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Genome-wide expression analysis of a rice mutant line under salt stress

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ABSTRACT. Salinity is a major environmental stress to plants. In this study, the ability of plants to tolerate salt was investigated by studying growth, physiological characteristics, and expression levels of genes related to the salt-stress response in the salt-tolerant rice mutant (Till-II-877), which was derived from γ-ray irradiation. Compared to plants grown under normal conditions, the height and root length of wild type (WT) were reduced by approximately 40 and 29% following exposure to salt stress for 3 weeks, whereas Till-II-877 line showed 29 and 23% reductions in plant height and root length, respectively. No significant changes were observed in total chlorophyll content, and the malondialdehyde content of the mutant increased less than that of the

WT under salt treatment. Gene expression was compared between the WT and mutant lines using microarray analysis. An unbiased analysis of the gene expression datasets allowed us to identify the pathways involved in salt-stress responses. Among the most significantly affected pathways, changes in gene expression were observed in α-linolenic acid and linoleic acid metabolism (in lipid metabolism), fructose and mannose metabolism and glycolysis-gluconeogenesis (in carbohydrate metabolism), cysteine and methionine metabolism (in amino acid metabolism), and carbon fixation (in the energy metabolism of photosynthetic organisms) under salt stress. These results show that the differential response of plants subjected to salt stress was due to changes in multiple metabolic pathways. These findings increase our understanding of the effects of salt stress in rice and may aid in the development of salt-tolerant rice cultivars.

Key words: Salt tolerance; Mutant; *Oryza sativa*; Microarray; Biochemical analysis

INTRODUCTION

Salinity is a major abiotic stress that affects plant growth and is a problem in many regions. The ability of plants to cope with salinity stress is an important determinant of crop distribution and productivity in many areas; thus, it is important to understand the mechanisms that confer tolerance to salty environments (Yu et al., 2016). Many parameters can be used to estimate salt tolerance in plant, including shoot and root growth, malondialdehyde (MDA) levels, and chlorophyll content (Hala et al., 2005; Kim et al., 2010). Salinity stress induces ionic perturbations, generation of reactive oxygen species (ROS), and cell damage. In particular, ROS generated by salt stress can cause cell damage (Hasegawa et al., 2000). MDA is a reactive and naturally occurring compound in plants, which acts as a marker of oxidative stress and these are used as makers of salt stress responses. Therefore, many studies have been carried out to the elimination of MDA content for selected salt tolerance mutants. Salinity can also cause a progressive loss of chlorophyll. Additionally, enhanced tolerance to salt stress can lead to changes in the chloroplast membrane, which mainly comprises the saturation of fatty acid (linoleic and alpha-linolenic acid) in many plants (Simopoulos, 2004).

Rice (*Oryza sativa* L.) is an important crop that feeds approximately half of the world's population (Goff et al., 2002). Additionally, rice is a significant resource for genetic research on the development of animal and plant species. In Korea, rice is grown on an area of about 80 million hectares, producing 432 million tonnes with an average yield of 5420 kg per hectare. Since the year 2000, 5132 varieties of rice have been collected, and more than 300 useful cultivars have been created for national plot testing each year (Qi et al., 2009). Despite significant output, there are many restrictions on tillage. To overcome the problems of salinity, various improvement strategies have been employed. However, these strategies have proven to be impractical due to their high costs and the low return on investments. The most practical approach is to develop tolerant varieties; thus, many researchers have considered breeding for salt tolerance (Qi et al., 2009; Kim et al., 2010). This trait requires genetic variability, screening parameters, and an understanding of the

genetic and physiological mechanisms of tolerance (Kikuchi et al., 2003; Moradi et al., 2003; Liu et al., 2015; Silveira et al., 2015; Yu et al., 2016).

Studies on rice genomic are very advanced because the rice genome (430 Mb) is the smallest among members of Poaceae, and rice is used as a model crop for cereal genomics research (Kondou et al., 2006). Recently, investigations into the genomes of many species and the assembly of cDNA collections have been actively promoted. In 2002, sequencing of the entire rice genome was completed, and more than 20,000 full-length cDNA clones were annotated (Goff et al., 2002). Thus, detailed analyses of genetic information have become possible using public databases, and the information obtained from cDNA is particularly useful for investigating the structure and function of genes (Kikuchi et al., 2003; Liu et al., 2015; Silveira et al., 2015; Yu et al., 2016).

Although rice is generally considered salt-sensitive, the development of cultivars with increased salt tolerance is required (Moradi et al., 2003; Kim et al., 2010). Utilization of genetic variation to improve the salt tolerance of rice may benefit from an improved understanding of the response of rice to salinity stress, and radiation-induced mutations have become an important method used to induce genetic variability (Kim et al., 2010). One aspect of the stress response occurs at the transcriptional level and involves alterations in gene expression (Moradi et al., 2003; Kondou et al., 2006). Owing to the large number of genes involved in the response to various abiotic stresses, microarrays are increasingly being used to monitor global changes in gene expressions in *Arabidopsis thaliana*, rice, and in other living organisms (Katsuhara et al., 2005; Kondou et al., 2006; Li et al., 2016; Luo et al., 2016).

