Expression of Antioxidant Isoenzyme Genes in Rice under Salt Stress and Effects of Jasmonic Acid and γ-Radiation

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Analysis of chlorophyll (Chl) fluorescence implicated treatment of 40 mM NaCl decreased maximal photochemical efficiency of photosystem II (PSII) (Fv/Fm), actual quantum yield of PSII (ΦPSII), and photochemical quenching (qP) in rice, but increased non-photochemical quenching (NPQ). Decreases in Fv/Fm, ΦPSII, and qP were significantly alleviated by 30 µM jasmonic acid (JA), while NPQ increase was enhanced. JA prevented salt-induced decrease of pCuZn-SOD gene expression, but caused greater decrease in mRNA levels of cAPXa and Chl_tAPX genes. Investigation of vacuolar Na+/H+ exchanger (NHX2) and 1-pyrroline-5-carboxylate synthetase (P5CS) gene expressions revealed transcription level of NHX2 gene was increased by JA, regardless of NaCl presence, while that of P5CS gene slightly increased only in co-presence of JA and NaCl. Unlike JA, γ-radiation rarely affected expressions of antioxidant isoenzyme, NHX2, and P5CS genes, except for increase in mRNA level of Chl_tAPX and decrease in that of pCuZn-SOD. These results demonstrate enhanced salt-tolerance in JA-treated rice seedlings may be partly due to high transcription levels of pCuZn-SOD, NHX2, and P5CS genes under salt stress.

Key words: rice, salt stress, antioxidant enzyme, γ-radiation, jasmonic acid

The ionic and osmotic stresses imposed by high salinity on plants may create secondary stresses. These secondary or derived stresses include the accumulation of toxic or unwanted compounds, perturbation in the cellular metabolism, and nutritional disorders,1 among which the oxidative stress is an important constraint for salt-tolerance.

Studies implicate that salt stress could generate reactive oxygen species (ROS) in plants, e.g., hydrogen peroxide (H2O2), superoxide anion (‘O2’), singlet oxygen (‘O2), and hydroxyl radical (‘OH’), thereby causing damages to plants.18 Superoxide dismutase (SOD) converts ‘O2 into H2O2, while ascorbate peroxidase (APX) and catalase (CAT) catalyze the breakdown of H2O2. These enzymes, which detoxify plants by scavenging the ROS, were found to be involved in salt-tolerance through studies of transgenic and mutant plants.16,17 Recently, several reports showed that salt stress affects activities of SOD, APX, and CAT differing in the salt-sensitive and salt-tolerant varieties of a plant species.8,9,10 However, the gene expression of the enzymes at the level of isoenzyme has been rarely investigated in plants under salt stress.

The vacuolar Na+/H+ exchanger (NHX) helps to maintain high concentration of K+ and low concentration of Na+ in the cytosol by sequestering Na+ in the vacuole. This physiological role of NHX has been reported to contribute to the salt-tolerance in Arabidopsis and rice.11,12 In contrast, proline and glycinebetaine increase by salinity,15 and their accumulation could contribute to the enhanced salt-tolerance by reducing the osmotic potential of the cytosol to facilitate water uptake, thereby protecting proteins from misfolding and alleviating the toxic effects of ROS generated by the salt stress.5,16-18 In fact, studies showed an increase in the expression of 1-pyrroline-5-carboxylate synthetase (P5CS), which controls the level of proline, resulted in the enhanced salt-tolerance.5,16

Jasmonic acid (JA) and methyl jasmonate (JA-Me) have been demonstrated to elevate the salt-tolerance by increasing the proline content and by protecting against reduction in the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity and the photosynthetic CO2 fixation.19,20 According to our preliminary data, JA treatment could partly mitigate growth inhibition in rice plants hydroponically cultured under salt stress, thus reducing electrolyte leakage, loss of relative water content, and lipid peroxidation. Similarly, these phenomena could be induced by γ-irradiation.

In the present study, we attempted to reveal a relationship between the expression of antioxidant isoenzyme gene and salt-tolerance. Accordingly, the transcription levels of SOD, APX, and CAT isoenzyme genes were investigated in rice...
seedlings under salt stress in the absence or presence of JA and/or γ-irradiation. Moreover, the expressions of NHX2 and P5CS were also investigated.

