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Efficacy of ascorbate-glutathione cycle for scavenging H_2O_2 in two contrasting rice genotypes during salinity stress

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Abstract

Twenty day-old seedling of Pokkali (salt-tolerant) and BRRI dhan 29 (salt-sensitive) rice genotypes were exposed to 0, 75 and 150 mM NaCl salinity levels for 7 days to investigate the efficacy of ascorbate-glutathione cycle for scavenging H_2O_2 during salinity stress. Results revealed that salinity caused a significant decrease in photosynthesis rate (A), stomatal conductance (g_s), chlorophyll content and shoot dry matter yield, with a greater reduction in the sensitive genotype, BRRI dhan 29. The activity of SOD (super oxide dismutase), an H_2O_2 generating enzyme increased significantly due to salinity in both the rice genotypes. Pokkali exhibited increased activities of Fe-SOD and Cu/Zn-SOD while Mn-SOD was in BRRI dhan 29. With increasing salinity levels, H_2O_2 and Thiobarbituric acid reactive substances (TBARS) content were increased significantly in both the genotypes with a higher magnitude in BRRI dhan 29. Both rice genotypes exhibited an increased activity of Peroxidase (POD) and a decreased activity of Catalase (CAT). The salt-tolerant genotype, Pokkali exhibited increased activity of ASC-GSH cycle enzymes (APX, MDHAR, DHAR and GR) during salinity stress. But salt-sensitive, BRRI dhan 29 exhibited a significant decreased activity of APX, increased activity of DHAR and unchanged activity of Monodehydroascorbate reductase (MDHAR) and GR during salinity stress. The results clearly indicated that ASC-GSH cycle responded differentially in salt-tolerant and salt-sensitive rice genotypes during salinity stress.

Keywords: Antioxidant enzymes, Ascorbate-glutathione cycle, Ascorbate peroxidase, Dehydroascorbate reductase, Salinity stress, Glutathione reductase, Oxidative stress.
Abbreviations: ASC_Ascorbate, GSH_Reduced Glutathione, SOD_Superoxide dismutase, CAT_Catalase, POD_Peroxidase, APX_Ascorbate peroxidase, MDHAR_Monodehydroascorbate reductase, DHAR_Dehydroascorbate reductase, GR_Glutathione reductase, BRRI_Bangladesh Rice Research Institute, TBARS_Thiobarbituric acid reactive substances, ABA_Abscisic acid.

Introduction

Salinity is one of the most important abiotic stresses that adversely affect agricultural productivity and causes significant crop loss worldwide (Munns and Tester, 2008). Adverse plant responses to salinity stress depend on the osmotic and toxic effects of salt since they can cause a serious metabolic impairment in the plant cell (Munns 2002; Vaidyanathan et al., 2003). Excess reactive oxygen species (ROS) generation is one of the outputs of such metabolic impairment during salinity stress (Dionisio-Sese and Tobita 1998; Noctor and Foyer 1998). Generally, ROS such as $O_2^{\cdot -}$ (superoxide radical), H_2O_2 (Hydrogen peroxide) and OH^- (Hydroxyl radical) are formed by successive reduction of molecular O_2 during aerobic metabolism in mitochondria (Mittler et al., 2004). In addition, plant cell also generates $^1O_2$ (singlet oxygen) in the chloroplast during photosynthesis (Asada, 2006; Muller et al., 2001) and H_2O_2 in the peroxisomes during photorespiration (Corpas et al., 2001). Reactive oxygen species are also generated at the plasma membrane level or extracellularly in the apoplast (Foyer and Noctor 2000; del Rio et al., 2002). Therefore, ROS generation is an inevitable consequence of plant metabolism and amplified in response to adverse environmental conditions. Since ROS are highly reactive, their excess accumulation may lead to the oxidative damage of various cellular components such as lipids, proteins, nucleic acids, chlorophyll and also may affect cell membrane properties (Mittler, 2002). During salinity stress, the excess generation of ROS results from impaired electron transport processes in chloroplast and mitochondria as well as from pathway such as photorespiration (Bauwe, 2010; Moradi and Ismail 2007; Vaidyanathan et al., 2003; Wang et al., 2009). Generally, plants are equipped with an array of non-enzymatic scavengers and antioxidant enzymes to reduce oxidative damage caused by excessive ROS production (Foyer and Noctor, 2005). Among the different antioxidant pathways, the ascorbate–glutathione (ASC–GSH) cycle has been regarded as the most important one (Noctor and Foyer, 1998). In this cycle, H_2O_2 is reduced to H_2O by ascorbate peroxidase (APX) using ASC as the specific electron donor (Smirnoff, 2000). At the same time, the ASC is regenerated from dehydroascorbate (DHA) by dehydroascorbate reductase (DHAR) at the expense of reduced glutathione (GSH), yielding oxidized glutathione (GSSG). Finally, GSSG is converted to GSH by receiving electron from NADPH due to the action of an NADPH-dependent GR. Both ASC and GSH are linked through the ASC-GSH cycle and are the most abundant low molecular weight non-enzymatic antioxidants in the plant cell. They can directly interact with $^1O_2$, $O_2^{\cdot -}$, and OH^- and thus contribute significantly to non-enzymatic ROS scavenging (Asada, 1999; Smirnoff and Wheeler, 2000). A differential antioxidant response and oxidative damage have been reported in salt-tolerant and salt-sensitive rice seedlings