To explain the response to salt stress, it is necessary to identify the genes involved in various metabolic pathways that undergo altered expression. The identification of differentially expressed genes (DEGs) in response to salt treatment could help us to elucidate the response to salt stress in rice. In our study on the changes that occur during the salt-stress response, we performed a microarray analysis to identify DEGs using the rice mutant line Till-II-877, which was induced by γ-ray irradiation.

MATERIAL AND METHODS

Plant materials and growth conditions

O. sativa L. Dongan (wild type, WT) were irradiated with γ-rays using a [^{60}Co] γ-irradiator (150 TBq capacity; ACEL, Ontario, Canada) at the Korea Atomic Energy Research Institute. Among the 2961 M_2 mutants derived from cv. Dongan (WT), a salt-tolerant γ-ray-irradiated mutant line, Till-II-877 (877), was selected based on the results of our previous study (Kim et al., 2010). Genetically fixed mutant lines (M_6 generation) with excellent agricultural characteristics were selected. Dongan (WT) and Till-II-877 lines were planted in trays in triplicate, with 15 seeds per pot for each of the lines. The trays were filled with soil, which was commercially available for use in rice culture (Sunghwa Inc., Korea). During this period, the plant culture was achieved when the plants were grown in a controlled greenhouse at 27 ± 1°C with a light/dark cycle of 16 h light and 8 h darkness. To compare the growth characteristics of WT and 877 lines, the plants were treated with 171 mM (1%, EC = 16 mS) NaCl for 3 weeks in triplicate. Following salt treatment, whole plants were collected and stored at -80°C until the RNA was extracted for microarray analysis (Figure 1).

Figure 1. Comparison of mutant (Till-II-877) and wild type (WT) plants (Dongan) grown under salt stress conditions. **A.** Till-II-877, **B.** WT. Photographs were taken after 3 weeks of salt stress (171 mM).

Biochemical measurements

To evaluate the physiological response of the rice mutant to salt stress, chlorophyll and MDA contents were analyzed in triplicate. Chlorophyll was extracted according to the method described by Lichtenaler (1987), and chlorophyll content was determined with the formulae described by Arnon (1949). Fresh leaf samples (0.5 g) were ground in 5 mL 100% acetone. The samples were centrifuged at 10,000 *g* at 4°C for 10 min. The supernatant fraction (1 mL) was removed and re-centrifuged for 5 min at 10,000 rpm. For pigment determination, the supernatant was diluted in 100% acetone. The absorbance of the mixture was measured at 470, 644.8, and 661.6 nm with a spectrophotometer (UVIKON 923, Bio-Tek instruments, USA).

Levels of lipid peroxidation products were estimated in triplicate, according to the method described by El-Moshaty et al. (1993). Briefly, the concentration of MDA (as an end-product of lipid peroxidation) was measured via a reaction with thiobarbituric acid (TBA). Fresh leaf samples (0.5 g) were ground in 5 mL 0.1% trichloroacetic acid (TCA). The samples were centrifuged at 10,000 *g* for 15 min. The supernatant fraction (1 mL) was collected and mixed with 4 mL 20% (w/v) TCA containing 0.5% (w/v) TBA. The mixture was incubated at 95°C for 30 min and quickly cooled in an ice bath for 15 min. After centrifugation at 10,000 *g* for 10 min, the absorbance of the extract was read at 532 nm. A correction for non-specific turbidity was made by subtracting the absorbance value obtained at 600 nm. Due to the limited specificity of this method, the concentration of TBA-reactive species (TBARS) was calculated using an extinction coefficient of 155 mM/cm, and the results were expressed as nanomoles TBARS per gram fresh weight (FW).

Microarray analysis

Total RNA was extracted from frozen material using the RNeasy Plant Mini Kit (QIAGEN, Germany), according to the manufacturer protocol. Total RNA was labeled and

used for microarray hybridization, according to the manufacturer protocol (Agilent Technologies, USA). cDNA was synthesized from total RNA and labeled with cyamine 3- or cyamine 5-CTP. This cDNA was then used for hybridization, which was performed at 60°C for 17 h in a hybridization chamber (Agilent Technologies). Following previously reported methods (Song et al., 2012), the products were washed with 6X sodium chloride/ sodium citrate containing 0.005% Triton X-120 for 5 min after hybridization. The microarray experiments were performed with an Axon 4000B scanner (Axon Instruments, Union City, CA, USA). A feature extraction software program (Agilent Technologies) was used to determine the signal intensities. To establish DEGs in response to salt stress in rice mutants, we performed a microarray analysis using the Affymetrix Gene Chip Rice Genome Array (Santa Clara, CA, USA).

Functional annotations

Functional clustering was performed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) Bioinformatics Resource software (v.6.7; http://david.abcc.ncifcrf.gov/home.jsp). A set of significant gene ontology (GO) terms were used as the input for the ReviGO tool to reduce the number of terms to a smaller meaningful set. The MapMan software (version 2.2.0) was used to annotate functional MapMan bin codes (BINs) of genes specifically expressed under salt stress. DEGs were assessed by fold change (FC) and log2 values of FC. Genes that exhibited an estimated absolute log2 ratio ≥1 (more than 2-fold change) were considered to be salt-affected gene expression were annotated in the MapMan ontology. Default settings were used to perform an uncorrected Wilcoxon rank test.

