Identification of the antioxidant defense genes which may provide enhanced salt tolerance in *Oryza sativa* L.

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Abstract Antioxidative mechanisms are important to protect cells from the hazardous effects of reactive oxygen species (ROS). Salt stress is one of the environmental stress factors that leads to accumulation of ROS at toxic levels. In this study, we analyzed the responses of two rice (*Oryza sativa* L.) cultivars against NaCl stress at enzymatic and transcriptional levels. In 14 day-old-seedlings, different antioxidant enzyme activities were observed. These findings were also supported by transcriptional analyses of the responsible genes. According to the results, Cyt-APX, CAT A, Cyt-GR1 and proline metabolism-related genes were differentially expressed between two rice varieties under different salt concentrations. Their regulational differences cause different salt sensitivities of the varieties. By this study, we provided an insight into understanding of the correlation between antioxidant defence genes and ROS enzymes under salt stress.

Keywords *Oryza sativa* L. · Salt stress · Antioxidant metabolism · Gene expression

Introduction

Rice (*Oryza sativa* L.) is one of the most economically important crops because it is a nutritional source of more than one-third of the world population (Kim et al. 2007). Also, it is an ideal model monocot species for the study of crop genomics due to relatively small genome size (approximately 430 Mb), diploid origin ($2n = 24$), and close relationship to other important cereal crops (Menezes-Benavente et al. 2004; Rabbani et al. 2003). High soil salinity is one of the most adverse abiotic stress factors and affects plant growth and productivity negatively (Barrera-Figueroa et al. 2013). As other crop plants, rice growth and yield is affected by salinity and is known to be most susceptible to salinity at the seedling stage (Kim et al. 2007; Zeng and Shannon 2000). We can observe stress effects on plants at morphological, physiological and molecular levels. The morphological and physiological changes including plant weight, leaf and root length, several biochemical and molecular effects of salt stress are nearly related to accumulation of reactive oxygen species (ROS) (Lee et al. 2001). Active oxygen radicals induce the chain-like peroxidation reactions of unsaturated fatty acids in the membranes therefore, lipid peroxidation of the membranes occurs (Hao et al. 2009). Several regulatory mechanisms play important roles to obtain salt tolerance in plants. Maintaining the homeostasis between Na^+ efflux and influx mediated by the transcriptional regulations of SOS pathway-related genes and Na^+ , K^+ /H exchangers were reported to be effective to improve the salt tolerance in several plants (Wu 2018). Salt tolerance also depends largely upon the level of coordination among the ROS scavenging mechanisms and levels of ROS. Some enzymatic and non-enzymatic defense mechanisms are evolved to scavenge the detrimental effects of ROS.

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Antioxidant enzymes and their genes have been studied in several plant species (Bian and Jiang 2009; Reddy et al. 2004; Scandalios 2005). It is important to demonstrate a relation between salt tolerance and antioxidant defenses. Transcriptional analysis of the genes that have role in antioxidative metabolism regulates the tolerance mechanisms. This regulation is required to define the tolerance-related mechanisms in rice. Fewer studies have focused to analyze this mechanism at protein or transcript levels (Menezes-Benavente et al. 2004).

Besides enzymatic scavenging mechanism containing superoxide dismutase (SOD), guaiacol peroxidase (POD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR), non-enzymatic scavenging mechanisms such as accumulation of different solutes/osmolytes like proline, glycine betaine and trehalose are also effective in providing tolerance to abiotic stresses (Celik and Atak 2012a; b; Sairam et al. 2002; Zhu et al. 1998). These osmolytes and non-reducing disaccharides offer protective roles via inducing osmotic adjustment, stabilizing cellular components and reducing the damages to the photosynthetic system elements. Proline is the most common osmolyte accumulating under salt stress. The proline accumulation rates show difference between the plant species and their stress tolerance capacities. Salt stress tolerance was obtained via transgenic improvements of overexpression of proline-metabolism related genes in several plants (Jung et al. 2010; Su and Wu 2004; Wang et al. 2003). Investigation of regulatory mechanisms of proline metabolism is important in abiotic stress tolerance applications. It is also known that while in some plants, overexpression of a single antioxidative enzyme couldn't provide protection against oxidative stress caused by salt stress, combined expression profiles may provide increased tolerance capacity (Lee et al. 2007). In this study, we aimed to reveal the expression profiles of antioxidative enzymes and proline metabolism-related genes in two different rice cultivars under different salt stress conditions to evaluate their potential usage as pre-selective molecular agents in salt breeding studies.

