Identification of genes involving in salt tolerance using GWAS data based on Na^+ content in local Thai rice leaves and Arabidopsis orthologous gene validation

Ratchata Chokwiwatkul[1], Natthaya Tantipirom[1], Noppkhun Khunpolwatatna[1], Apichart Imyim[2], Duangjai Suriya-aroonroj[3], Teerapong Buaboocha[1,4,5], Monnat Pongpanich[5,6], Luca Comai[7], Supachittra Chadchawan[1,5]

[1]Center of Excellence in Environment and Plant Physiology, Department. of Botany, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[2]Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[3]Nakhon Ratchasima Rice Research Center, Bureau of Rice Research and Development, Nakhon Ratchasima 30110, Thailand.
[4]Department of Biochemistry, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[5]Omic Science Center, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[6]Department of Mathematics and Computer Science, Faculty of Science, Chulalongkorn University, Bangkok 10330, Thailand.
[7]Department of Plant Biology, UC Davis Genome Center, UC Davis, Davis, CA 95616, USA.

**Corresponding author: s_chadchawan@hotmail.com; Supachitra.C@chula.ac.th*

ABSTRACT

High salinity is one of the most abiotic stresses that can adversely affect plant growth, development and productivity in rice for rice grown in the Northeastern part of Thailand. To overcome this problem and improve crop yield under salt stress conditions, it is important to improve salt tolerance in crops. This research aims to validate if the SNPs predicted by Genome-Wide Association Study (GWAS) is consistent with the QTL for salt tolerant trait that was previously reported. GWAS based on Na^+ content in leaf tissues of local Thai rice varieties after six days of salt stress revealed the involvement of SNPs from 10 loci, located on chromosomes 1, 2, 5, 10, 11 and 12. Five loci are in the reported salt tolerant QTL. To determine if these loci contributing to salt tolerant phenotype, the orthologous genes in Arabidopsis were used as the representatives. Homozygous Arabidopsis mutant lines, each of which contains the T-DNA in the orthologous genes of the predicted loci, were analyzed in comparison with wild type for the physiological responses, fresh weight, dry weight, chlorophyll *a*, chlorophyll *b* and carotenoids contents, after 7 days of 250 mM NaCl treatment. All of the mutant lines had the significant lower levels of chlorophyll *a* and chlorophyll *b* contents in normal condition, while under salt stress condition, all of them showed the significant difference in stability index of photosynthetic pigment contents. These indicate that all of these genes are involved in salt tolerance, which implies the impact of the predicted loci from GWAS in salt tolerant ability of rice. Our data show the consistency between GWAS and QTL studies and suggested that GWAS can be used to identify the precise loci that are responsible for the trait of interest.

Keywords: genome-wide association study (GWAS); Na^+/K^+ ratio; salt stress; QTL; Arabidopsis mutant lines

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the world's most crucial food crops. Rice has previously been reported to be susceptible to salt stress at both seedling and reproductive stages, which leads to grain yield reduction (Zeng *et al.*, 2001; Moradi and Ismil, 2007). It affects physiological processes, for instance, Na^+ uptake, exclusion, and ion imbalance (Na^+/K^+ ratio) (Siringam *et al.*, 2011). Salinity causes two main problems; osmotic stress which reduces the capacity of the plant to absorb water and decreases soil water potential (Zhu *et al.*, 1997), and ionic stress which is induced by an excessive concentration of salt, especially sodium chloride (NaCl) from the soil (Jouyban, 2012). Excess of Na^+ in plant cells causes damage in membrane systems and organelles, resulting in plant growth reduction and abnormal development (Davenprot *et al.*, 2005; Quintero *et al.*, 2007). Moreover, high Na^+ concentration inhibits uptake of K^+

ions which is an essential element for growth, development and enzyme activities. Eventually, these culminate in lower productivity and may even lead to death (Luan, 2009; Rubio *et al.,* 1995).

Salt tolerance is a quantitative trait, controlled by multiple loci in the genome. Several studies have reported quantitative trait loci (QTL) responsible for this trait (Dixit *et al.,* 2015; Koyama *et al.,* 2001; Dell'Acqua *et al.,* 2015). Moreover, genome wide association study (GWAS) was also studied for salt tolerance trait in rice (Kumar *et al.,* 2015). However, the regions in the genome responsible for this trait identified by these two approaches were not consistent.