(Dionisio-Sese and Tobita, 1998; Moradi and Ismail, 2007; Vaidyanathan et al, 2003; Wang et al., 2009). This cycle plays an important role in keeping the equilibrium between H_2O_2 generation and scavenging in the chloroplast (Asada 2006) since H_2O_2 at a concentration of 10μM in the chloroplasts has been reported to cause a net reduction in photosynthesis in plants by over 50% (Kaiser, 1979). However, scant attention was paid to focus the relevancy of ASC-GSH cycle and its key components involved in oxidative stress tolerance in rice. Our present hypothesis is that the ASC–GSH cycle components may respond differentially in salt-tolerant and salt-sensitive rice seedlings during salinity stress. Therefore, the current study is designed to investigate the efficacy of ASC-GSH cycle for scavenging H_2O_2 and to identify the key components of the cycle involved in oxidative stress tolerance in rice.

Results

Seedling growth

Effect of salinity stress on shoots dry matter (DM) yield in two rice genotypes, namely Pokkali (Salt-tolerant) and BRRI dhan 29 (Salt-sensitive) was shown in the Table 1. Shoots D.M. content reduced in both genotypes in the presence of 75 and 150 mM NaCl in the culture solution. Pokkali displayed 10 and 20% reduction in shoot DM yield over the control while it was 20 and 43% in BRRI dhan 29 after 7 days exposure to 75 and 150 mM NaCl salinity. The result indicated that Pokkali was more efficient in shoots D.M. production than BRRI dhan 29 during salinity stress.

Photosynthesis rate and Stomatal conductance

Table 1 showed the changes in photosynthesis rate (A) and stomatal conductance (g_s) after one-week of imposition to salinity stress. The rate of photosynthesis declined in both genotypes at 75 and 150 mM NaCl salinity stress condition. Pokkali maintained 93% (average) photosynthesis of the control under salinity stress. In contrast, BRRI dhan 29 produced 80 and 61% photosynthesis of the control under 75 and 150 mM NaCl salinity stress, respectively. The results indicated that salinity impacted on photosynthesis rate seriously in the salt-sensitive, BRRI dhan 29. Genotypic difference was also observed in stomatal conductance (g_s) under salinity stress and the trend was similar to that of photosynthesis rate. A positive correlation between A and g_s (R^2= 0.96) was observed in both rice genotypes (data not shown).

H_2O_2, TBARS and Chlorophyll content

Among the reactive oxygen species (ROS), H_2O_2 is relatively long-lived molecule that can diffuse some distances from its production site and the rice genotypes significantly varied in H_2O_2 concentration in leaf tissues both under salinity stressed and non-stressed condition (Table 1). At 75 and 150 mM NaCl salinity levels, H_2O_2 content was always higher in BRRI dhan 29 than in Pokkali. The level of lipid peroxidation, expressed by TBARS content did not vary in the rice genotypes under control condition. But genotypic variation was evident upon exposure to salinity (Table 1). In fact, the magnitude of lipid peroxidation was greater in BRRI dhan 29 than in Pokkali after one-week exposure to 75 and 150 mM NaCl salinity stress. In the control plants, chlorophyll content was lower in Pokkali than in BRRI dhan 29. But 150 mM NaCl caused a significant decrease in chlorophyll content in both genotypes and Pokkali exhibited less chlorophyll damage than BRRI dhan 29, as expressed by chlorophyll content.

H_2O_2 metabolizing enzymes (SOD, CAT and POD) activities

Superoxide dismutase (SOD) activity in the leaves of the control seedlings was similar in the both rice genotypes. At 150 mM NaCl salinity level, SOD activity increased by 136% when compared to that of the respective controls but genotypic difference was insignificant (Fig. 1a). However, genotypic difference existed in the activity of its different isoforms, such as Fe-SOD, Cu/Zn-SOD and Mn-SOD. Both at 75 and 150 mM NaCl salinity levels, Pokkali exhibited stronger activity of Fe-SOD and Cu/Zn-SOD than BRRI dhan 29 (Figs. 1b and 1c). In contrast, BRRI dhan 29 displayed a stronger activity of Mn-SOD than Pokkali under salinity stress (Fig. 1d). Catalase activity in the seedlings of two rice genotypes was measured under salinity stress. CAT activity declined by 12 and 22% in Pokkali, and 20 and 40% in BRRI dhan 29 at 75 and 150 mM NaCl salinity, respectively (Fig. 2a). Peroxidase activity increased due to salinity and it was 3.3 folds higher than that in the control at 75 mM NaCl salinity in both the genotypes. However, the genotypes showed a significant difference of POD activity at 150 mM NaCl salinity and it was 4.4 and 4.7 folds higher over the control in Pokkali and BRRI dhan 29, respectively (Fig. 2b). Both ASC and GSH were also involved in scavenging of different ROS via non-enzymatic and enzymatic means. Figure 2c and 2d showed that salinity stress enhanced the level of ASC and GSH in the leaf tissues of Pokkali while a remarkable reduction was observed in BRRI dhan 29.