RESULTS

Physiological responses of Till-II-877 and Dongan (WT) rice to salt stress

Height and root length of the 877 and the WT under salt- and non-salt conditions for 3 weeks are given in Table 1. The height of the WT and 877 line was 37.7 and 37.5 cm under non-salt conditions and 22.8 and 26.5 cm under salt conditions, respectively. The height of the WT under the salt condition was reduced by approximately 40% compared to that of the WT under the non-salt condition, while the height of 877 line was reduced by 29%. The root length of the WT grown under the salt condition was reduced by approximately 29% when compared to that of the WT under the non-salt condition, while the 877 line exhibited a 23% reduction, respectively.

Table 1. Growth characteristics of wild type (WT) and 877 line grown under salt and non-salt conditions for 21 days.

	Treatment	Plant height (cm) ***	Root length (cm)***
Wild type	NSF*	37.7[a]	8.3[a]
	SF**	22.8[c]	6.0[c]
Till-II-877	NSF	37.5[a]	8.9[a]
	SF	26.5[b]	6.9[b]

*NSF: Non-salt treatment, **SF: salt treatment (171 mM) ***a, b, ab, c: significant differences at the 5% level (Duncan's multiple range tests; N = 3).

Till-II-877 (γ-ray irradiated mutant line, 877) and WT (cv. Dongan) plants showed different morphological responses when subjected to salt stress for 3 weeks (Figure 1). The leaves of the 877 line remained green, whereas those of the WT changed from green to yellow. The lines also differed in their chlorophyll contents (Figure 2). The WT showed an increase in chlorophyll a (5.8%) and a decrease in chlorophyll b (15.9%) contents, whereas the levels of chlorophyll a and b were unaltered in the 877 line (Figure 2A and B). Total chlorophyll (a + b) levels were not altered in the 877 line, whereas in WT, total chlorophyll was decreased by approximately 4.2% (Figure 2C). The carotenoid content was increased in the 877 line (16.6%) but not in the WT (Figure 2D). The MDA content differed between WT and 877 line (Figure 3); MDA was increased by approximately 18.8% in 877 line, and by 128.3% in WT when subjected to salt stress for 3 weeks.

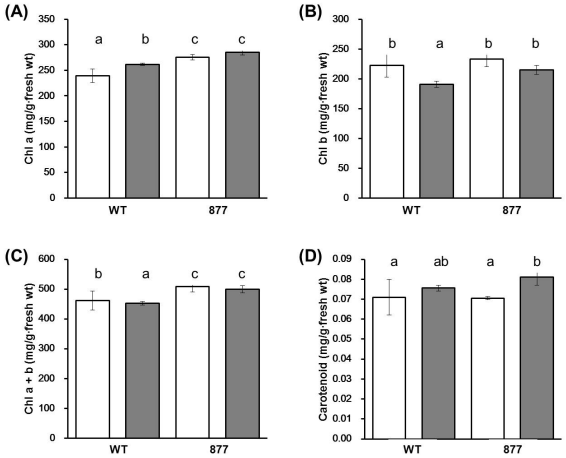

Figure 2. Changes in chlorophyll a (**A**), chlorophyll b (**B**), total chlorophyll (a + b; **C**), and carotenoid (**D**) contents in response to salt stress for 3 weeks. White and gray boxes represent non-treatment and salt treatment, respectively. WT, wild type; 877, mutant line. a, b, ab, c: significant differences at the 5% level (Duncan's multiple range tests; N = 3).

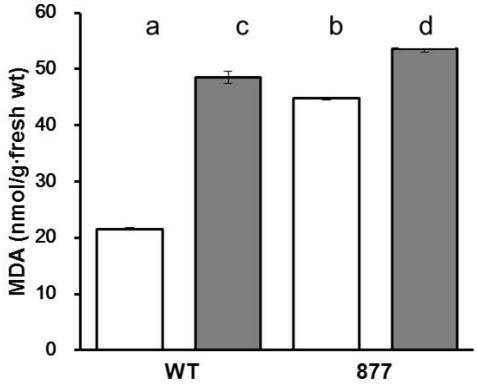

Figure 3. Changes in malondialdehyde (MDA) contents following 3-weeks salt stress. White and gray boxes represent non-treatment and salt treatment, respectively. WT, wild type; 877, mutant line. a, b, ab, c: significant differences at the 5% level (Duncan's multiple range tests; N = 3).

Overview and comparison of global gene expression among rice mutant lines and the original cultivar under salt stress

DEG analysis between mutated line (877) and WT under salt and non-salt conditions were developed based on log2 ratio (Figure 4). Among 57,381 genes represented on the Affymetrix Gene Chip, 630 were up- or down-regulated 2-fold under non-treatment conditions and 1563 genes were up- or down-regulated under the salt stress condition. We found that 436 genes were up-regulated and 194 were down-regulated under non-treatment conditions, whereas 772 genes were up-regulated and 791 were down-regulated under the salt stress condition in 877. Among the DEGs, 40 up-regulated and 11 down-regulated genes were common in plants subjected to non-treatment and salt stress conditions. The expression patterns of 110 genes were altered under salt stress. Sixteen genes switched from being down-regulated to being up-regulated, whereas 94 genes switched from being up-regulated to being down-regulated in a comparison between WT and 877.