Materials and methods

Plant material and salt stress treatment

In this study, we selected two rice varieties (Osmancik-97 and Mevlutbey) which are improved and widely planted in Turkey. Osmancik-97 is the most planting rice variety in Turkey and it shows high productivity in all regions of Turkey. Mevlutbey variety has shown the higher productivity than Osmancik-97 in Black Sea region of Turkey, therefore, these varieties were selected to compare their

salt tolerances. Seeds of rice (*Oryza sativa* L.) cultivars Osmancik-97 and Mevlutbey, were obtained from Black Sea and Thracia regions (Black Sea Agricultural Research Institute, Samsun and Thracia Agricultural Research Institute, Edirne). Four hundreds seeds of each variety were sown into the plastic pots containing perlite and grown under controlled conditions (16/8 h, light/dark period at 25/20 °C, relative humidity of 60–70%). The plants were irrigated regularly for 14-days with the Yoshida nutrient solution (Cock et al. 1976). Salinity responses of two different rice varieties were investigated at seedling stage applying 0, 30, 90, 150, and 210 mM NaCl to the full-strength nutrient solution for 7 days. The electrical conductivities of the solutions were 0.648 dSm⁻¹, 4 dSm⁻¹, 8 dSm⁻¹, 12 dSm⁻¹ and 17 dSm⁻¹, respectively. Seedlings which were irrigated with nutrient solution without NaCl served as the control group. Each treatment were replicated three times and each replicate contained 50 seedlings. At the end of the seven days, the leaves were harvested and stored at – 80 °C for enzyme analyses and RNA isolation.

Growth parameters

After seven days of NaCl treatment, 50 plants for each group were taken randomly and leaf and root explants were separated from seedlings. The fresh weights of leaves were weighed, and shoot and root lengths were measured.

Lipid peroxidation

Thiobarbituric acid (TBA) method as described by Stewart and Bewley (1980) was used to determine the lipid peroxidation. 0.1 g of leaf sample was homogenized with extraction buffer containing 0.5% (w/v) TBA and 20% (w/v) trichloroacetic acid (TCA). The mixture was measured at 532 nm and 600 nm. The lipid peroxidation rate was calculated by extinction coefficient of 155 mM⁻¹ cm⁻¹.

Soluble protein extraction

0.5 g of leaf samples were homogenized at 4 °C. Homogenates were then centrifuged at 4 °C. Soluble protein content was calculated with respect to Bovine Serum Albumin (BSA) (Bradford 1976).

Determination of free proline

0.5 g frozen leaf tissue was used to measure the free proline content according to Bates et al. (1973). The proline concentration was calculated using a standart curve prepared by using L-proline and defined as μmol proline g⁻¹ FW.

Enzyme extractions and assays

All enzyme extraction procedures were performed at 4 °C and 0.1 g of leaf samples were homogenized in sodium-phosphate buffer (pH 7.0) for the extractions.

Guaiacol peroxidase (GPX; EC 1.11.1.7) activity was defined for a unit as $\mu\text{mol H}_2\text{O}_2 \text{ min}^{-1} \text{ g}^{-1} \text{ FW}$ decomposed per minute (Seever et al. 1971).

Ascorbate peroxidase (APX, EC 1.11.1.11) activity was measured at 290 nm and one unit of APX activity was calculated by extinction coefficient ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$) due to method of Nakano and Asada (1981).

Catalase (CAT, EC 1.11.1.6) activity was measured with the reduction levels of H_2O_2 (Aebi 1984). 1U of CAT activity of the leaf samples was measured as a reduction rate of 1 mmol $\text{H}_2\text{O}_2 \text{ ml}^{-1} \text{ min}^{-1}$ at 240 nm.

Glutathione reductase (EC 1.6.4.2) activity was measured due to the method of Foyer and Halliwell (1976). The reduced GSSG per min was determined due to the glutathione-dependent oxidation of NADPH (extinction coefficient $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$).

%50 inhibition of reduction reaction of nitroblue tetrazolium salt (NBT) was used to evaluate the SOD (EC 1.15.1.1) activities of the plants. The photoreduction reactions of NBT were performed due to the method of Beauchamp and Fridovich (1971) at room temperature. The enzyme activity was calculated as unit protein per mg fresh weight of the leaves.

RNA extraction and quantitative RT-PCR (qRT-PCR)

Total RNAs of individual leaves was extracted using the RNeasy® Plant Mini Kit (Qiagen, Germany). First-strand cDNA was synthesized in a total volume of 25 μl using the iScript cDNA Synthesis Kit (BioRad, USA). In the qRT-PCR experiment, three technical replicates for each reaction and three biological replicates for each condition were carried out. The synthesized cDNA was subjected to quantitative RT-PCR analysis using Eva Green Supermix (Bio-Rad, USA) in an LightCycler®480 Real-Time PCR System (Roche, Switzerland). The primers specific to antioxidative genes, Mn-SOD, Cu/Zn-SOD, Fe-SOD, Cytosolic APX, Thylakoid-bound APX, Stromal APX, Cytosolic GR, CatA, P5CS, P5CR, P5CDH and PDH obtained from Kim et al. (2005) and used to investigate salt response of two different rice varieties (Table 1). Thermal-cycling parameters were as follows: after an initial denaturation at 95 °C for 5 min, samples were subjected to a cycling regime of 40 cycles at 95 °C for 10 s, 52–54 °C for 30 s and 72 °C for 30 s. The elongation factor EIF-4A was used as internal control. The raw Ct values obtained from the LightCycler®480 Software release 1.5.0 were

converted into relative quantities via the delta–delta Ct method (Pfaffl et al. 2003) and the different expression levels between salt-treated and untreated rice varieties were determined.