ASC-GSH cycle enzymes

In Pokkali, APX activity was 1.2 and 1.8 folds higher at 75 and 150 mM NaCl salinity stress when compared to that of the control (Fig. 3a). However, APX activity was 85% of the control at 150 mM NaCl salinity stressed seedlings of BRRI dhan 29. A significant difference of MDHAR activity was observed between the salt-tolerant and the salt-sensitive rice seedlings under salinity stress (Fig. 3b). MDHAR activity increased by 120 and 155% when seedlings of Pokkali were exposed to 75 and 150 mM NaCl containing nutrients solution for 7 days. However, no significant difference in MDHAR activity was observed between salinity stressed and non-stressed seedlings of BRRI dhan 29. Dehydroascorbate reductase activity significantly increased in Pokkali in response to both salinity levels (Fig. 3c). At high salinity level (150mM NaCl), Pokkali showed 1.7 folds higher DHAR activity than its activity found in the control. In case of BRRI dhan 29, DHAR activity increased by 1.4 folds at 150 mM NaCl salinity level compared to the control. Glutathione reductase activity increased in the leaves of the tolerant (Pokkali) due to salinity and it was 1.5 and 2-folds higher at 75 and 150 mM NaCl salinity level, respectively compared to its activity in the control (Fig. 4d). In contrast, GR activity in the leaves of BRRI dhan 29 did not change under both salinity levels.

Discussion

Photosynthesis and Seedling growth

Salinity stress significantly decreased shoot dry matter yield in both rice genotypes in the present study (Table 1). At both salinity levels, shoot DM yield reduction was greater in

Table 1. Effect of salinity stress on shoot dry weight (mg seedling^{-1}), photosynthesis rate (µmolCO_2 m^{-2}s^{-1}), stomatal conductance (mmol m^{-2}s^{-1}), total chlorophyll (mgg^{-1}FW), H_2O_2 (µmol g^{-1}FW) and TBARS (nmol g^{-1}FW) content of two rice genotypes. Twenty one (21) days old seedlings were exposed to treatment solution for 7 days. Results are mean of four replicates ± standard errors (SE).

Parameter	Pokkali			BRRIdhan-29		
	Salinity levels (mM NaCl)					
	0	75	150	0	75	150
Shoot dry weight	445 ±7.19	401 ±8.21	356±8.31	435 ±3.71	348±5.23	248 ±7.80
Photosynthesis rate	16.26 ±0.67	15.57 ±0.53	14.88±0.44	18.85 ±0.58	15.05 ±0.80	11.43 ±0.38
Stomatal conductance	0.41 ±0.03	0.38 ±0.01	0.36 ±0.03	0.45±0.05	0.36±0.02	0.32 ±0.01
H_2O_2	5.0±0.23	6.5±0.52	7.5±0.65	5.4±0.39	7.7±0.55	11.10±0.27
TBARS	64±2.31	70±1.12	85±2.02	63±2.11	85±1.57	97±1.35
Total chlorophyll	1.63±0.04	1.39±0.05	1.36±0.05	1.78±0.03	1.41±0.04	1.23±0.03

