG, Car, Cur

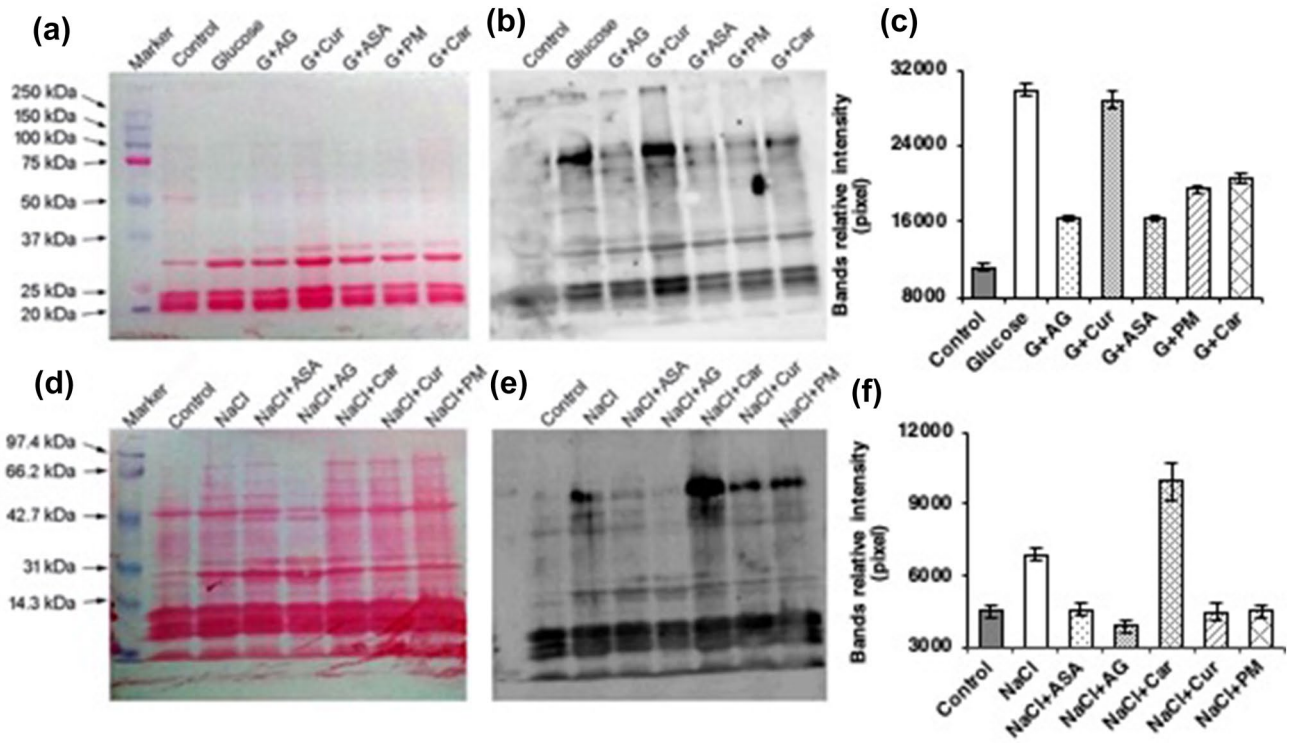

Fig. 7 Small molecule treatments reduces the advanced glycation end products—carboxymethyl lysine (AGE-CML) under 4% glucose and 150 mM NaCl stress. The seedlings were treated with distilled water (control), glucose or NaCl and small molecules, after 2-days of exposure, the total protein was isolated and equal amount of protein was loaded on the gel and immobilized on PVDF membrane. **a**, **d** total protein stained by Ponceau stain. **b**, **e** advanced glycation end products-carboxymethyl-lysine (AGE-CML) detected on western blot using Anti-CML antibodies. **c**, **f** bar graphs representing the figures **b**, **e** with relative intensities of AGE-CML quantified using Image J tool and expressed in pixels. (G = 4% glucose; ASA = 50 µM acetylsalicylic acid; AG = 10 µM aminoguanidine; Car = 10 µM carnosine; Cur = 1 µM curcumin; PM = 10 µM pyridoxamine)

