

# Identification of Salt-responsive Biosynthesis Genes in Rice via Microarray Analysis

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## Abstract

We used a multiple screening process to identify genes involved in salt tolerance-specific biosynthesis and metabolism in rice (*Oryza sativa* L.). We selected 8,275 salt tolerance-related candidate genes using expression profiles generated across four stages on a microarray containing 135,000 probes (135 K microarray) in *Oryza sativa*. Using our method, we screened 342 ortholog genes, and 74 pathway genes associated with salt-response-related biosynthesis. Finally, we identified six genes by comparison of pathway-network genes and orthologous genes. The six genes were anchored to the chromosomes of rice to characterize their genetic-map positions and were used to construct the phylogenetic tree. The results were verified by reverse transcription-polymerase chain reaction.

**Keywords:** Microarray analysis; Salinity; Salt-responsive genes

## Introduction

In rice, abiotic stresses are responsible for most of the reduction that differentiates yield potential from harvestable yield. Salinity is a major abiotic stress that plants encounter in irrigated agricultural land, and salt stress may induce alterations in biochemical pathways and physiological responses [1]. Salt stress can lead to changes in development, growth, and productivity. Indeed, soil salinity causes severe losses in crop productivity worldwide mainly because it inhibits biomass yield [2-4]. To minimize the deleterious effects of salt stress, plants need to coordinate the activation and effects of several molecular mechanisms [5]. In many important plants, salt stress negatively affects germination, root development, and biomass production, resulting in the significant yield loss [6]. Using stress-resistance mutants, many studies have discovered the mechanisms by which plants mediate and attenuate salt stress. In transgenic overexpression of specific genes, homologous genes responsible for mediating salt tolerance have been identified [7], and pathways involved in detoxification and growth regulation have been found [8]. Thus, components of salt-stress signaling [9] stimulate the activation of specific signaling pathways in the cell [10]. High salt-tolerant mutant rice has been generated by transgenic modulation of salt-response gene expression [11]. However, although these studies have provided specific information about the underpinnings of salt tolerance, salt tolerance is a complex trait controlled by many quantitative trait loci. Thus, it is very difficult to define the genes whose products comprise a salinity-resistance signaling pathway [12]. Thus, it is necessary to apply high-throughput technologies to identify genes related to salt-responsive biosynthesis [13,14].

Rice (*Oryza sativa* L.), the most consumed grain in Asia, is a significant staple for feeding much of the world's population, and its productivity is greatly affected by high salinity. This study, modification of rice strains to tolerate and thrive in high salt concentrations will allow inhabitants in these high-saline areas to also thrive. Thus, the further elucidation of the genes and proteins involved in promoting high-salt tolerance is vitally important. Recently, microarray analyses of the salt-stress response of many plant species have been performed that examined cell cultures, whole plants, or specific plant organs. Several microarray-based platforms are available for abiotic stress-related

transcriptome studies of plants [15,16]. In this study, we examined the effects of high salt concentrations on gene expression responses in rice to better understand the genetic regulation of growth and metabolism in a high salinity environment. We identified candidate genes for the regulation of the salt stress expression network pathway using a microarray containing 135,000 probes (135 K microarrays), Clusters of Orthologous Groups (COGs), pathway, gene mapping, and phylogenetic tree analyses.

## Materials and Methods

### Rice

For this study, we selected two Ds mutant lines, *Salt10* and *Salt23*, which showed increased salt tolerance as compared with their original variety, Dongjin. The high salt-tolerance mutants were generated with the Ac/Ds insertional gene trap system using Dongjin cultivar [17]. These mutant lines showed salt stress tolerance degree of 5~6 in two independent seedling salt stress tests using Yoshida nutritional solution [18] including 0.6% NaCl, while Dongjin's degree was 9 in nine grade evaluation method which regards most resistant reference line as 1 and most susceptible line as 9. We expected both mutant cultivars to be useful in the analysis of gene expression specific to salt-responsive biosynthesis because these mutants have a similar genetic background and exhibit greater salt tolerance than the control Dongjin.

### Experimental design

We assessed gene expression between inbred control and mutants of high salt-tolerant rice during four different salt treatment stages.