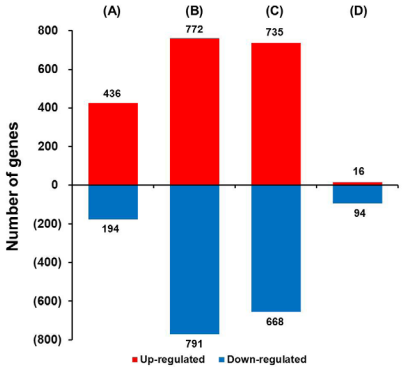

Figure 4. Number of genes that were differentially expressed between WT and 877 line during salt treatment. **A.** Non-treatment, **B.** salt treatment, **C.** genes specifically expressed under salt treatment, **D.** genes exhibiting altered expression levels between treatments.

GO analysis revealed several classes of genes that are affected by salt stress

Figure 5 is a ReviGO representation summarizing GO biological processes that were detected by DAVID as overrepresented ($P \leq 0.01$) in the set of specifically expressed genes, and those that showed altered expression under salt stress. Functional categories that were enriched in specifically expressed genes included 'fatty acid biosynthesis' (fatty acid metabolism, fatty acid biosynthesis, oxylipin biosynthesis, oxylipin metabolism, organic acid biosynthesis, and carboxylic acid biosynthesis), 'carbohydrate catabolism' (carbohydrate catabolism, aromatic compound catabolism, hemicellulose metabolism, cell-wall polysaccharide metabolism, xylan metabolism, phenylpropanoid metabolism, L-phenylalanine metabolism, L-phenylalanine catabolism, and aromatic amino acid family catabolism), 'M phase' (cell-cycle phase, cell-cycle process, M phase of the mitotic cell cycle, M phase, mitotic cell cycle, nuclear division, and mitosis), 'lipid transport' (lipid localization and lipid transport), 'oxidation-reduction process' and 'cell cycle' (Figure 5A). Genes with altered expression represented the functional categories 'oligopeptide transport' (peptide transport and oligopeptide transport), 'oxidation-reduction process', and 'glutamine family amino acid metabolism' (Figure 5B).

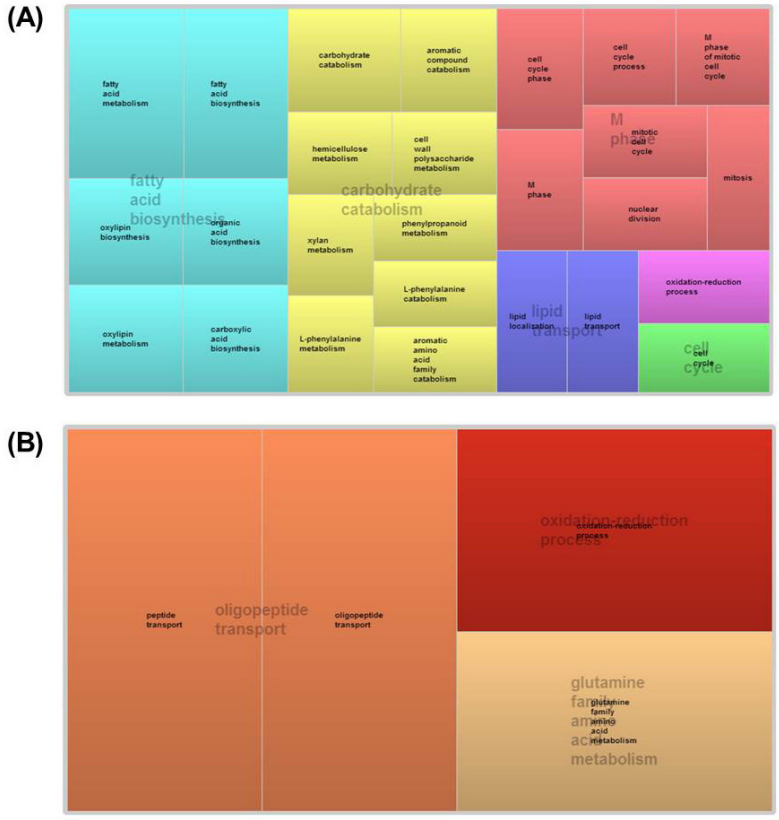

Figure 5. Representation of the gene ontology (GO) biological processes significantly enriched with genes specifically expressed (**A**) and genes showing altered expression levels (**B**) under salt stress in the mutant rice line.