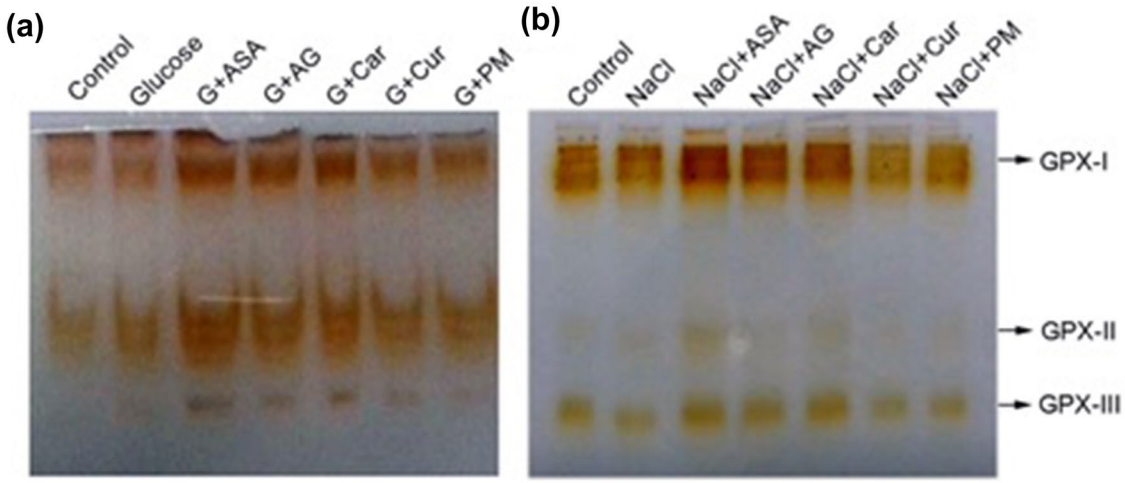

Fig. 8 Small molecules maintain the Guaiacol peroxidase (GPX) enzyme activity under carbonyl stress induced by 4% glucose, 150 mM NaCl. After 2 days of exposure, the total protein was isolated, equal amount of protein was loaded on the gel and in-gel assay was performed with guaiacol as a substrate. **a** gel profile showing Guaiacol peroxidase enzyme isoforms under 4% glucose and **b** 150 mM NaCl stress conditions. (G = 5% glucose; NaCl = 150 mM NaCl; ASA = 50 µM acetylsalicylic acid; AG = 10 µM aminoguanidine; Car = 10 µM carnosine; Cur = 1 µM curcumin; PM = 10 µM pyridoxamine)

and PM treated seedlings under stress clearly suggests that small molecules potentially alleviate the glycation-induced stress by scavenging RCC.

The role of these small molecules to improve tolerance against diverse abiotic stresses has been well documented (Senaratna et al. 2000; Mooney and Hellmann 2010; Colina et al. 2016). ASA treatment in tomato has been shown to improve seed germination under heat, cold and drought stress (Senaratna et al. 2000). PM plays an essential role in plant development and metabolism and improved response to stress in diverse species (Colina et al. 2016; Mooney and Hellmann 2010). The role of AG in improving stress tolerance was demonstrated in faba bean under hypoxia and NaCl stress (Yang et al. 2015). AG has significant potential to react with amadori compounds in Maillard reaction and it has been extensively studied both in vitro and in vivo (Brownlee et al. 1986; Corbett et al. 1992). The exogenous application of Car to *Arabidopsis* under salt and H_2O_2 stress showed reduced cell death. Car inhibits the formation of oxylipin carbonyls which are derived from oxygenated lipids and fatty acids to prevent the programed cell death (PCD) under stress conditions in tobacco BY2 cells and *Arabidopsis* roots (Biswas and Mano 2015). Car supplementation to rats was found to increase the activities of enzymatic antioxidants SOD and GPX and reduced lipid peroxide levels (Kim et al. 2011).

RCC form adducts with the proteins to form PCs and thus affect the function of many enzymes. The seedlings treated with the small molecules could maintain higher antioxidant enzyme Guaiacol peroxidase (GPX) activity in glucose and NaCl stresses. The reduction of AGE-CML levels in small molecules treated samples signifies the relevance of managing RCC as evidenced by higher activity of GPX. Our earlier studies showed that AKRs protect proteins from RCC damage (Vemanna et al. 2017; Nisarga et al. 2017). We showed that overexpression of AKR1 in tobacco protected glutathione, APX, P5CS enzymes in NaCl stress (Vemanna et al. 2017). The study shows that the small molecules can scavenge the RCC and protect the enzymes from RCC damage under stress conditions and rescue the cucumber seedlings from stress-induced carbonyl stress. These molecules could be exploited in early seed germination to improve growth and productivity of crops.

Conclusion

Plants have evolved potential mechanisms to scavenge these reactive substances by the enzymatic and non-enzymatic processes. The transgenic tobacco expressing AKRs showed improved tolerance to stress. The small molecules ASA, AG, Car, Cur and PM scavenge or react with RCC and convert to harmless products could efficiently rescue cucumber seedlings from stress-induced growth inhibition. The small molecules could be a potential option to improve stress tolerance of crops.

Acknowledgements Authors thank Department of Science and Technology – Fund for Improvement of Science and Technology (SR/FST/LSI-051/2015). VR thanks Ramanujan fellowship (SB/S2/RJN-046/2016), and DBT-innovative young Biotechnologist award (BT/010/IYBA/2016/09).

Author Contributions MUK, VR, and MJK conceived the concept and wrote the manuscript. VR and ARV developed transgenics and did the assays. AN, SS and AV did the small molecule related work. RB performed CML western blots. MUK and VR edited and finalized the manuscript.

Funding This work was supported by the NASI—senior scientist platinum jubilee fellowship, SERB-EMR Grant (EMR/2016/002078) to MUK.

Compliance with Ethical Standards

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

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