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We grew seedlings of these two mutant lines and Dongjin on water for the first week after germination and on Yoshida nutrient solution for the next two weeks in a growth chamber with 15 hour day-length and 30°C/20°C (day/night) temperature. Subsequently, salt treatment was conducted by transferring rice seedlings to Yoshida nutrient solution including 0.6% NaCl. The plants were subjected to salt stress conditions for the indicated time periods [12], upper parts of rice seedlings were taken at 0, 1, 3, and 6 hours after salt treatment for RNA extraction. After salt treatment, we cut the leaves on each plant at four different stages. We froze the harvested samples in liquid nitrogen and extracted the total RNA from the leaf tissues by using RNeasy® plant mini-kits (Qiagen Inc., Valencia, CA, USA) with DNase (Qiagen Inc.) to remove residual DNA, and quantified RNA by using a Nanodrop ND-1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, NC, USA). For transcriptome analysis, we used 135K *Oryza sativa* microarrays [19] to perform a total of 36 experiments during four salt stress-treatment stages with two replicates.

### Ortholog and pathway analysis

In order to identify ortholog, we classified the genes identified in our microarray experiments by comparing them to those in the eukaryotic NCBI/COGs database (<http://www.ncbi.nlm.nih.gov/COG/>). To identify interaction networks, our microarray experiment data represented potential interactions between expressed genes and the salt-responsive pathway. We performed an enriched-pathways analysis and calculated p-values with Fisher's exact test to determine the most significant signaling network responses using Pathway Studio® software 8.0 (Ariadne Inc., Rockville, MD, USA).

### Phylogenetic with gene mapping analysis

In the phylogenetic analysis, amino-acid sequences of the salt tolerance-related genes were aligned using Clustal Omega (EMBL European Bioinformatics Institute, <http://www.ebi.ac.uk/Tools/msa/clustalo/>) Multiple Sequence Alignment program using the default options. In addition, we mapped all of the selected salt tolerance-related genes onto the 12 chromosomes of rice. To find the best mapping position, we used the BLASTN program with cutoff value  $\leq 2.0 \times 10^{-10}$  using the FSTVAL (G.G. BioTech Inc., Yongin, Korea, <http://www.ebi.ac.uk/Tools/msa/clustalo/>). The phylogenetic trees were constructed using the maximum likelihood algorithm in Molecular Evolutionary Genetics Analysis version 5 program (MEGA5, <http://www.megasoftware.net/>).

### Reverse-transcription-polymerase chain reaction analysis (RT-PCR)

We used 5 µg RNA from each sample to synthesize complementary DNA, employing the EcoDry Premix Oligo dT kit (TaKaRaBio, Otsu, Shiga, Japan). To validate our RT-PCR results, we performed each experiment three times. We performed PCR with 10 pmol of each primer under the following conditions: 94°C for 5 min; 24 cycles of 94°C for 30 sec; 58°C for 30 sec; 72°C for 1 min; and a 10 min extension at 72°C. To measure the different expression of each target gene, we used Ubiquitin as a loading control for the primer sequences (Table 1).

## Results and Discussion

### Candidate genes associated with salt-responsive biosynthesis genes

We performed a total of 36 microarray experiments, analyzing three cultivars at four stages with three replications. Using the signal-

Identification	Forward primer 5' - 3'	Reverse primer 5' - 3'	Expected size (bp)
AK107809	TGGATGAGTGGCTGCC-GACG	GGCGGCGCAGAA-CACAATTC	444
J065130P18	GGTTGCCTTGGATCTCCAG-GTGG	CCGAGGATTGCTG-CAGGATTGTAG	392
J065183J16	TCTCTGGATGGCGCT-GCTTCGTA	AGGAGAACAG-GTAGTCCACAT-GACATG	450
AK069955	AGGTTTCGACAAGCAGATG-GACCTC	CCGACAGGGACTG-GAGGAGATTT	482
AK110629	ACACTTAGAAAAATCGCCCG-GCT	CCAGCGAAAAT-CATCCCAG-GAAAAATA	729
AK067929	AAACCGGAAGCAGCAAGAC-CA	AGAGGAACATT-GATATCCCGGACC	424
Ubiquitin	AATCAGCCAGTTTGGTG-GAGCTG	ATGCAAATGAG-CAAATTGAGCACA	218