This research aims to identify the SNPs involving in Na^+ content in rice using GWAS approach, and validate the involvement of the genes in salt tolerance if they were in the regions of salt tolerant QTL that were previously reported. For validation of gene functions in salt tolerance, the Arabidopsis's orthologous genes of the predicted rice loci were analyzed. The homozygous mutant lines containing T-DNA insertion at the orthologous genes were evaluated for the phenotypes grown in normal and salt stress conditions in comparison with the wild type. The gene mutations that result in significant difference between the wild type and the mutant line under salt stress condition would be considered as the salt tolerant-related genes.

MATERIALS AND METHODS

Plant Materials and growing conditions

For the GWAS experiment, 75 local Thai rice varieties (Table 1) were grown in 4-inch pots, containing 800 g of soil for 14 days in natural condition. Four replications of each variety per condition were used in this experiment. Then, seedlings were separated into 2 groups, one was treated with 115 mM NaCl solution, which caused soil salinity to 8-9 $dS.m^{-1}$, and the other was treated with filtered water with the same volume. After 6 days of the treatment, leaf tissues were collected and weighed and dried until used for Na^+ content measurement.

Arabidopsis T-DNA insertion lines (Alonso *et al.,* 2003) were used for the validation of the orthologous gene involvement in salt tolerance. The mutant lines containing the T-DNA insertion at the orthologous genes of the predicted rice loci from GWAS results were ordered from the Arabidopsis Biological Resource Center (ABRC).

Arabidopsis mutant line seeds were sterilized with 7.85% w/w of sodium hyperchlorite and 0.01% of Tween20 for 12 minutes. Then, seeds were washed with sterile water for 5 times. Sterile Arabidopsis seeds were placed on Murashige and Skoog (MS) media plates (Murashige and Skoog, 1962) and incubated in 4°C for 48 hours. After that, the seeds were grown on 18°C for 7 days. Then, Arabidopsis seedlings were transferred to soil (peat moss: lite: vermiculite, 3: 1: 1, respectively) and grew for another 7 days before leaf tissue collection for homozygosity determination. The seeds from the homozygous plants were collected for the physiological response analysis. If it was the heterozygous plant, the seeds were collected and replanted. Then, the screening was done again until the homozygous line was obtained.

Genome Wide Association Study (GWAS)

Rice genomic DNA was extracted from leaf tissues and subject to high-throughput sequencing. Then, SNP calling was done as followed. The Illumina paired-end reads were mapped to the rice reference genome, IRGSP-1.0.21 (Kawahara *et al.,* 2013) using Burrows Wheeler Aligner software (BWA version 0.7.10) (Li and Durbin, 2009). Variants from rice varieties were called using Genome Analysis Toolkit (GATK) (McKenna *et al.,* 2010). SNPs were filtered using the following criteria: minimum coverage in order for a position to be called homozygous was 5; minimum coverage of each of the two observed major basecalls to be called heterozygous was 5; and minimum percentage of each of the two observed major basecalls in order to be called heterozygous is 20.

Association analysis was performed with the following condition. SNPs with more than 40% missing data were removed from the analysis. Ungenotyped SNPs were then inferred by genotype imputation method using Beagle (Browning and Browning, 2016). Principle Component Analysis based on SNPs in all accessions was conducted using the EIGENSOFT package (Patterson *et al.,* 2006). Association analysis was performed using the mixed model implemented in the software GEMMA (Zhou and Stephens, 2012). The significance threshold, set at p values from Wald test less than 10^{-13}, was used in this research.

Screening of homozygous T-DNA insertion lines of Arabidopsis

Total genomic DNA of Arabidopsis was extracted from leaf tissues according to Chabi et al (2015). Then, the homozygous insertion at the target genes were screened using the specific primer set, LP+RP and BP+RP. LP and RP are the primers specific

to the sequence of the target gene at the 5'end and 3'end of the T-DNA insertion, which will be about 900 bp apart from each other. BP primer is specific to the sequence of T-DNA fragment. If there is the T-DNA insertion at the target gene, the positive band (about 400 bp) from the amplification with LP and BP primers will be detected, if no insertion, no amplification will be obtained. Moreover, the amplification with LP and RP will be detected (about 900 bp) in the case of no T-DNA insertion. If T-DNA is inserted, LP and RP will not be able to amplify any fragment as the length of amplification is not appropriate with the amplification cycle (Figure 1). The wild type (WT) Arabidopsis, ecotype Columbia-0 (Col-0), was used as the control.