Materials and Methods

Plant materials, γ-irradiation, and treatments of NaCl and JA. Rice (Oryza sativa L. cv. Ilpoombyeo) plants were hydroponically cultivated in one-half-strength Murashige and Skoog (MS) nutrient solution under greenhouse conditions for 2 weeks during the late spring season (May to June). The plants were then irradiated with γ-rays at 4 Gy using a γ-iradiator (60Co, ca. 150 TBq of capacity, Atomic Energy of Canada Limited) in Korea Atomic Energy Research Institute (KAERI). The control and irradiated plants were allowed to further grow in an MS-NaCl solution supplemented with 40 mM NaCl. One day later, JA was treated to half of these plants by directly adding to the MS-NaCl solution to a final concentration of 30 μM. All plants were cultivated for 7 days after the treatment of NaCl.

Chlorophyll fluorescence analysis. Chlorophyll (Chl) fluorescence was measured using a Chl fluorometer (IMAGING-PAM, Walz, Germany) as described in the operation manual. Leaf samples were excised (0.5 cm in diameter) from the fully expanded leaves detached 7 days after the treatment of NaCl. Readings were taken after the samples were dark-adapted for 15 min at room temperature. The variable fluorescence (Fv) was obtained by subtracting the initial Chl fluorescence (Fo) from the maximum yield of fluorescence (Fm). The ratio of Fv/Fm served as a measure of the maximal photosynthetic efficiency of photosystem II (PSII).22)

The parameters for photochemical (qP) and non-photochemical quenching (NPQ) were obtained by analysis of Chl fluorescence quenching using the same fluorometer. The calculations of the qP and NPQ parameters were based on the equations of van Kooten and Snel23) as follows: qP = (Fm’ – Ft)/(Fm’ – Fo) and NPQ = (Fm – Fm’)/Fm’, where Fm’ is the maximum yield of fluorescence at the steady-state level reached during the application of a saturation pulse onto the light-acclimated leaves, Ft is the steady-state fluorescence level under the continuous actinic illumination, and Fo’ is Fm/ (Fv/Fm + Fo/Fm) estimated using the approximation of Oxboorugh and Baker.24) The actual quantum yield of PSII (ΦPSII) was calculated according to the equation set by Genty et al.25) as follows: ΦPSII = (Fm’ – Ft)/Fm’.

RNA extraction and reverse transcription (RT)-PCR. Total RNA was extracted from the whole seedlings except roots 7 days after the treatment of NaCl using RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA, USA) according to the supplier’s recommendation. One microgram total RNA was reverse-transcribed in an RT system, AccuPower RT Premix (Bioneer, Daejeon, Korea) for 60 min at 42°C using 0.5 μg anchored oligo(dT)18V primers. Gene-specific primers for the genes of SOD, APX, and CAT isozymes previously designed by Kim et al. were used.26) The sense/antisense primers for NHX2 (accession number: AY360145) and P5CS (accession number: AY574031), 5’-CTGGTTCATAACTCGGCAGGGTGGTG-3’ and 5’-CCAGACAAATGGAGAGTGGGTCTT-3’ respectively, were generous gifts from Dr. In-Seok Lee (Radiation Mutant Breeding Lab., KAERI, Daejeon, Korea). The primers for actin and rRNA, used as internal controls, were 5’-TCCAATGTGACATCTC CAG-3’/5’-GTACCCATCATCGGCATCTG-3’27) and 5’-CTT CGGGATCGGATGTAATGA-3’/5’-AACAATAAAGCGGCC TGCAC-3’,26) respectively, while those for LIP5 (accession number: AB011368) and DHN1 (accession number: AY786415), used as positive controls, were 5’-CGAGCACAAGGAAG AAGG-3’/5’-ATCATCTGCAGCATACCTG-3’ and 5’-GAA GACATGAGAGGACTTGGT-3’/5’-CTCCTTGAACGCCC TTCT-3’. The subsequent PCR was carried out with 3 μL of a total 20 μL RT reaction mixture in a PCR system, Perfect Premix ver. 2.0 (Takara Korea Biomedical Inc., Seoul, Korea) as follows: denaturation at 94°C for 5 min, 24 or 35 cycles of 94°C (45 s) - 58°C (45 s) - 72°C (1.5 min), and an extension at 72°C for 7 min26) in all genes except for NHX2, P5CS, LIP5 and DHN1, which were subjected to denaturation at 95°C for 5 min, 30 cycles of 95°C (80 s) - 57°C (1 min) - 72°C (1.5 min), and an extension at 72°C for 10 min. The resultant RT-PCR products were electrophoresed and analyzed on a 2.0% (w/v) agarose gel after staining with ethidium bromide (EtBr).