**Table 1:** The primer sequences of reverse transcription-polymerase chain reaction used for verification of the six candidate genes.

intensity values from the microarrays, we screened 8,275 genes to identify those with at least a 3-fold difference in expression between the two groups. We assumed that the observed differences in expression were related to the transcriptional regulation of salt-responsive genes because we based them on three separate signal-intensity ratios, two high salt-tolerance cultivars compared with the one weak salt-tolerance cultivar, and all of the cultivars experienced the same controlled experimental conditions. To identify conserved orthologous genes, we performed COGs analyses using the 8,275 candidate genes. In the COGs analyses, we placed 1,795 genes into functional categories on which we performed BLAST searches for each of the four stages. We screened a total of 342 orthologous genes using the COGs analyses, which use ortholog similarities to categorize conserved genes.

### Pathway associated with salt-responsive genes

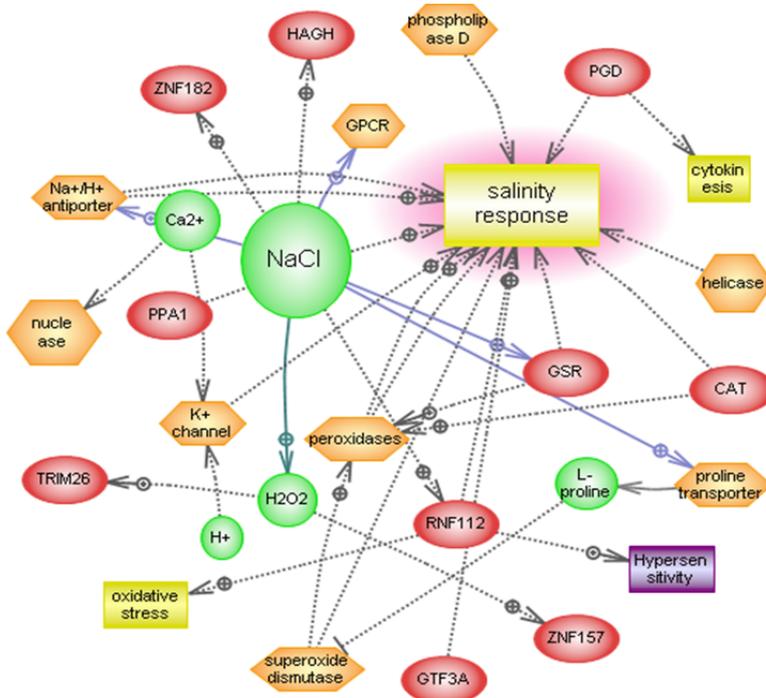
To identify genes involved in specific salt-responsive related pathways among the 8,275 differentially expressed genes, we performed an enriched-pathways analysis. We acquired a total of 729 microarray candidate genes for which evidence was found for involvement of salt tolerance in rice. Using a Fisher's exact test to determine the most significant responses genes, we found 74 interconnected genes linked to specific salt-responsive pathways. Finally, we used these genes to construct an interconnected network to contain the salinity response, including Na<sup>+</sup>/H<sup>+</sup> antiporter, and K<sup>+</sup> channel pathways (Figure 1). These results illustrate the functional diversity of proteins that may be activated during increasing salt tolerance.