Statistical analysis

The data of this paper were the mean $\pm$ SD independent experiments and 3 replicates of biochemical tests. All the statistical analyses were performed by ANOVA and the Duncan's multiple range test was applied to compare the results ($P < 0.05$) (Compton 2006; Duncan 1955). Relationships between relative gene expression levels of salt stressed rice varieties and NaCl treatments and also enzyme activities which were determined biochemically, were statistically evaluated by Pearson's correlation analysis. For each of variables, the interactions among all antioxidative metabolism-related genes were also evaluated using Kuskal–Wallis test and post-test Dunn's multiple comparison test.

Results

Growth parameters

In both rice varieties, all growth parameters were decreased with increasing salt concentration. The reduction rates of plant height, root length and fresh weight were recorded as 26.64%, 14.78% and 58.82% for Osmancık variety whereas 27.33%, 24.54% and 48.38% for Mevlütbey at 210 mM NaCl treatment, respectively. The changes in growth parameters caused by NaCl treatment were given in Table 2. In Mevlütbey variety, 150 and 210 mM NaCl treatments showed statistically significance in reduction rates of plant height whereas 90 mM and higher doses were found statistically significant in Osmancık-97. The reduction rates of root lengths and fresh weight were found statistically significant at 90 mM and higher doses in Mevlütbey while in Osmancık-97 variety, 30 mM and higher doses were found significant ($P < 0.05$).

Lipid peroxidation

Membrane lipid peroxidation in the leaves of two rice varieties was assessed as the MDA content. The effect of salt stress on MDA formation in the leaves of both varieties against different concentrations of salt stress is given in Fig. 1a. In both varieties, lipid peroxidation rates were increased with increasing NaCl concentrations. At 210 mM NaCl treatment, the lipid peroxidation rate increased by 1.47-fold in Osmancık-97 variety, while 2.69-fold increase

Table 1 Primer list for qRT-PCR analysis of two Turkish rice varieties

Accession code	Gene	Nucleotide sequences (5′–3′)	Annealing temperature (°C)	Product size (bp)
D85239	Cu/Zn-SOD	Forward Primer: CAATGCTGAAGGTGTAGCTGAG Reverse Primer: GCGAAATCCATGTGATACAAGA	54	300
AB014056	Fe-SOD	Forward Primer: TGCACTTGGTGATATTCCACTC Reverse Primer: CGAATCTCAGCATCAGGTATCA	52	297
L34038	Mn-SOD	Forward Primer: GGAAACAACCTGCTAACCAGGAC Reverse Primer: GCAATGTACACAAGGTCCAGAA	54	297
D45423	Cytosolic APX	Forward Primer: GACAAGAAACCCTCTGCAGTTT Reverse Primer: GTAGTCTGCTGGTTCACACTGG	54	305
AB114856	Thylakoid-bound APX	Forward Primer: ATTTTCACTGGACGATGAACCA Reverse Primer: GGAAGTAGTTGGACTGCAGAGG	54	320
AB114855	Stromal APX	Forward Primer: GTCTGGAGCACATACTTGGGA Reverse Primer: TTAACCGTCCAACGTGAATCCC	54	352
D85751	Cytosolic GR1	Forward Primer: CAGATGCACCAGAGATTATCCA Reverse Primer: TCCTTTTTACCGTTCCAGGTAG	52	300
D29966	CAT A	Forward Primer: GAAGATTGCGAATAGGCTCAAC Reverse Primer: GTGGCATTAAATACGCCAGTACA	52	305
D49714	P5CS	Forward Primer: CAAATGCTCCTTTTAGCCTGTT Reverse Primer: GCGTTGGTACACAAGTTCTCAG	52	260
EF576184.1	P5CR	Forward Primer: AATAGAGGCCATGGCTGATG Reverse Primer: AATGCACCCCTTCTCAAGCTC	54	541
NC_008398.2	P5CDH	Forward Primer: CCAGTGGGTGTTGAAGGTCT Reverse Primer: ACACGACATCCTTGTCAACCA	55	250
NC_008403.2	PDH	Forward Primer: ATTGCTCTCGTCTTCCTCCT Reverse Primer: ATGACTCGATCGCTTCACTC	54	295
KC140119.1	eIF4A	Forward Primer: TGGAAGCTCGACACTCTTTG Reverse Primer: CCAATGCGATGGAGGTAGTT	52	250

was observed in Mevlütbey variety. In both rice varieties, all salt treatment were caused statistically significant increase in lipid peroxidation rates but salt-induced

increase in lipid peroxidation was found significantly higher in salt sensitive Mevlütbey in comparison with Osmancık-97 ($P < 0.05$).