MapMan analysis of salt-affected genes

To permit a visual comparison of the WT and mutant lines under salt stress, specifically expressed genes and genes with altered expression were imported into MapMan. The 'metabolism overview' and 'cellular response overview' are shown in Figure 6. The metabolism overview of specifically expressed genes revealed that genes from the 877 line were involved in primary metabolism that significantly up-regulated compared with the WT line (Figure 6A). Specifically, genes involved in photosynthetic light reactions and the Calvin cycle of the photosynthesis system, starch synthesis, and sugar degradation in major carbohydrate (CHO) metabolism, glycolysis, fermentation, cellulose synthesis, pectinesterases, and cell-wall proteins involved in cell-wall metabolism, and genes involved in fatty acid (FA) synthesis, and FA elongation in lipid metabolism were more highly expressed in the mutant compared with the WT. Genes related to waxes showed increased expression, although other genes involved in secondary metabolism were down-regulated under salt stress. In the cellular response overview, genes involved in drought/salt abiotic stresses, heme in redox reactions, cell division, and the cell cycle were found to be more highly expressed in the mutants than in the WT (Figure 6B).

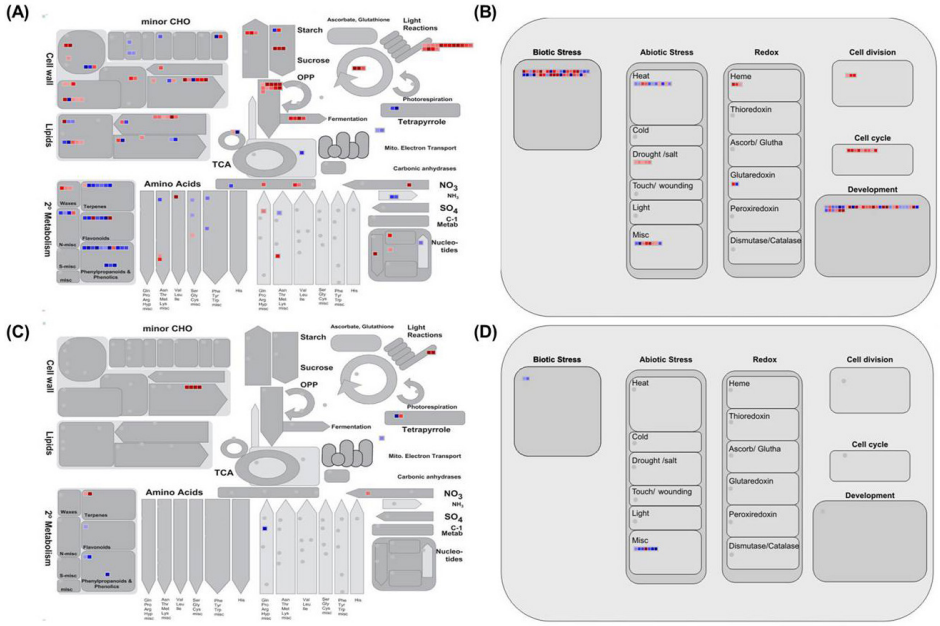

Figure 6. Metabolic pathways affected by the transcriptional changes that occur during salt stress. **A. C.** Metabolism overview of genes specifically expressed under salt treatment (**A**) and genes exhibited altered expression (**C**). **B. D.** Cellular response overview of genes specifically expressed under salt treatment (**B**) and genes exhibiting altered expression levels (**D**).

Among the genes with altered expression, polypeptide subunits in photosystem II, pectatelyases and polygalacturonases involved in cell-wall metabolism, and isoprenoids and terpenoids involved in secondary metabolism switched from being down-regulated under non-treatment conditions to being up-regulated under salt-stress conditions (Figure 6C). However, the genes showing changes in expression were not involved in the drought/salt stress response in the cellular response overview (Figure 6D).

Differential gene expression in metabolic pathways under salt stress

To identify expression changes in genes involved in metabolic pathways, genes that were specifically expressed under salt stress were added to the gene list of the KEGG pathway detected by DAVID functional annotation (Table 2). The specifically expressed genes were involved in α-linolenic acid and linoleic acid metabolism (in lipid metabolism), fructose and mannose metabolism and glycolysis-gluconeogenesis (in carbohydrate metabolism), cysteine and methionine metabolism (in amino acid metabolism), carbon fixation in photosynthetic organisms (in energy metabolism), biosynthesis of alkaloids, biosynthesis of plant hormones, and DNA replication. The expression of lipoxygenase, which is involved in lipid metabolism, was decreased in the 877 line as compared to that in the WT. Genes involved in carbohydrate metabolism and DNA replication were more highly expressed in the 877 line. Most genes involved in cysteine and methionine metabolism were up-regulated, with the exception of Os01g0720700 and Os01g0578000 compared to WT.

Table 2. Genes involved in the salt-stress response with Kyoto encyclopedia of genes and genomes (KEGG) pathway information.