Results and Discussion

Changes in Chl fluorescence parameters. Chl fluorescence analysis can be used to assess the salt-tolerance in crop plants in vivo.28) Dionisio-Sese and Tobita29) have suggested that the salt-sensitivity in rice is associated with increased shoot sodium levels, decreased photosynthetic efficiency of PSII, and enhanced NPQ using Chl fluorescence parameters. One of these parameters, the maximal photochemical efficiency of PSII or Fv/Fm, has been reported to decrease with increasing salt stress in pea, rice, and sorghum.30,31) Similarly, our results showed that the Fv/Fm values of rice were slightly decreased by the treatment of 40 mM NaCl, and this decrease was alleviated in the presence of JA (Fig. 1A). The alleviatory effect of JA on the salt stress-induced decrease of the photosynthetic efficiency was also confirmed by the actual quantum yield of PSII, ΦPSII, and the parameter for photochemical quenching, qP. Studies have revealed that salinity decreases these parameters in rice, sorghum, and so on.15,32) However, the ΦPSII and qP values were significantly higher in the JA-treated plants under salt stress than in the control ones (Figs. 1B and C). In contrast, the parameter for non-photochemical quenching, NPQ, was slightly increased by the treatment of NaCl, and this increase was significantly enhanced in the presence of JA (Fig. 1D). Although Oh et al.16) reported a simultaneous decrease in the Fv/Fm, qP, and NPQ in rice, NPQ appears to generally
increase under salt stress as a defense mechanism.\(^{30,31,32}\) Therefore, the JA treatment could help reduce the excitation pressure on photosystems through an increase in the thermal energy dissipation under salt stress and maintain the photosynthetic efficiency.

**Expression of genes of SOD isozymes.** Expression of SOD isozyme genes was analyzed in the JA-treated and/or \(\gamma\)-irradiated rice seedlings under salt stress. Transcript levels of two cytosolic isozymes, cCuZn-SOD1 and cCuZn-SOD2, one plastidic isozyme of CuZn-SOD, pCuZn-SOD, one isozyme of Fe-SOD, Fe-SOD, and two isoforms of Mn-SOD, Mn-SOD1 and Mn-SOD2, used in the report of Kim et al.\(^{26}\) were investigated by RT-PCR analysis. This methodological approach was adopted to reveal expression of antioxidant isozyme genes difficult to be subjected to Northern blot and quantitative PCR analyses due to the high sequence homology among genes and the number of genes to be analyzed.

The treatment of NaCl increased the expression of cCuZn-SOD2 gene; however, this increase was less noticeable in the presence of JA (Fig. 2). Hernandez et al.\(^{20}\) reported that in pea (*Pisum sativum* cv. Puget) higher concentrations of NaCl (110-130 mM) enhance the activity of cCuZn-SOD2. According to our preliminary data, 50 mM NaCl could also induce an increase in the total activity of SOD in rice (*O. sativa* L. cv. Ilpoombyeo) seedlings (data not shown). Thus, the increased expression of cCuZn-SOD2 gene might contribute to the high activity of SOD in the salt-treated rice seedlings. In contrast, the transcripts of pCuZn-SOD gene were markedly decreased by the treatment of NaCl, and this decrease could be protected by the JA treatment only, and not by \(\gamma\)-irradiation. This effect of JA appears to be associated with the protective functions of JA-Me as described by previous studies.\(^{19-21}\) Interestingly, the transcription level of pCuZn-SOD gene was synergistically decreased by both the JA treatment and \(\gamma\)-irradiation in the absence of NaCl. The genes of cCuZn-SOD1, Fe-SOD, Mn-SOD1, and Mn-SOD2 were rarely affected by JA, NaCl, and \(\gamma\)-radiation. However, Parida et al.\(^{36}\) reported that salinity induces a preferential enhancement of Mn-SOD in the mangrove, Bruguiera parviflora. This discrepancy can be explained by the difference in the NaCl concentration and plant species used.