### Anchored genes involved in salt-responsive biosynthesis

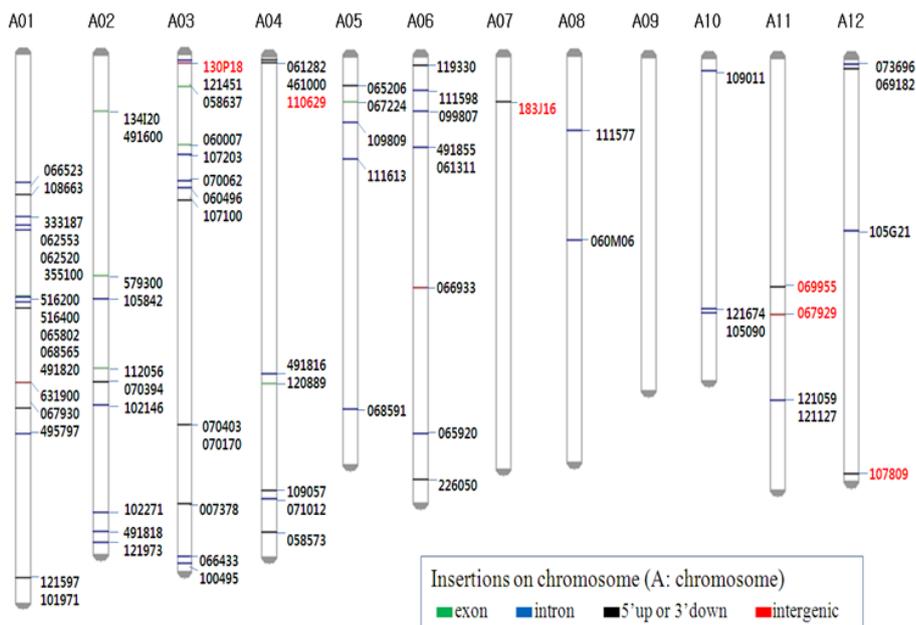
To screen the final candidate genes, we compared the 74 pathway genes and the 342 ortholog genes. We found six genes that were differentially expressed between the high- and weak-tolerance cultivars continuously during the four different stages. The putative proteins encoded by the three up-regulated genes (AK107809, J065130P18 and J065183J16) are a hypothetical protein, a wound-induced protein, and a conserved hypothetical protein, respectively. The three down-regulated genes (AK069955, AK110629 and AK067929) are nod factor-binding lectin-nucleotide phosphohydrolase, a conserved hypothetical protein, and a hypothetical protein, respectively. However, these genes identified by our method did not include any of the well-known genes in the salt-responsive biosynthesis pathway which in response to salt stress, salt-related genes are up- or down-regulated. We assume that this lack of identification might have resulted because many of the previously

identified genes are not expressed continuously during the four salt treatment stages or might only be strongly expressed during a few of the four salt-treatment stages. To reveal the chromosome position relationship between the previously known genes and those identified by our analysis, we mapped the 74 pathway genes and anchored six

genes onto the 12 chromosomes of the genome sequence of rice (Figure 2). The six genes were observed on five chromosomes (A03, A04, A07, A11 and A12). The mapping results of the six genes on the rice genome are presented with related mapping information in Table 2.



**Figure 1:** Pathway diagram of the differentially regulated genes from the 74 screened genes in the high salt-tolerance cultivars, as compared with the weak-expression cultivar (*Dongjin*). The pathway networks of salt-responsive salinity response show the biological interactions among different cellular processes.



**Figure 2:** Map of the 12 rice chromosomes showing the 74 salt-responsive biosynthesis related genes. The color of position lines shows the insertion type such as exon, intron, 5'upstream, and intergenic region. In the gene names, black text of the taxon name presents the 68 genes from predicted salt-tolerance cultivars, and red text indicates the six genes identified.

A phylogenetic analysis was performed to identify relationships between anchored genes and the others salt responsive-related genes based on the maximum likelihood method. Using the six anchored genes and 68 pathway genes, phylogenetic analysis was performed. All salt tolerance-related genes were clustered into three groups (Groups I to III). Intriguingly, Group I contain only down-regulated genes and up-regulated genes were found in Groups II and III (Figure 3). Thus, the six anchored genes identified included one wound-stress gene, four hypothetical protein genes, and one nod factor-binding function gene. These results suggest that the six genes of rice do not share the same genetic background with the previously known salt responsive-related pathway genes.