Probe ID	Gene symbol	Gene name	DEG (log_2 ratio)
α-Linolenic acid metabolism			
Os.8266.1.A1_at	Os03g0767000	Allene oxide synthase 1, chloroplastic	-1.49
Os.15829.1.S1_at	Os02g0194700	Lipoxygenase	1.66
Os.53604.1.S1_at	Os03g0738600	Lipoxygenase 2; Lipoxygenase	-1.38
Os.4416.1.S1_at	Os08g0508800	Lipoxygenase 7, chloroplastic; Lipoxygenase	-2.12
OsAffx.26361.1.S1_x_at	Os04g0447100		-1.86
OsAffx.26361.1.S1_at	Os04g0447100		-1.67
Os.7513.1.S1_at	Os11g0605500		-1.05
Os.2371.1.S1_at	Os12g0448900		-1.87
Linoleic acid metabolism			
Os.15829.1.S1_at	Os02g0194700	Lipoxygenase	1.66
Os.53604.1.S1_at	Os03g0738600	Lipoxygenase 2; Lipoxygenase	-1.38
Os.4416.1.S1_at	Os08g0508800	Lipoxygenase 7 chloroplastic; Lipoxygenase	-2.12
OsAffx.26361.1.S1_x_at	Os04g0447100		-1.86
OsAffx.26361.1.S1_at	Os04g0447100		-1.67
Biosynthesis of alkaloids			
Os.4167.1.S1_at	Os02g0236000	Aspartate aminotransferase	1.40
Os.27606.1.S1_at	Os03g0850400	Aspartokinase	1.52
Os.5725.1.S1_at	Os11g0171300	Fructose-bisphosphate aldolase, chloroplastic; Fructose-bisphosphate aldolase	1.04
Os.47303.1.S1_at	Os01g0743500	Malic enzyme	-1.42
Os.47303.1.S1_s_at	Os01g0743500	Malic enzyme	-1.48
Os.57475.1.S1_x_at	Os02g0601300		1.42
Os.23560.1.S1_at	Os04g0543900		-1.35
Os.52150.1.S1_x_at	Os04g0543900		-1.47
Os.12014.1.S1_s_at	Os05g0524400		2.12
OsAffx.27312.1.S1_at	Os05g0524400		1.91
Os.3350.1.S1_a_at	Os10g0390500		1.21
Os.3350.1.S1_at	Os10g0390500		1.53
Os.52536.1.S1_at	Os04g0518400	Phenylalanine ammonia-lyase	-1.69
Os.57474.1.S1_x_at	Os04g0677500	Pyruvate kinase	1.22
Biosynthesis of plant hormones			
Os.8266.1.A1_at	Os03g0767000,	Allene oxide synthase 1, chloroplastic	-1.49
Os.4167.1.S1_at	Os02g0236000,	Aspartate aminotransferase	1.40
Os.27606.1.S1_at	Os03g0850400,	Aspartokinase	1.52
Os.5725.1.S1_at	Os11g0171300,	Fructose-bisphosphate aldolase, chloroplastic; Fructose-bisphosphate aldolase	1.04
Os.15829.1.S1_at	Os02g0194700,	Lipoxygenase	1.66
Os.53604.1.S1_at	Os03g0738600,	Lipoxygenase 2; Lipoxygenase	-1.38
Os.4416.1.S1_at	Os08g0508800,	Lipoxygenase 7, chloroplastic; Lipoxygenase	-2.12
Os.10543.1.S1_at	Os02g0109100,		1.02
Os.57475.1.S1_x_at	Os02g0601300,		1.42
Os.28127.2.S1_at	Os03g0231700,		2.07
Os.28127.1.S1_at	Os03g0231700,		1.90
Os.53086.1.S1_at	Os03g0810100,		2.86
OsAffx.26361.1.S1_x_at	Os04g0447100,		-1.86
OsAffx.26361.1.S1_at	Os04g0447100,		-1.67
Os.49507.1.S1_at	Os04g0578000,		-2.01
Os.12014.1.S1_s_at	Os05g0524400,		2.12
OsAffx.27312.1.S1_at	Os05g0524400,		1.91
Os.56273.1.S1_at	Os06g0225800,		-1.22
Os.46633.1.A1_at	Os10g0533500,		-1.74
Os.12743.1.S1_at	Os11g0525200,		1.05
Os.7513.1.S1_at	Os11g0605500,		-1.05
Os.52536.1.S1_at	Os04g0518400,	Phenylalanine ammonia-lyase	-1.69
Os.57474.1.S1_x_at	Os04g0677500,	Pyruvate kinase	1.22
Carbon fixation in photosynthetic organisms			
Os.4167.1.S1_at	Os02g0236000	Aspartate aminotransferase	1.40
Os.5725.1.S1_at	Os11g0171300	Fructose-bisphosphate aldolase, chloroplastic; Fructose-bisphosphate aldolase	1.04
Os.47303.1.S1_at	Os01g0743500	Malic enzyme	-1.42

Continued on next page

Table 2. Continued.