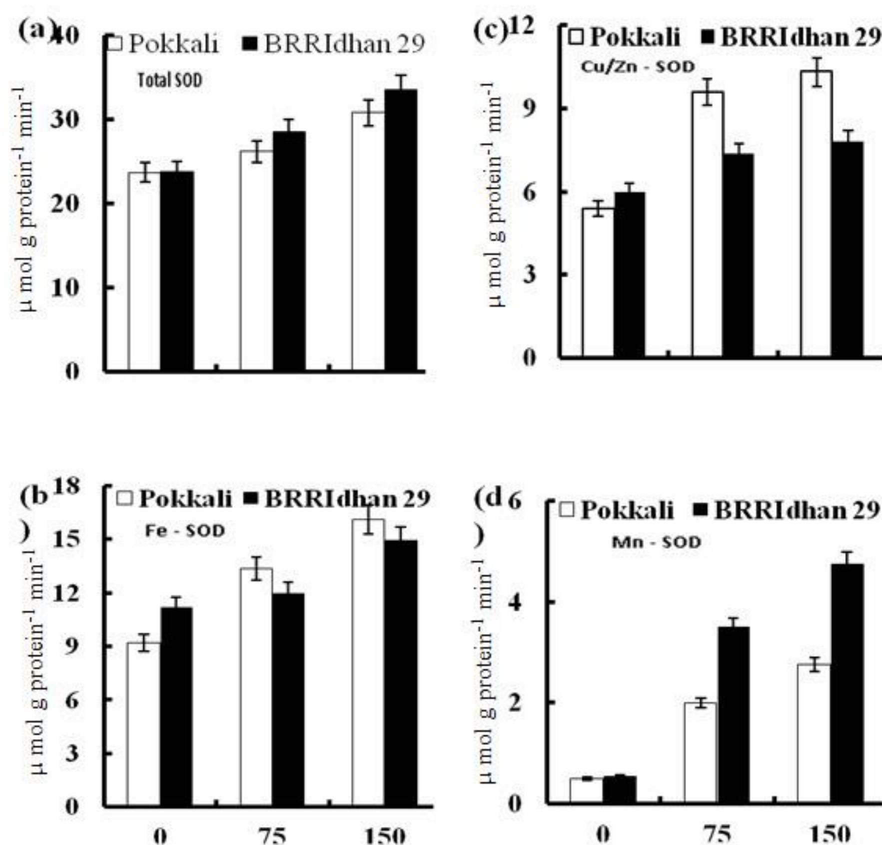

Fig 1. Effect of salinity on H_2O_2 generating enzymes (a) total SOD, (b) Fe-SOD, (c) Cu/Zn-SOD and (d) Mn-SOD activities in the leaves of two rice genotypes. Twenty one (21) days old seedlings were exposed to different treatment solutions (0. 75, 150 mM NaCl) for 7 days. Columns represent the mean of four replicates ± standard errors (SE).

BRRI dhan 29 than in Pokkali. Generally, DM yield is the output of photosynthesis in the plant cell (Zhu et al. 2010). Therefore, DM yield reduction during salinity stress might be the results of decreased rate of photosynthesis (Table 1). However, photosynthesis rate is directly regulated by stomata (Damour et al., 2010). Actually, stomatal conductance (g_s) is an important factor, which regulates CO_2 assimilation rate in plants (Lawlor and Tezara, 2009). Salinity stress declines g_s to CO_2 since ABA-mediated stomatal closure is dominant and widely accepted event in salinity stressed plants, especially glycophytes (Munns and Tester, 2008; Munns, 2002). Ultimately, salinity diminishes net CO_2 assimilation as g_s falls (Munns 2005). In the current study, Pokkali always maintained the greater photosynthetic rate (*A*) along with higher stomatal conductance (g_s) than BRRI dhan 29 under salinity stress and it was reflected in the shoot DM yield (Table 1). Salinity not only diminishes leaf CO_2 fixation rate (*A*) but also affects normal photosynthetic electron flow (Munns 2005; Vaidyanathan et al., 2003). Generally, excited chlorophyll of PSII passes electrons (e^-) to the PSI and forms reduced ferredoxin (Fd_{red}). Simultaneously, electron (e^-) is removed from H_2O and passes to PSII (Lawlor and Tezara 2009). Ultimately, electron transport system from H_2O to ferredoxin is completed. Then, the reduced ferredoxin (Fd_{red}) transfers electrons (i) to CO_2 for CH_2O (Calvin cycle), (ii) to O_2 in the Mehler reaction (photoreduction of O_2), and (iii) to Cytochrome b_6f complex to complete cyclic electron flow around PSI (cyclic photophosphorylation). Under water deficit condition, restricted CO_2 availability due to stomatal closure may lead to excess electron flow to O_2 at the reduction site of PSI and generates excess ROS such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (OH^-) (Tezara et al. 2008). It is generally accepted that the photosynthetic electron transport system