Table 2 Growth profiles of Mevlütbey and Osmancık-97 rice varieties in response to different concentrations of NaCl

Variety	NaCl concentration (mM)	Height (cm) (mean $\pm$ SD)	Root length (cm) (mean $\pm$ SD)	Average weight (g) (mean $\pm$ SD)
Mevlütbey	Control	40.29 $\pm$ 3.38 ^a	7.62 $\pm$ 1.79 ^a	0.93 $\pm$ 0.26 ^a
	30	39.54 $\pm$ 3.29 ^a	7.54 $\pm$ 1.62 ^a	0.73 $\pm$ 0.20 ^b
	90	34.44 $\pm$ 4.07 ^a	7.33 $\pm$ 1.09 ^b	0.69 $\pm$ 0.19 ^b
	150	30.99 $\pm$ 2.66 ^b	7.11 $\pm$ 1.01 ^c	0.60 $\pm$ 0.16 ^c
	210	29.28 $\pm$ 3.49 ^b	5.75 $\pm$ 0.99 ^d	0.48 $\pm$ 0.10 ^d
Osmancık-97	Control	40.31 $\pm$ 2.91 ^a	7.58 $\pm$ 1.31 ^a	1.19 $\pm$ 0.34 ^a
	30	35.10 $\pm$ 6.36 ^a	6.96 $\pm$ 1.79 ^b	0.98 $\pm$ 0.24 ^b
	90	33.23 $\pm$ 2.90 ^b	6.60 $\pm$ 1.12 ^c	0.96 $\pm$ 0.23 ^b
	150	32.75 $\pm$ 4.02 ^b	6.52 $\pm$ 1.55 ^c	0.63 $\pm$ 0.16 ^c
	210	29.57 $\pm$ 3.77 ^c	6.46 $\pm$ 1.52 ^d	0.49 $\pm$ 0.15 ^d

The data are represented as the mean $\pm$ SD and are derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

Fig. 1 Lipid peroxidation (a), total protein content (b) and proline content (c) changes of Osmançik-97 and Mevlütbey rice varieties under 0, 30 mM, 90 mM, 150 mM and 210 mM NaCl stress. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

Soluble protein content

Total protein content results were given in Fig. 1b. The total protein content of the control plants was higher by 2.076-fold in Mevlütbey in comparison with Osmancık-97. The results show that leaves of Osmancık-97 subjected to NaCl stress up to 30 mM showed increase in protein content. The protein level determined at 90 mM didn't show any statistically significant difference from 30 mM treatment. When treated with 150 mM NaCl, total protein amount decreased to control levels but a 59.72% increase was observed after 210 mM NaCl treatment. However, we didn't observe statistically significant difference between 30 and 90 mM treatments ($P < 0.05$). In Mevlütbey variety, an increase was observed upto 90 mM NaCl treatment, but there was no statistical significance between 90 and 150 mM NaCl treatments. At 210 mM NaCl treatment, the soluble protein content was found to be lower than the control in Mevlütbey variety ($P < 0.05$).

Free proline content

The changes in proline contents of leaves from both varieties treated with 30–210 mM NaCl are shown in Fig. 1c. While a gradual increase was observed with increasing NaCl concentrations in Osmancık-97, Mevlütbey variety showed a statistically significant sharp increase in proline accumulation at 90 mM in response to salt stress. The major increase in proline content was observed in both varieties treated with 210 mM NaCl ($P < 0.05$). While Osmancık-97 variety showed proline accumulation by 2.55-fold, Mevlütbey variety showed 11.66-fold greater proline accumulation compared to controls.

Antioxidant enzyme activities

Activity of antioxidative system enzymes are important to evaluate the tolerance mechanisms of plant species. Therefore, the GPX, APX, CAT, SOD and GR activities of 21 day-old rice seedlings were determined in both cultivars during the *in vivo* salinity stress are given in Fig. 2a–e. GPX and APX activities significantly increased, whereas a gradually decrease was determined in CAT activity in both varieties at all NaCl concentrations.

While Osmancık-97 control group showed higher GPX activity than Mevlütbey variety, Mevlütbey variety showed 9.25-fold increase at 90 mM NaCl treatment and showed higher activity than Osmancık-97. At higher doses, both varieties showed increasing GPX activity profile however, the increment rates were found higher in Mevlütbey variety. At 210 mM NaCl doses, GPX activity of Mevlütbey significantly increased by 10.6-fold with respect to control ($P < 0.05$) (Fig. 2a).

A sharp increase in APX activity was determined in both rice cultivars (Fig. 2b). Both varieties showed the same increasing patterns with different rates at 150 mM NaCl treatment. APX activity was increased by 6.0 and 6.71-fold at 150 mM NaCl treatment in Osmancık-97 and Mevlütbey rice varieties, respectively ($P < 0.05$). The increasing salt concentration caused increased APX activities in Osmancık-97 and Mevlütbey varieties with the increasing rates of 8.2 and 8.57-fold, respectively at 210 mM NaCl treatment ($P < 0.05$).