Probe ID	Gene symbol	Gene name	DEG (log_2 ratio)
Os.47303.1.S1_s_at	Os01g0743500	Malic enzyme	-1.48
Os.3350.1.S1_a_at	Os10g0390500		1.21
Os.3350.1.S1_at	Os10g0390500		1.53
Os.57474.1.S1_x_at	Os04g0677500	Pyruvate kinase	1.22
Os.10959.1.S2_at	Os12g0292400	Ribulose bisphosphate carboxylase small chain	1.37
Os.12423.1.S1_at	Os01g0841600	Triosephosphate isomerase	1.99
Cysteine and methionine metabolism			
Os.4167.1.S1_at	Os02g0236000	Aspartate aminotransferase	1.40
Os.27606.1.S1_at	Os03g0850400	Aspartokinase	1.52
Os.10215.1.S1_at	Os02g0105400	L-lactate dehydrogenase	1.54
Os.31913.1.S1_at	Os06g0104900	L-lactate dehydrogenase	1.68
Os.49507.1.S1_at	Os04g0578000	Os04g0578000	-2.01
Os.57351.1.S1_at	Os01g0720700	Probable serine acetyltransferase 1	-1.15
DNA replication			
Os.7052.1.S1_at	Os02g0829100		1.44
Os.54130.1.S1_at	Os04g0476400		1.14
Os.53549.1.S1_at	Os05g0160800		1.04
Os.54267.1.S1_at	Os05g0235800		1.14
Os.12974.1.S1_at	Os11g0484300		1.14
Fructose and mannose metabolism			
Os.5725.1.S1_at	Os11g0171300	Fructose-bisphosphate aldolase chloroplastic; Fructose-bisphosphate aldolase	1.04
Os.12866.1.S1_at	Os02g0714200		1.05
Os.12014.1.S1_s_at	Os05g0524400		2.12
OsAffx.27312.1.S1_at	Os05g0524400		1.91
Os.10991.1.S1_at	Os06g0247500		1.66
Os.12423.1.S1_at	Os01g0841600	Triosephosphate isomerase	1.99
Glycolysis_gluconeogenesis			
Os.57464.1.S1_x_at	Os11g0210300	Alcohol dehydrogenase 1	1.49
Os.5725.1.S1_at	Os11g0171300	Fructose-bisphosphate aldolase chloroplastic; Fructose-bisphosphate aldolase	1.04
Os.10215.1.S1_at	Os02g0105400	L-lactate dehydrogenase	1.54
Os.31913.1.S1_at	Os06g0104900	L-lactate dehydrogenase	1.68
Os.57475.1.S1_x_at	Os02g0601300		1.42
Os.313.2.S2_at	Os02g0730000		1.43
Os.12014.1.S1_s_at	Os05g0524400		2.12
OsAffx.27312.1.S1_at	Os05g0524400		1.91
Os.5319.1.S1_at	Os10g0189100		1.00
Os.57474.1.S1_x_at	Os04g0677500	Pyruvate kinase	1.22
Os.12423.1.S1_at	Os01g0841600	Triosephosphate isomerase	1.99

DISCUSSION

Rice plants are highly sensitive to salinity during the early growth stage (Kim et al., 2010; Yu et al., 2016); therefore, we investigated the salt tolerance of 877 and WT. The plant height and root length of 877 line were much higher than those of WT. Chlorophyll and carotenoid levels are considered to be indicators of salt stress in plants. Because the levels of the main photosynthetic pigments, chlorophyll a, chlorophyll b, and carotenoids, are significantly affected by salt stress, these are used as parameters to select tolerant and sensitive cultivars of crop plants (Eryilmaz, 2007; Rahdari et al., 2012). Besides, the level of MDA is a measure of membrane lipid peroxidation damage caused by abiotic stresses and it is an important indication of oxidative damage under conditions of salt stress (Liang et al., 2003). MDA is a final product of lipid peroxidation in bio-membranes, and the MDA content reflects the level of lipid peroxidation while indirectly reflecting the extent of membrane injury (Wang et al., 2009). In the present study, the chlorophyll content of the rice mutant line Till-II-877 was

altered compared with that of the WT line. The total chlorophyll content was not significantly altered in 877 line subjected to salt stress for 3 weeks, although WT showed a 4.2% decrease in total chlorophyll levels. Specifically, chlorophyll b levels were markedly decreased (15.9%) in WT, whereas no significant change was observed in 877 line. The accumulation of MDA in seedlings increased with prolonged salinity stress in all plants including those of the 877 and WT lines; however, a greater increase was observed in WT (128.3%) compared with 877 (18.8%) line. Based on the changes in chlorophyll and MDA contents, we suggest that mutant rice is more tolerant to salt stress than WT rice.

Ion cytotoxicity and osmotic stress adversely affect plant growth under salt stress, and lead to metabolic imbalances and oxidative stress (Zhu, 2002; Hussain et al., 2008). Thus, under stress conditions, lipid metabolism and carbohydrate metabolism occur, and the accumulation of ROS (such as superoxide, hydrogen peroxide, and hydroxyl radicals) is induced (Hasegawa et al., 2000). In the present study, GO analysis showed that the GO terms 'fatty acid biosynthesis', 'carbohydrate catabolism', 'M phase', 'oxidation-reduction process', and 'cell cycle' were significantly enriched in specifically expressed genes, and 'oligopeptide transport', 'oxidation-reduction process', and 'glutamine family amino acid metabolism' were significantly enriched in genes exhibiting altered expression under salt stress (Figure 5). Plants of the mutant line 877, showed similar metabolic responses under salt stress as previously described. 'Oligopeptide transport' and 'glutamine family amino acid metabolism' in genes with altered expression were also reported to be related to salt stress in previous studies (Jung et al., 2010).