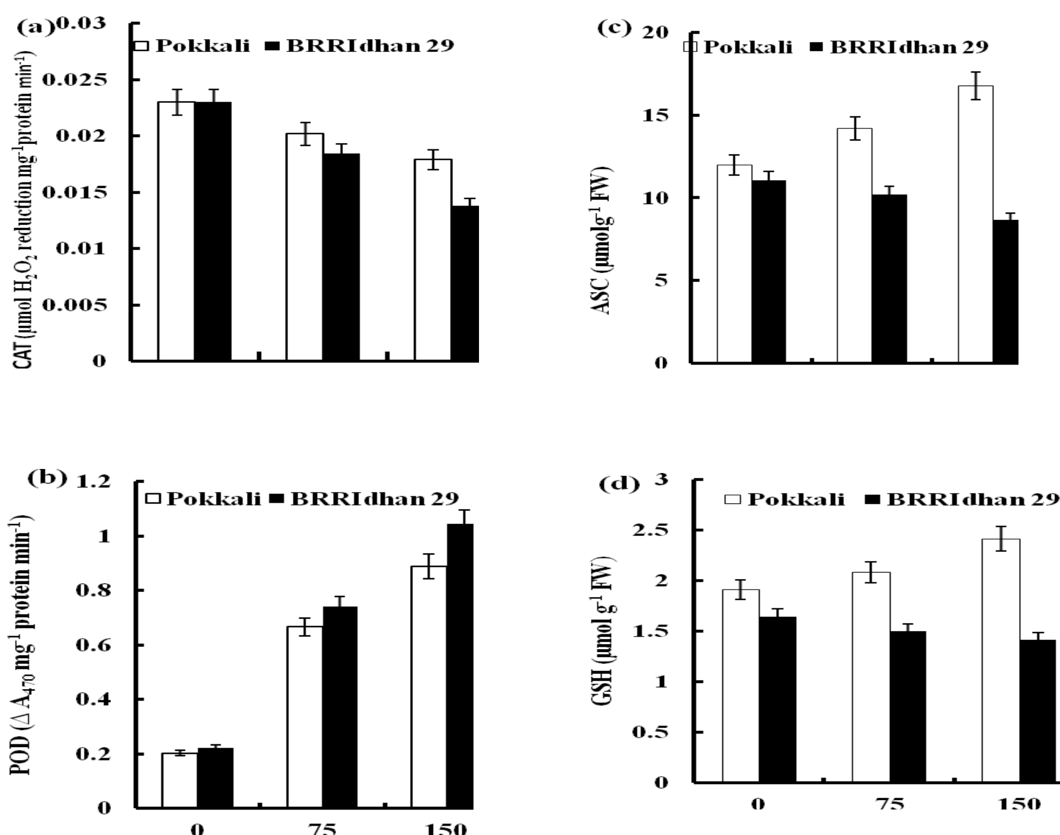

Fig 2. Effect of salinity on H_2O_2 scavenging enzymes (a) Catalase activity, (b) Peroxidase activity, and non-enzymatic antioxidants (c) ASC and (d) GSH content in the leaves of two rice genotypes. Twenty one (21) days old seedlings were exposed to treatment solution for 7 days. Results are mean of four replicates ± standard errors (SE).

produces excess ROS when CO_2 fixation is limited by environmental stresses (Asada 1999). In the present study, increased activity of SOD (Fig. 1) coupled with excess H_2O_2 content (Table 1) in the salinity treated rice leaves for both genotypes, suggested that a large proportion of the electron flux is diverted from CO_2 assimilation to O_2 reduction. The results are in agreement with the findings of several previous studies (Moradi and Ismail, 2007; Singh et al. 2007).

Effect of H_2O_2 on biomolecules

Being long lived molecule, excess H_2O_2 in the cell can also accelerate processes like Haber-Weiss / Fenton reaction, resulting the generation of hydroxyl radicals (OH^-) that can cause lipid peroxidation (Vranova et al., 2002). The measurement of TBARS content was taken as an index of lipid peroxidation in our study and the greater extent of lipid peroxidation was observed in BRRI dhan 29 under both salinity levels (Table 1). In contrast, seedlings of Pokkali exhibited a lower degree of lipid peroxidation under salinity stress. The excess H_2O_2 can also cause serious damage to organelles such as plasma membrane, chloroplast and mitochondria (Mittler, 2002). Chlorophyll content of the seedlings of control and salt-treated plants was taken as an index of photo-oxidative damage of chloroplast in this investigation and damage was always higher in BRRI dhan 29 than in Pokkali under salinity stress (Table 1). The results suggest that the sensitive genotype, BRRI dhan 29 mainly suffers from toxic level of H_2O_2 possibly due to the lack of efficient H_2O_2 detoxification mechanisms. It meant that rice genotypes under present investigation may have a differential antioxidant defense capacity against H_2O_2. In case of BRRI dhan 29, excess H_2O_2 may also inhibit the activities of Fe-SOD and Cu/Zn-SOD (Fig. 1b and 1c) since both are sensitive to H_2O_2 (Casano et al. 1997). Again, concentrations of H_2O_2 as low as 2 µM inactivate chloroplastic APX within several seconds when the level of ASC is too low (Miyake and Asada 1996). The H_2O_2 content (Table 1) and APX activity (Fig. 3a) along with ASC content (Fig. 2c) in BRRI dhan 29 during salinity stress could be harmonized with the aforesaid statement.

H_2O_2 scavenging via non-enzymatic route

Two non-enzymatic antioxidants, such as ASC and GSH for plant cells to dispose of H_2O_2 in some cellular compartments (Ashraf 2009; Chao et al. 2010; del Rio et al. 2002; Hossain et al 2012). For the study of non-enzymatic defense, we measured ASC and GSH in leaf tissues under salinity stressed condition. The increased ASC content in the leaves of salinity stressed Pokkali (Fig. 2c) might be accounted for increased activities of MDHAR and DHAR (Figs. 3b and 3d). The results suggested that sufficient ASC was present for scavenging H_2O_2 effectively by APX or non-enzymatic ways. Similarly, increased GSH in the leaves of Pokkali (Fig. 2d) may be accounted for increased activity of GR (Fig. 3d),