The CAT activity in each rice varieties under different NaCl concentrations was evaluated and the changes were given in Fig. 2c. Statistically significant decreases in CAT activity were observed in both varieties compared to control plants at all NaCl concentrations ($P < 0.05$). At 210 mM NaCl stress treatment, Mevlütbey variety showed 74.74% decrease in CAT activity, while a 78.42% decrease was observed in Osmancık-97 variety.

In both rice varieties, a gradual increase in GR enzyme activities in both varieties in accordance with increasing NaCl doses was observed. After 210 mM NaCl treatment, the enzyme activity was increased by 3.14-fold in Mevlütbey variety, whereas 3.53-fold increase was recorded in Osmancık-97 rice variety. These changes in GR enzyme activity were found statistically significant ($P < 0.05$) (Fig. 2d).

The SOD activity was increased with increasing NaCl doses in both rice varieties and the changes of SOD activity were found statistically significant ($P < 0.05$) and were given in Fig. 2e. The highest SOD activity was observed at 210 mM NaCl treatment. Mevlütbey variety showed higher increase at the highest salt exposure than Osmancık-97 rice variety.

Expression of antioxidant defense genes

Transcript levels of antioxidative metabolism-related genes in two salt stressed rice varieties (Osmancık-97 and Mevlütbey) were compared in this study. Fold changes of the genes involved in the antioxidative metabolism under salt stress are determined. NaCl treatment caused changes in expression profiles of the genes that have role in scavenging mechanism.

Expression of genes encoding APX enzyme

The transcript levels of APX cytoplasmic (cyt), stromal (str) and thylakoid (thy) genes were given in Fig. 3. Cyt-APX expression showed a significant induction in all treatment groups of Osmancık-97 variety with respect to control group ($P < 0.05$). The expression levels of Cyt-APX gene increased after 30 and 90 mM NaCl treatments in Mevlütbey rice variety, and the levels decreased at 150 and 210 mM NaCl exposure (Fig. 3a).

Fig. 2 The effect of salt stress on **a** Guaiacol peroxidase, **b** Ascorbate peroxidase, **c** Catalase, **d** Glutathione reductase, **e** Superoxide dismutase enzyme activities in salt stressed Osmancık-97 and Mevlütbey rice varieties under 0, 30 mM, 90 mM, 150 mM and

210 mM NaCl stress. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The enzyme activities were evaluated and compared with the levels in the control group. The differentially given letters represent significance at the 0.05 level

Expression of *str*-APX was up-regulated in all salt concentrations in both rice varieties. It induced by 22-fold at 150 mM NaCl treatment in Osmancık-97 variety, but at higher NaCl concentrations, it decreased sharply ($P < 0.05$). In Mevlütbey variety, the expression was slightly up-regulated at 210 mM in comparison with Osmancık-97 (Fig. 3b).

Thy-APX expression was down-regulated in Mevlütbey variety at all treatments. In contrast, the expression increased after 210 mM NaCl treatment in Osmancık-97, while showing a decrease pattern at other concentrations (Fig. 3c).

Expression of genes encoding CAT and GR enzymes

The expression patterns of CAT A was down-regulated at all salt concentrations in both varieties (Fig. 4) with respect to control ($P < 0.05$). CAT A transcript levels were found

in relation with NaCl treatments in Osmancık-97 variety ($P < 0.05$) (Fig. 9c) in contrast to Mevlütbey variety.

The expression pattern of gene encoding enzyme Cyt-GR1 showed a gradual up-regulation in response to increasing NaCl stress in Mevlütbey variety (Fig. 5). Transcript levels of Cyt-GR1 showed positive correlation with NaCl treatments (Fig. 8d) ($P < 0.05$). On the other hand, in Osmancık-97 rice variety, the transcript level was down-regulated at 30 mM. At higher NaCl stress treatments, Cyt-GR1 gene expressions were up-regulated ($P < 0.05$).

Expression of genes encoding SOD enzyme

Expressions of three types of SOD genes, Fe-SOD, Cu-Zn/SOD and Mn-SOD, under salt stress were investigated. Transcript level of Fe-SOD markedly decreased after

Fig. 3 The relative expression levels of **a** *Cyt-APX*, **b** *Str-APX* and **c** *Thy-APX* genes in salt stressed Mevlütbey and Osmancık-97 rice varieties treated with 30 mM, 90 mM, 150 mM and 210 mM NaCl for 7 days. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

treatment in both rice varieties (Fig. 6) ($P < 0.05$). The other SOD gene Cu–Zn/SOD was down-regulated in Osmancık-97 at all NaCl concentrations, whereas 1.36-fold up-regulation was observed only at 90 mM NaCl treatment in Mevlütbey variety in contrast to decrease at other salt concentrations. Additionally, Mn-SOD expressions were

found in correlation with NaCl concentrations in Mevlütbey (Fig. 8a) and Osmancık-97 varieties (Fig. 9a) ($P < 0.05$).