There were differences in the expression of genes involved in α-linolenic acid metabolism and linoleic acid metabolism between WT and 877 line in the present study (Table 1 and **Figures S1** and **S2**). Among these, Os08g0508800 (13-LOX, -2.12) and Os03g0738600 (9-LOX, -1.38) showed decreased expression levels in the mutant compared with the WT. The products of these two genes, 9-lipoxygenase and 13 lipoxygenase, cause the enzymatic oxygenation of polyunsaturated fatty acids (PUFAs) (Liavonchanka and Feussner, 2006). PUFAs are highly susceptible to oxidative attack (Hasegawa et al., 2000). We hypothesized that the lower expression levels of these two LOX genes in 877 line caused a decrease in lipid peroxidation and enhanced the defense against salt stress in this mutant line. This hypothesis was verified by the MDA content data (Figure 3).

Romero et al. (2001) suggested that cysteine biosynthesis is induced by salt stress in plants in order to protect against damage caused by high Na^+ concentrations. Cysteine determines the cellular glutathione (GSH) concentrations, and GSH is an important factor produced by plants in response to environmental stress, including oxidative stress (Marrs, 1996; Ruiz and Blumwald, 2002). The results of the present study showed there were changes in the expression levels of genes related to cysteine and methionine metabolism (Table 1 and **Figure S3**). Among these, Os01g0720700 (probable serine acetyltransferase 1) expression was down-regulated more than 2-fold ($\log_2$ ratio, -1.15) in 877 line. This is consistent with previous results reported by Ruiz and Blumwald (2002).

Photosynthesis is modulated by the enzyme RuBisCO activase (Tezara et al., 1999; Mott and Woodrow, 2000). Under salt stress, RuBisCO activity is decreased, which leads to a reduction in the photosynthetic rate (Tezara et al., 1999; Wingler and Roitsch, 2008; Wang et al., 2009). In our study, there was differential expression of genes related to carbon fixation in photosynthetic organisms between WT and 877 line. Among these, Os12g0292400 (ribulose bisphosphate carboxylase small chain) was more highly expressed in the 877 line than in the WT ($\log_2$ ratio, 1.37) (Table 1; **Figure S4**). Additionally, with the exception of two genes

related to malic enzyme, all other genes showed higher expression levels than WT. Regarding genes involved in photochemical reactions (**Figure S5**), genes related to photosystems I and II and the redox chain were all more highly expressed in 877 than in WT . These responses in 877 line appeared to protect the plant against attack by ROS.

Six genes involved in fructose and mannose metabolism were more highly expressed in 877 than in WT. Altered sugar content is a common response to environmental stress (Wingler and Roitsch, 2008). Sugar plays important roles as an osmoprotectant and as an immediate energy source in plants (Wingler and Roitsch, 2008). Many studies have reported increased sugar contents in plants under salt stress (Brosché et al., 2005; Gong et al., 2005; Sanchez et al., 2008; Li et al., 2016; Luo et al., 2016).

All genes involved in glycolysis were more highly expressed in 877 line compared with WT in this study (Table 1 and **Figure S6**). Specifically, Os05g052440 (log_2 ratio, 2.12 and 1.91) and Os04g67750 (log_2 ratio, 1.22) are phosphofructokinase (PFK) and pyruvate kinase (PK), respectively. Long-term salt stress can increase the activities of PFK and PK, which contribute to increased glycose catabolism for energy (Suzuki et al., 2005). Ashihara and Sato (1993) examined the roles of PFK and phosphoenolpyruvate carboxylase (PEPC) in alternative glycolysis, which may be particularly important in tissues where biosynthesis is most affected. Following exposure to salt stress, PFK and PK activity increases with salt stress. The level of PEPC was also stimulated by salt. As well as CO_2 fixation in C4 and Crassulacean acid metabolism plants, PEPC has several roles in plant metabolism (Ashihara and Sato 1993; Suzuki et al., 2005).

Salt stress causes huge losses in agricultural productivity worldwide (Moradi et al., 2003; Yu et al., 2016). Therefore, strategies aimed at overcoming severe environmental stresses need to be quickly and fully implemented. In this study, a mutant rice line was examined for its increased salt tolerance using microarray and gene-network analyses, and we found that its differential response under salt stress was due to changes in the expression of components of various metabolic pathways. This salt-tolerant mutant rice line (Till-II-877) will further our understanding of the effects of salt stress in rice and may aid the development of salt-tolerant rice cultivars.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

Figure S1. Differential gene expression in alpha-linolenic acid metabolism. Red and blue represent up- and down-regulation, respectively.

Figure S2. Differential gene expression in linoleic acid metabolism. Red and blue represent up- and down-regulation, respectively.

Figure S3. Differential gene expression in cysteine and methionine metabolism. Red and blue represent up- and down-regulation, respectively.

Figure S4. Differential gene expression in carbon fixation in photosynthetic organisms. Red and blue represent up- and down-regulation, respectively.

Figure S5. Differential gene expression in photochemical reactions using Mapman. Red and blue represent up- and down-regulation, respectively.

Figure S6. Differential gene expression in glycolysis and gluconeogenesis. Red and blue represent up- and down-regulation, respectively.