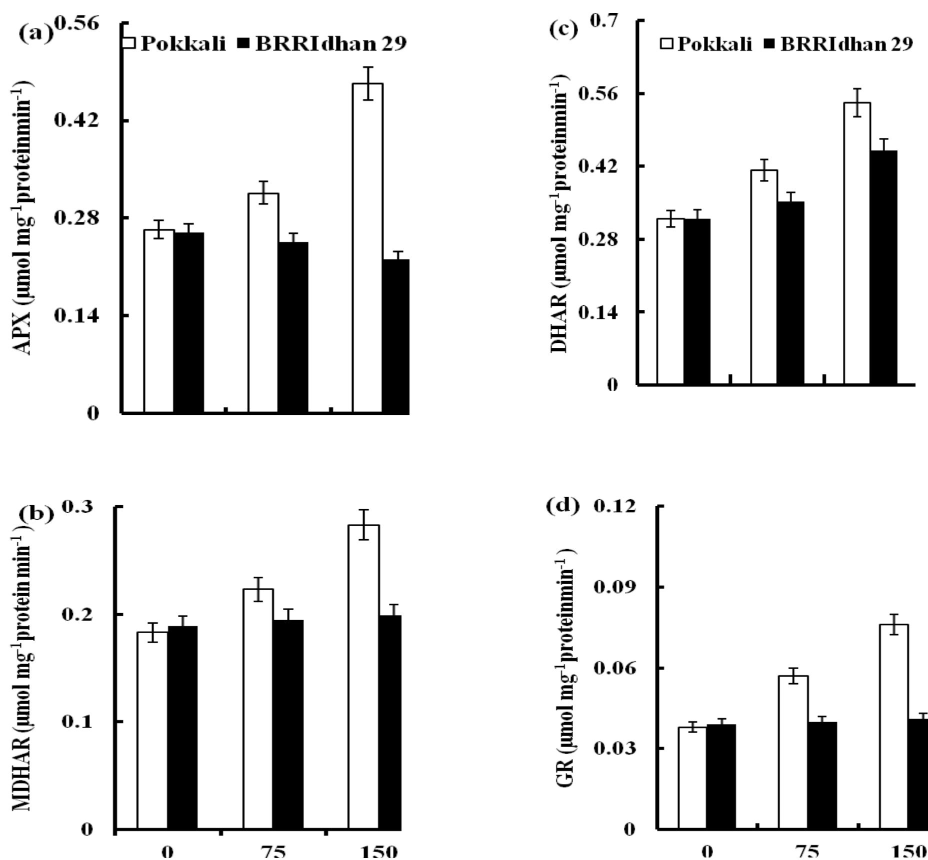

Fig 3. Ascorbate-glutathione cycle enzymes (a) APX, (b) MDHAR, (c) DHAR and (d) GR activities in leaves of 21days old rice seedlings were grown for 7 days at 0, 75 and 150 mM NaCl containing nutrients solution. Columns represent the mean of four replicates ± standard errors (SE).

which actively participates for scavenging H_2O_2 in co-ordination with ASC (Asada, 1999). Hence, H_2O_2 content (Table1) was decreased in Pokkali leaves during salinity. In contrast, salinity declined ASC content in the sensitive rice, BRRI dhan 29 (Fig. 2c). Previously some researchers reported that salt stress leads to a decrease in ASC content in salt-sensitive cultivars (Mittova et al., 2003; Shalata and Neumann, 2001). It is reasonable since the regeneration of ASC under salinity is insufficient or ASC synthesis is lower than ASC catabolism (Amor et al., 2006; Shalata and Neumann, 2001).

H_2O_2 scavenging via enzymatic ways

For the study of enzymatic defense, we measured some H_2O_2 detoxifying enzymes (CAT and POD) activities, which are not involved in the ASC-GSH cycle. In our study, CAT activity was decreased by 12 and 22, 20 and 40% in Pokkali and in BRRI dhan 29, respectively at 75 and 150 mM NaCl salinity levels (Fig. 2a). When CAT activity is depressed, the endogenous level of H_2O_2 is enhanced (del Rio et al., 2002) and increased H_2O_2 level in the leaves of salt-treated rice seedlings (Table 1) in the present investigation could be explained in the same way. However, POD activity (another H_2O_2 detoxifying enzyme) increased in both genotypes (Fig. 2b). Since the magnitude of POD activity was greater in BRRI dhan 29 than in Pokkali, it does not mean that BRRI dhan 29 was efficient as like as Pokkali to detoxify H_2O_2. This implies that increased POD activity alone could not compensate for the decreased activity of CAT. In other words, it is assumed that other H_2O_2 scavenging mechanisms might be present in the plant cell.