Expression of proline metabolism-related genes

In both rice varieties, the transcript levels of proline metabolism-related genes (P5CS, P5CR, PDH and P5CDH) were investigated. The expression level of P5CS gene was up-regulated with increasing NaCl in both varieties (Fig. 7a–b). In Mevlütbey variety, NaCl treatments were found in relation with increasing P5CS gene expression ($P < 0.05$) (Fig. 8e). P5CR gene expression was significantly up-regulated by 1.6 fold at 210 mM NaCl treatment in Osmancık-97 variety. In contrast, the expression of P5CR was up-regulated by 1.8-fold at 150 mM NaCl, then down-regulated to 30 mM levels at 210 mM in Mevlütbey variety. The highest decreased PDH gene expression level was observed at 30 mM NaCl treatment in Mevlütbey variety. By subjecting to higher concentrations of NaCl, the expression level of PDH showed 25% and 34% increase in Mevlütbey variety at 150 and 210 mM NaCl treatments, respectively ($P < 0.05$). In Osmancık-97 rice variety, we observed 23% and 49% decreased profile at 150 mM and 210 mM salt treatments, respectively ($P < 0.05$). P5CDH expression was down-regulated in both varieties ($P < 0.05$). Transcript levels of P5CR and PDH genes showed positive correlation with NaCl treatments for Osmancık-97 variety (Fig. 9e) ($P < 0.05$).

Discussion

Salt stress inhibits plant growth and development through several processes. When plants were subjected to different levels of salt stress, photosynthetic mechanism is the primary target with reduced activities leading to decreased growth and productivity (Wang et al. 2003). Salt tolerance is a multigene trait and different group of genes are functional for the tolerance of the plants (Belghith et al. 2018; Sytar et al. 2017). This is the first study evaluating the antioxidative profile of both local varieties having different salt tolerance capacities. In this study, we systematically investigated antioxidative defence related enzyme activities and gene expression profiles of two rice cultivars, Osmancık-97 and Mevlütbey.

We observed that the different concentrations of salt differentially affected the two rice cultivars. Salt stress is a complex stress including osmotic stress, specific ion effect, nutrient deficiency. As an expected result of the ionic stress, various ROS species were generated. ROS can affect the plant photosynthetic apparatus (Zhang et al. 2013). Sodium chloride content in the nutrient solution inhibited

Fig. 4 The relative expression levels of *CAT A* gene in salt stressed Mevlütbey and Osmancık-97 rice varieties treated with 30 mM, 90 mM, 150 mM and 210 mM NaCl for 7 days. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

Fig. 5 The relative expression levels of *Cyr-GR1* gene in salt stressed Mevlütbey and Osmancık-97 rice varieties treated with 30 mM, 90 mM, 150 mM and 210 mM NaCl for 7 days. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

plant growth through reduction in plant height, root length and fresh weight.

Higher lipid peroxidation rate is evaluated as an sign of oxidative damage at cellular level (Liu et al. 2014) caused by salt stress, although some salt tolerant rice and tomato species were reported to have lower lipid peroxidation ratios (Bor et al. 2003). The increase in membrane permeability or loss of integrity of membrane causes sensitivity to salt stress due to increasing lipid peroxidation (Azevedo Neto et al. 2006).

Soluble protein content is an important indicator of changes in metabolism of a plant to reveal the effects of different kind of stressors (Luo et al. 2011). Protein contents were reported to vary in different salt stressed plants (Abdul Qados 2011). Protein content was affected by salt stress differently in two different rice cultivars depending on the NaCl concentrations. In Mevlütbey variety, an increase was observed between 30 and 150 mM NaCl while a significant decrease was observed at 210 mM NaCl treatment. In Osmancık-97 variety, total protein content was increased at 30 and 90 mM NaCl treatments with respect to control but we didn't observe any significance between treatments. After 150 mM NaCl treatment, the total protein level was decreased to the control level while

an increase was observed at 210 mM NaCl treatment ($P < 0.05$). These results were consistent with the results of Abdul Qados (2011), who also determined the protein content to decrease with the increased salt stress in shoot tissues of bean.