### RT-PCR analysis of selected genes

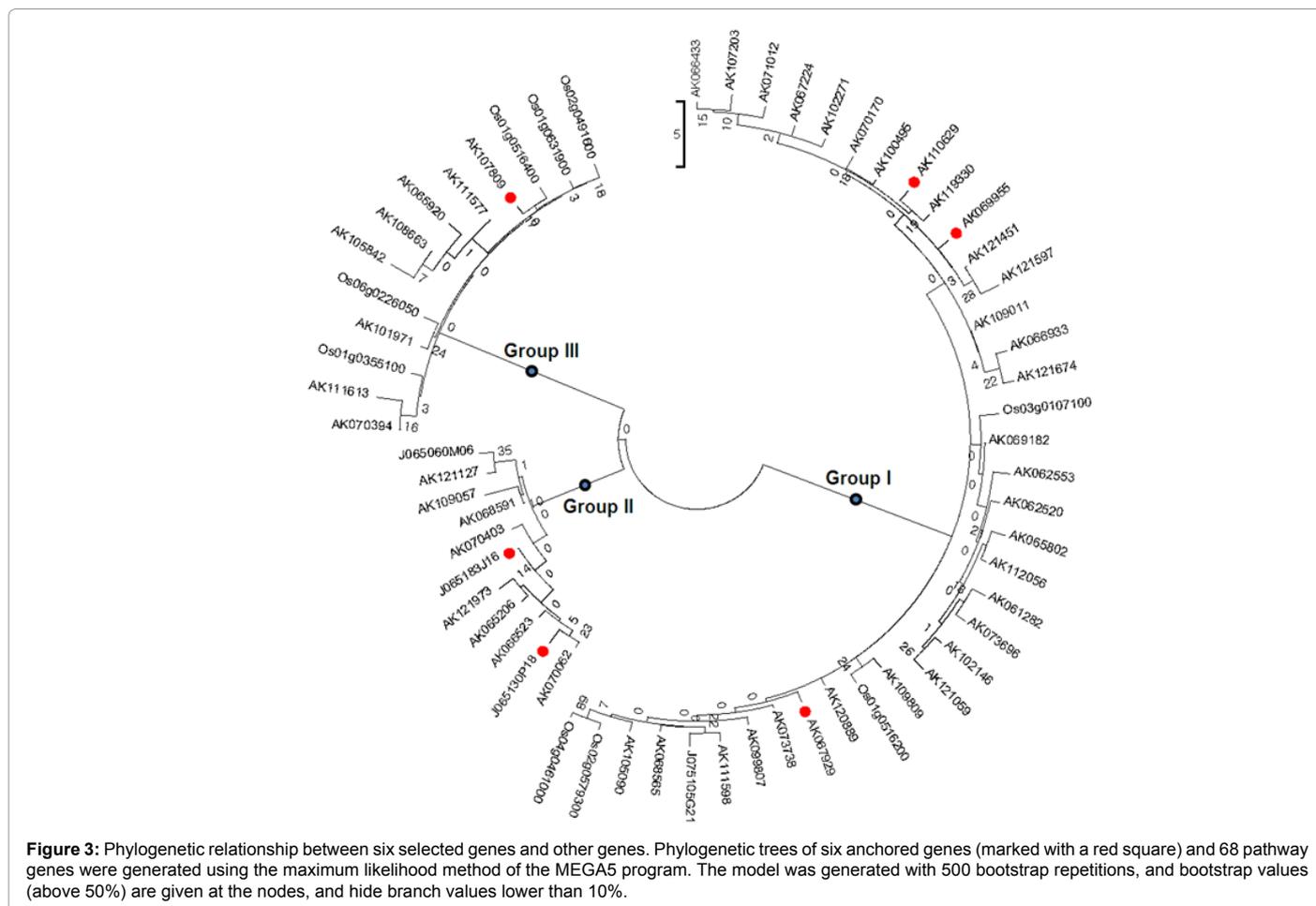
To verify the six hypothetical genes identified by COGs and