H_2O_2 scavenging via ASC-GSH cycle

Generally, ASC-GSH cycle involves in scavenging H_2O_2 in plant cell (Anjum et al. 2011) and hence, we also studied it. Four enzymes are included in the ASC-GSH cycle, namely APX, MDAR, DHAR and GR. Among them, APX enzyme directly participates in detoxifying H_2O_2 into H_2O and O_2 (Maruta et al. 2010). In our study, rate of APX activity in Pokkali was greater than that in BRRI dhan 29 under both salinity levels (Fig. 3a), indicating that some H_2O_2 was detoxified by APX using ASC as an electron donor. For regeneration of reduced ASC, two enzymatic reactions can run simultaneously (Asada 1999). The MDHAR, is one of the major enzymes of the ASC–GSH cycle, functions as a defensive enzyme against oxidative damage by ROS (Noctor and Foyer 1998). It utilizes NADPH for the regeneration of ASC (Asada 1999; Shalata and Neumann, 2001). The increase in MDHAR activity increases ASC regeneration, which can serve electron to H_2O_2 for its reduction in the presence of APX. Results in this study revealed that the increased activity of MDHAR (Fig. 3b) coupled with increased activity of APX in Pokkali (Fig. 3a) was accompanied by enhanced level of ASC (Fig. 2c) successfully detoxify H_2O_2 (Table 1) under salinity stress. In contrast, unchanged MDHAR activity (Fig. 3b) may be

responsible for lower regeneration of ASC in salt-sensitive BRRI dhan 29 under salt-stress conditions (Fig. 2c) and increased H_2O_2 content (Table 1) and thus unable to prevent photo-oxidative damage of chlorophyll and lipid peroxidation (Table 1) in our present investigation. And the DHAR, another important enzyme of ASC-GSH cycle for regeneration of ASC. We observed that BRRI dhan 29 displayed an increased DHAR activity (Fig. 3c) in response to salt stress accompanied by a decreased level of ASC (Fig. 2c) seems to be an exception to the general rule. The results suggested that ASC may be involved in H_2O_2 generation via non-enzymatic route particularly in BRRI dhan 29 under salinity stress since non-enzymatic route for conversion of $O_2^{\cdot -}$ to H_2O_2 using antioxidants like ASC is evident (Noctor and Foyer 1998). The result is in support of Anjum et al. (2010), who noticed an enhancement of DHAR activity in Cd-exposed plants that could not maintain ASC since ASC was utilized by some other metabolic functions. Similarly, an up-regulated DHAR activity was detected in drought sensitive wheat cultivar (Secenji et al., 2010). However, increased activity of DHAR leads to increased GSSG content in plant cells, which is regarded as oxidative stress and increased GR activity regarded as protective role (Moradi and Ismail 2007). Our study showed that GR activity was unchanged in BRRI dhan 29 at 75 and 150 mM NaCl salinity levels (Fig. 3d), suggesting that GSSG was not sufficiently reduced to GSH and thus decreased GSH content (Fig.2d). This clearly indicates that GR activity in salinity stressed seedlings of BRRI dhan 29 was insufficient to maintain the glutathione pool in its reduced form (GSH). In contrast, Pokkali exhibited increased activity of GR under salinity stress (Fig. 3d) and maintained sufficient GSH (Fig.2d), which was further oxidized to GSSG by enhanced activity of DHAR (Fig. 3c) activity thus yielding ASC. Then, ASC was utilized by APX for the detoxification of H_2O_2 and decreased H_2O_2 and lipid peroxidation as well (Table 1). Since over expression of GR increases antioxidant activity by maintaining sufficient GSH pools and improves tolerance to oxidative stress (Noctor et al., 1998; Moradi and Ismail 2007), our results could be explained in the same way.

Materials and methods

Plant material

Two rice (*Oryza sativa* L.) genotypes contrasting in salinity stress tolerance [Pokkali; salt-tolerant and BRRI dhan 29 (Bangladesh Rice Research Institute dhan 29); salt-sensitive] were selected for this investigation. Rice seeds of both genotypes were treated with 1% sodium hypochlorite (NaOCl) solution for 10 min and rinsed with deionized water for several times. Seeds were dipped in 3 mM $CaSO_4$ solution for 3 hours for accelerating germination. Then, seeds were soaked in water for 48 hours in the dark. Pre-germinated seeds of each genotype were sown in holes made on Styrofoam sheets with a nylon net bottom. Two seeds were sown per hole, with 50 holes per entry. The sheets were first floated on distilled water in 11 L plastic trays for 3 days, after which a nutrient solution (Yoshida et al. 1976) was used until the plants were 21 days old. A control treatment (nutrient solution + 0 mM NaCl) and two salinity stress conditions (nutrient solution + 75 mM NaCl, and nutrient solution + 150 mM NaCl) were imposed to the seedlings and the seedlings were harvested after one-week salinization. The culture solution was renewed once with the pH adjusted daily to 5.5 by adding either NaOH or HCl. This experiment was conducted in a growth cabinet with light intensity of about 700 µmol $m^{-2}s^{-1}$ and 12 hours duration, 70 % relative humidity and 29/22°C day/night temperature.

Growth measurements

For the measurements of seedling growth, 20 seedlings from each replication of control and salinity treatments were randomly selected and gently up-rooted. The seedlings were separated into roots and shoots. Then shoots were dried in an aerated oven at 70°C for 48 h and shoot dry weights were recorded.