Plants have different scavenging mechanisms against extreme conditions like salinity (Barkla et al. 2013). Free proline accumulation is one of these mechanisms to act as a non-enzymatic antioxidant. It prevents oxidative damage by scavenging of hydroxyl radicals as a singlet oxygen quencher. Proline metabolism is under balance between control of biosynthesis and degradation reactions. Proline is considered both as a symptom of a injury and an indicator of salt tolerance (Çelik and Atak 2012b; Lutts et al. 1999). Salt sensitive rice genotypes were reported to accumulate more proline than tolerance genotypes (Demiral and Türkan 2005; Lutts et al. 1999). In the present study, the salt tolerance capacities of two rice cultivars were evaluated according to their proline accumulations and lipid peroxidation rates. Osmancık-97 variety was found to be more salt tolerant as it exhibited lower lipid peroxidation rate and lower proline accumulation than Mevlütbey-97 variety. In both rice varieties, transcript levels of P5CS was induced with increasing salt

Fig. 6 The relative expression levels of *SOD* genes in salt stressed Mevlütbey (a) and Osmancık-97 rice varieties (b) treated with 30 mM, 90 mM, 150 mM and 210 mM NaCl for 7 days. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

concentrations. P5CS is one of the biosynthetic genes in proline pathway. It produces NADP⁺ and stimulate pentose phosphate pathway to control redox potential in the cell (Kim et al. 2007; Silva-Ortega et al. 2008). The main role of P5CS is argued to be more than a basic proline metabolism. It is suggested to have a more significant role against salt stress. Proline accumulation is thought to accumulate as a result of salt stress (Kim et al. 2007). P5CS reduces glutamate to glutamyl-5-semialdehyde (GSA) and it is converted to pyrroline-5-carboxylate (P5C) and it is then reduced to proline by P5CR (Silva-Ortega et al. 2008; Zhang et al. 2014). According to our results, P5CR transcript level was increased in both rice varieties as a consequence of increasing salt concentrations, in consistent with P5CS expressions. The second alternative mechanisms is inhibition of proline degradation pathway. It is controlled by PDH and P5CDH enzymes in mitochondria. Synergetic actions of induction of biosynthetic genes and inhibition of degradative genes are observed in both rice varieties during stress treatments. We statistically

evaluated the relationships among proline metabolism related gene expressions and NaCl concentrations by Pearson correlation analysis, respectively. The responses of two different rice varieties were found different. While only P5CS gene expression level was associated with NaCl treatment for Mevlütbey variety, transcript levels of P5CR and PDH genes were found in relation with NaCl treatment in Osmancık-97 rice variety ($P < 0.05$). The reason of this differentiation is the salt responsive mechanisms between rice varieties. In Osmancık-97, up-regulation of anabolism related genes of proline metabolism, P5CS and P5CR, beside catabolism-related gene PDH, indicates difference in salt stress response of this variety. P5CS seems to be main salt-responsive gene in proline synthesis in Mevlütbey variety, whereas, the relation between P5CR and PDH expressions seem to maintain proline balance in Osmancık-97 in response to salinity.

Every kind of cell has a dynamic balance between production and elimination of ROS. This equilibrium can be perturbed by abiotic stresses such as salinity. Increased

Fig. 7 The relative expression levels of proline mechanism-related genes in salt stressed Mevlütbey (a) and Osmancık-97 rice varieties (b) treated with 30 mM, 90 mM, 150 mM and 210 mM NaCl for 7 days. The data are represented as the mean $\pm$ SD and derived from 3 replicates. The differentially given letters represent significance at the 0.05 level

ROS affects cellular redox balance and oxidative stress improves. Several rice varieties were biochemically evaluated for their antioxidant capacities (Demiral and Türkan 2005; Lee et al. 2001, 2013). Understanding of antioxidant responses to salt stress at transcriptomic level is essential to suggest new strategies to improve abiotic stress tolerances. Different isoforms of antioxidative genes might be differentially regulated under extreme environmental conditions (Ara et al. 2013; Chakraborty and Bhattacharjee 2015). SODs are elementary enzyme systems in ROS defense. SOD enzymes were placed in different parts of the plants and their expression is regulated by developmental and environmental signals (Menezes-Benavente et al. 2004). In this study, we investigated the prime antioxidant transcript levels of two rice varieties which were subjected to different NaCl concentrations at seedling stage. 14 day-old seedlings were exposed to NaCl stress for 7 days and Fe-SOD and Cu–Zn/SOD transcripts were decreased while

Mn-SOD transcripts were up-regulated. Cu–Zn/SOD isoenzyme transcripts were reported to be up-regulated by 0.25 M NaCl treatment (Menezes-Benavente et al. 2004). We observed down-regulation of Cu–Zn/SOD and Fe-SOD genes as a consequence of increasing salt stress treatments. Fe-SOD expression was reported to remain stable during salt stress although, up-regulation of Cu–Zn/SOD gene was observed in time-dependent manner. (Kim et al. 2007). The induction of Fe-SOD and Mn-SOD isoforms were found important in tolerant genotypes of Cucurbita species against heat stress (Ara et al. 2013). Fernández-Ocaña et al. (2011) reported that all of the SOD genes were differentially regulated in response to environmental stimuli in sunflower. In both varieties, we determined positive correlation between increasing NaCl concentrations and the transcript level of Mn-SOD gene ($P < 0.05$). Mn-SOD has been known to be induced by salt stress (Wang et al. 2004). Mn-SOD genes could be evaluated as an indicator and a

Fig. 8 Relationships between NaCl concentrations, antioxidative system enzymes, proline content and the metabolism-related genes of salt stressed Mevlütbey rice variety, which exposed to 30, 90, 150

and 210 mM NaCl stress. The statistically significant relations were indicated by ★

potential sensor to warn the cells to express other SOD genes as an early signal (Fernández-Ocaña et al. 2011). In our study, the up-regulation of Mn-SOD seemed to trigger increased SOD activity determined in biochemical analyses in both rice varieties.