**Expression of genes of APX isozymes.** Our preliminary data also indicated that the treatment of NaCl up to 100 mM induces an increase in the activity of APX in rice plants (data not shown). Similarly, many studies have revealed that the salinity enhances the activity of APX in various plant species, e.g., pea, rice, and beet.\(^{9,10,35}\) Therefore, we investigated mRNA levels of two cytosolic and two chloroplastic (stromal and thylakoid) isozymes of APX, cAPXa, cAPXb, Chl_sAPX, and Chl_tAPX, respectively.\(^{20}\) The expression of cAPXb gene was noticeably decreased by the treatment of NaCl, and the mRNA levels of cAPXa and Chl_tAPX were slightly lower in the JA-treated groups under salt stress (Fig. 3). Interestingly, the expression of Chl_tAPX gene was slightly elevated by \(\gamma\)-irradiation without salt stress, while that of Chl_sAPX gene remained unaffected by JA, NaCl, and \(\gamma\)-radiation. Yoshimura et al.\(^{77}\) reported that the cytosolic APX activity of spinach increased in parallel with the transcript abundance during high-light stress, while the protein level was not altered. Accordingly, the present data may indicate no direct correlation between the gene expression and enzyme activity.
of APX under salt stress.

Expression of genes of CAT isoforms. Together with APX, CAT plays an important role in the breakdown of H$_2$O$_2$ in cells. Many experimental evidences have shown that salt stress induces a significant increase in the activity of CAT in salt-tolerant rice cultivars. However, in the salt-sensitive cultivars or other plant species, the activity of CAT was almost unaffected or decreased with increasing concentration of NaCl.9,10,38) In the present study, the transcription levels of two CAT isoforms, CATb and CATc, were decreased by the treatment of NaCl, while Actin and rRNA were amplified as internal controls.

Fig. 3. Expression of APX isoenzyme genes. The RT-PCR conditions are described in ‘Materials and Methods’ and Fig. 2. All genes except LIP5 and DHN1 had 24 cycle reactions. LIP5 and DHN1 were used as positive controls inducible by salt stress, while Actin and rRNA were amplified as internal controls.

Fig. 4. Expression of CAT isoenzyme genes. The RT-PCR conditions are described in ‘Materials and Methods’ and Fig. 2. All genes except LIP5 and DHN1 had 24 cycle reactions. LIP5 and DHN1 were used as positive controls inducible by salt stress, while Actin and rRNA were amplified as internal controls.

Fig. 5. Expression of NHX1 and P5CS genes. The RT-PCR conditions are described in ‘Materials and Methods’ and Fig. 2. The subsequent PCR was performed as follows: denaturation at 95°C for 5 min, 30 cycle reactions of 95°C (80 s) - 57°C (1 min) - 72°C (1.5 min) and an extension at 72°C for 10 min. LIP5 and DHN1 were used as positive controls inducible by salt stress, while Actin and rRNA were amplified as internal controls.

of compatible osmolytes.39) Until now, we have demonstrated that the expressions of SOD, APX, and CAT isoenzyme genes are differentially regulated in response to JA, NaCl, and γ-radiation. However, besides pCuZn-SOD, none of the isoenzyme genes tested could be positively associated with the higher salt-tolerance in the JA-treated seedlings (Fig. 1). Therefore, we attempted to assess the expressions of NHX2 and P5CS genes belonging to the third and fourth categories, respectively. Like NHX1, NHX2 is localized to the vacuole in plant cells, facilitating Na$^+$/H$^+$ exchanger.11,13,14,40) JA treatment increased the expression of NHX2 gene, regardless of the presence of NaCl and γ-radiation, while that of P5CS gene appeared to slightly increase only in the co-presence of NaCl and JA. These results are in good agreement with our preliminary data, indicating that the JA treatment helps to elevate the proline content, and partially protect against electrolyte leakage and reduction of relative water content under salt stress (data not shown).

In conclusion, the present study clarified that JA can induce enhanced salt-tolerance in rice under salt stress using Chl fluorescence parameters. The transcription levels of SOD, APX, and CAT isoenzyme genes were differentially modulated under salt stress. Furthermore, our results suggest that the enhanced salt-tolerance in the JA-treated rice seedlings may be partly due to the high transcription levels of pCuZn-SOD, NHX2, and P5CS genes under salt stress.

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References

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