Measurement of Photosynthesis rate and stomatal conductance

Gas exchange measurements i.e., net CO_2 assimilation/ photosynthesis rate (A) and stomatal conductance (g_s) were measured on the youngest fully expanded leaf at 7 days after treatment in experiment using a LI-6400 portable gas exchange system (LI-COR, Licoln, NE, USA). Each measurement of fully expanded leaves was repeated with leaves of similar age of three plants, between 9.00 and 15.00 hour. Leaf temperature was maintained at 25°C, light intensity was set at 1150 µmol photons $m^{-2}s^{-1}$ with a red/blue light source, CO_2 was set at 400 µmol m^{-1} (Changhai et al 2010).

Chlorophyll content

The total Chlorophyll content was measured according to the method of Arnon (1949) and expressed as mg g^{-1} fresh weight.

Lipid peroxidation

Thiobarbituric acid reactive substances (TBARS) were estimated as described by Hodges et al. (1999). The lipid peroxides was expressed as µmol TBARS g^{-1} FW by using an extinction coefficient of 155 $mM^{-1}cm^{-1}$.

Determination of H_2O_2

H_2O_2 concentration in the leaves of Pokkali and BRRIdhan 29 was measured spectrophotometrically using potassium Iodide (KI) as described by Alexieva et al. (2001).

Extraction and analysis of ascorbate and glutathione

Leaf samples were prepared for ascorbate (ASC) and reduced glutathione (GSH) analyses by homogenizing 1.0 g leaf material (FW) in 10 ml of cold 5 % meta-phosphoric acid (Gossett et al. 1994). The homogenate was centrifuged at 22000 g for 15 min at 4°C, and the supernatant was collected for analyses of ASC and GSH. Ascorbate (ASC) was measured according to Zhang and Kirkham (1996). Total ascorbate was determined after reduction of DHA to ASC with DTT, and the concentration of DHA was estimated from the difference between (ASC+ DHA) and ASC. Content of ASC was expressed as µmol g^{-1} FW. GSH and GSSG were assayed according to the methods of Griffith (1985). Content of GSH was expressed as µmolg^{-1}FW.

Preparation of enzyme extracts

Enzyme extraction was performed according to the method as described by Hossain et al. (2005) with slight modification.

In brief, freshly harvested leaves (0.5 g) were crushed into fine powder in a mortar and pestle under liquid N_2.

Soluble protein was extracted by homogenizing the powder in 10 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA, 1% polyvinylpyrrolidone and cocktails of protease inhibitors (1 mM PMSF, 5 mM DTT and 5 μg mL^{-1} leupeptin) with the addition of 1 mM ASC in case of APX assay. The homogenate was centrifuged at 15000 g for 20 min at 4°C and the supernatant was used for the following enzyme assays. We measured all enzymes activities that are present as in soluble protein extract. Protein content was determined according to the method used by Bradford（1976）using Bio-Rad reagent with bovine serum albumin as standard.

Assays of H_2O_2 metabolizing enzyme activities

Total superoxide dismutase (SOD; EC1.15.1.1), Cu/ZN-SOD, Fe-SOD and Mn-SOD activities were determined spectrophotometrically by recording the absorbance at 560 nm according to the method of Singh et al. (2007). Catalase (CAT; EC1.11.1.6) activity was determined by following the method as described by Chance and Herbert (1950). Peroxidase (POD; EC1.11.1.7) activity was determined based on the method as described by Rao et al. (1997). Ascorbate peroxidase (APX; EC1.11.1.11) activity of enzyme extract was determined as described by Rao et al. (1997). Monodehydroascorbate reductase (MDHAR; EC1.6.5.4) activity was determined by the method of Hossain et al. (1984). Dehydroascorbate reductase (DHAR; EC1.8.5.1) activity was determined by the procedure described by Nakano and Asada (1981) with some modifications. The reaction buffer solution contained 50mM potassium phosphate buffer (pH 7.0), 2.5mM GSH, and 0.1mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. Glutathione reductase (GR; EC 1.6.4.2) activity was assayed following the method as described by Hernandez et al (1999).

Statistical analysis

The experimental design was a completely randomized design (CRD) arrangement in 2x3 factorial with four replications. Two rice genotypes (as first factor) were studied in three salinity levels of irrigation water including 0, 75 and 150 mM as second factor. In all the figures values are shown as error bars representing standard errors of the means. The significance of differences between mean values was compared by Student's T-test. Differences at $P \leq 0.05$ were considered significant.

Conclusion

It may be concluded that Pokkali maintained the higher levels of antioxidants metabolites, like GSH and ASC, in comparison to sensitive BRRI dhan 29 during salinity stress. Our results also revealed that ASC-GSH cycle works more efficiently in Pokkali than in BRRI dhan 29 for the scavenging of H_2O_2. However, CAT and POD activities play an important complementary role in scavenging H_2O_2 in both rice genotypes and thus a differential response was observed in their lipid peroxidation levels during salinity stress in the present study.

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