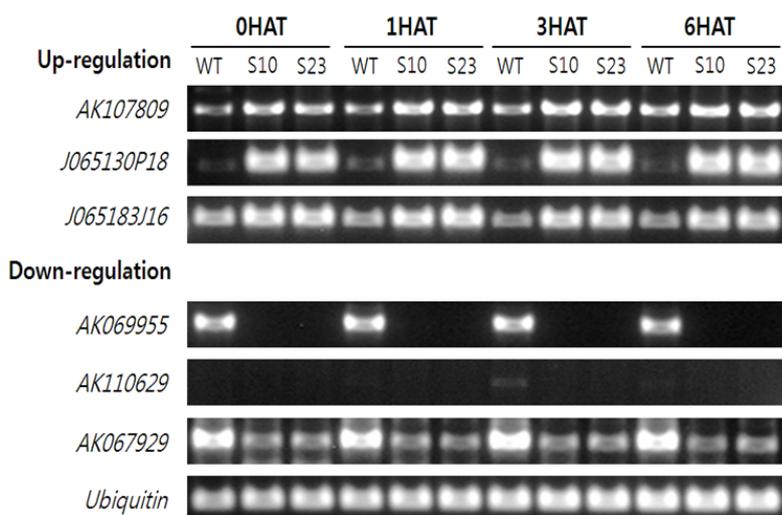
pathway analyses, we performed RT-PCR using the same RNA samples used in the microarray experiments. Three of six genes were up-regulated (AK107809, J065130P18, and J065183J16) and three genes were down-regulated (AK069955, AK110629, and AK067929) during all of the treatment stages tested (Figure 4). Among the six genes expressed at higher levels in the Dongjin cultivar, AK107809, J065130P18, and J065183J16 were expressed during all of the examined developmental stages. Therefore, these genes may play a role in salt tolerance or are related to salt stress-resistance increasing which are encoded a hypothetical protein, a wound-induced protein, and a conserved hypothetical protein, respectively. The three down-regulated genes were expressed at lower levels in the Dongjin cultivar that lacked salt tolerance or salt stress-resistance. Therefore, these genes may have additional functions other than salt stress biosynthesis. It is likely that the three up-regulated genes either play regulatory roles in increasing

Query ID	C <sup>a</sup>	MR <sup>b</sup>	ML <sup>c</sup>	Query		E-value	Score	Type
				Start	End			
AK067929	A11	100	736	485	1220	0	1459	Intergenic
AK069955	A11	100	431	1	431	0	854	5'Upstream
AK107809	A12	100	476	377	852	0	884	5'Upstream
AK110629	A04	100	523	86	608	0	1037	5'Upstream
J065130P18	A03	100	1279	1	1279	0	2535	5'Upstream
J065183J16	A07	100	1245	1	1245	0	2468	5'Upstream

<sup>a</sup>Chromosome; <sup>b</sup>matching ratio (%); <sup>c</sup>matching length.

**Table 2:** The mapping information of the six genes screened on the rice genome.





**Figure 4:** RT-PCR analysis of the salt tolerance–related genes identified. The expression of the six candidate genes was assessed in three up-regulated expression cultivars and three down-regulated expression cultivars during four stages. The 0HAT, 1HAT, 3HAT, and 6HAT columns represent hours after salt treatment. WT=*Dongjin* (wild type with weak salt tolerance expression), S1=*Salt10* (cultivar with high salt tolerance expression), S2=*Salt23* (cultivar with high salt tolerance expression), HAT=hours after salt treatment.

salt tolerance or are related to salt stress–resistance signaling during metabolite biosynthesis, whereas the down-regulated genes likely inhibit salt tolerance–resistance biosynthesis.

## Conclusions

We identified six putative genes with different functions that appear to be related to the regulation of salt-responsive biosynthesis and/or metabolism in rice, using 135K microarrays. Using a screening method to combine ortholog, pathway, gene mapping, and phylogenetic analyses, we performed a multi-processes analysis to screen salt tolerance–related biosynthesis genes during four salt-treatment stages in rice. After this analysis, we identified six genes that are most likely involved in regulation of either salt tolerance biosynthesis or metabolism. Our results suggest that these six genes participate in the production or control of salt tolerance during all of the treatment stages. Although there is no direct evidence, we suggest our methodology aided the identification of candidate genes after microarray experiment in the rice. While these genes will require further study to support the findings of our microarray analyses, our screening method does demonstrate a new and robust approach with which to identify genes involved in salt tolerance in rice.

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