Many studies have reported that salt stress induces the activity of APX in various plant species. Therefore, we evaluated the expression profiles of APX isoforms (cytoplasmic (Cyt-APX), stromal (Str-APX) and thylakoid (thy-APX)). Treatment with NaCl increases the activity of APX enzyme in both rice varieties. Several studies have showed that the salinity induces APX activity in different plant species like other abiotic stresses. Morita et al. (2011) observed increase in expression profile of cytosolic APX of rice in response to chilling stress. Cytosolic APX and chloroplastic thylakoid APX were found down-regulated under salt stress in rice in contrast to our findings (Kim et al. 2005).

APX and CAT enzymes act together to convert H_2O_2 to H_2O and O_2 . CAT A is known to have role in detoxification of hydrogen peroxide during the process of photorespiration. The main reason is supposed to be the gradual increasing H_2O_2 content in the cells under increasing NaCl stress as a toxic product during metabolic processes. Induction of CAT A by salt stress was reported by Menezes-Benavente et al. (2004). Liu et al. (2014) reported increased CAT activity together with SOD under drought-stress treated tobacco plants. The CAT activity reduced in rice in response to salt stress (Kim et al. 2007). Yin et al. (2014) reported the CAT A isoform as the main H_2O_2 scavenging enzyme in the aged rice seeds which have specific role in redox balance. In our study, we observed reduction in CAT A gene expression with increasing NaCl treatments which is correlated with decreased CAT enzyme activity findings determined biochemically in Osmancık-97 rice variety ($P < 0.05$).

Fig. 9 Relationships between NaCl concentrations, antioxidative system enzymes, proline content and the metabolism-related genes of salt stressed Osmancik-97 rice variety, which exposed to 30, 90,

150 and 210 mM NaCl stress. The statistically significant relations were indicated by ★

One of the key steps of ROS detoxification mechanisms which is aimed to decrease cellular ROS accumulation, is ascorbate–glutathione cycle. GR enzyme activity is important in glutathione regeneration in this cycle. GR enzyme transforms glutathione disulfide (GSSG) to glutathione. One cytosolic and two chloroplastic GR isoforms have been identified in rice (Wu et al. 2013). The importance of chloroplastic GRs in stress tolerance has been showed by several studies (Ding et al. 2012; Shu et al. 2011). In present study, we determined the expression of cytosolic GR in response to salinity. Mevlütbey variety showed increased GR1 activity with increasing salt concentrations. Osmancik-97 rice variety regressed the increased transcript level of GR1 at 210 mM NaCl treatment and the rate was found below the 150 mM NaCl. Kim et al. (2007) reported that the regulation of GR enzyme is differentially controlled by different subcellular parts of the rice leaves. Yin et al. (2014) didn't observe any change in transcript level of GR1. The expressional differences of

cytosolic GR gene between rice varieties is suggested to be important to determine the possible role of functional GR1 in salt tolerance. The increases in expression level of GR1 was correlated with GR enzyme activity and increasing NaCl concentration for Mevlütbey variety ($P < 0.05$). The cytosolic GR activity is thought to be the reason of this profile. Cytosolic (GR2) and other chloroplastic GR (GR3) were reported to be induced by salt stress in rice seedlings by Wu et al. (2013). They also reported steady-state transcript number of GR1 under salt-stressed rice roots and embryos which is contrary to our findings.

Conclusion

In conclusion, the results indicate that the salt responsive mechanisms between two rice varieties are different. The expression patterns and correlation analysis reveal that some key regulatory gene families involved in abiotic

stress are differentially expressed in Osmancık-97 and Mevlütbey rice varieties. The transcriptional regulation of these genes suggests adaptive reactions of rice plants to salinity. We observed some key genes which can be functional in determining the tolerance capacities of the varieties. Cyt-GR1 and P5CS gene expression levels were found statistically significant in Mevlütbey variety in response to salt treatments, while the relation between NaCl and CAT A, P5CR, PDH transcript levels were found significant in Osmancık-97. In our study, Osmancık-97 variety had higher proline content than Mevlütbey variety in control plants. In parallel with accumulated proline, proline-metabolism related genes were differentially regulated in response to salt stress in both rice varieties. In accordance with increasing NaCl concentration, we observed an induction in catabolic pathway genes for Mevlütbey variety. The transcriptional homeostasis between two pathways were obtained in Osmancık variety. Our study preliminarily indicated the salt tolerance capacities and underlying antioxidative responses in two rice varieties. The studies focusing on these subjects will certainly prime the tolerance metabolism genes in salt tolerance breeding studies.

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