Table 1 Information of rice varieties used in the GWAS experiment

No.	Rice varieties	GS No.	Type of rice	Salt tolerance level*	Source of rice variety
1	IR29	-	Lowland rice	Salt sensitive standard variety	Philippines
2	POKKALI	-	Lowland rice	Salt tolerant standard variety	India
3	RD 73	-	Lowland rice	The latest salt tolerant cultivar from Rice Department, Thailand	Thailand
4	LPT123	868	Lowland rice	2	Thailand
5	GWIAN HAK	13201	Lowland rice	1	Thailand
6	JAO RAHK HAENG	12494	Lowland rice	2	Thailand
7	CHIANG PHATTHALUNG	21964	Lowland rice	2	Thailand
8	LEB NOK	3979	Lowland rice	2	Thailand
9	SETTI	5678	Lowland rice	2	Thailand
10	NIAW DAM LAI	21240	Lowland rice	1	Thailand
11	LEUANG TIA	6448	Lowland rice	4	Thailand
12	LEUANG GAEW	6812	Lowland rice	4	Thailand
13	LEUANG BAI JAEK	14155	Lowland rice	2	Thailand
14	LEUANG BAI LOD	14154	Lowland rice	4	Thailand
15	LEUANG KWAI LAH	5551	Lowland rice	2	Thailand
16	LEUANG KWAI LAH	7285	Lowland rice	4	Thailand
17	LEUANG HUAN	7303	Lowland rice	4	Thailand
18	LEUANG PRATEW	5681	Lowland rice	2	Thailand
19	LEUANG PLAH GIM	7293	Lowland rice	2	Thailand
20	GAEN JAN	22653	Lowland rice	3	Thailand
21	JAE GAN	12507	Lowland rice	3	Thailand
22	DAENG NAH	22850	Lowland rice	1	Thailand
23	RD10	4790	Lowland rice	3	Thailand
24	RD12	21628	Lowland rice	1	Thailand
25	RD17	3999	Deep water rice	3	Thailand
26	RD19	4000	Deep water rice	2	Thailand
27	RD21	4791	Lowland rice	2	Thailand
28	RD25	4793	Lowland rice	3	Thailand
29	RD27	7125	Lowland rice	3	Thailand
30	RD31	24533	Lowland rice	2	Thailand
31	GAM FEUANG	4490	Lowland rice	1	Thailand
32	PRACHIN BURI 1	23406	Deep water rice	2	Thailand
33	KHAO SAMER	3370	Deep water rice	2	Thailand
34	KHAO GAEW	6152	Lowland rice	3	Thailand
35	KHAO TAENG MO	3810	Lowland rice	3	Thailand
36	KHAO KOD	12503	Lowland rice	1	Thailand
37	KHAO TAH JEUA	3808	Lowland rice	2	Thailand
38	KHAO TAH CHEUA	12270	Lowland rice	2	Thailand

Table 1 (continued)

No.	Rice varieties	GS No.	Type of rice	Salt tolerance level*	Source of rice variety
39	KHAO BAHN POD	19877	Lowland rice	1	Thailand
40	KHAO PUANG	9362	Lowland rice	3	Thailand
41	KHAO LUANG	7282	Lowland rice	2	Thailand
42	KHAO LUANG	5533	Lowland rice	2	Thailand
43	KHAO' HAO	5625	Lowland rice	1	Thailand
44	KHITOM KHAO	21708	Lowland rice	2	Thailand
45	KAN NAH	6442	Lowland rice	2	Thailand
46	JAMPAH TAWNG	5211	Lowland rice	2	Thailand
47	CHAW PLI KHAO	9742	Lowland rice	2	Thailand
48	CHAW MA GAWK	2042	Deep water rice	2	Thailand
49	CHAI NAT 1	20712	Lowland rice	3	Thailand
50	CHUMPAE 60	16235	Lowland rice	1	Thailand
51	SEW MAE JAN	4001	Upland rice	3	Thailand
52	DAWK KHAH	12160	Lowland rice	3	Thailand
53	TAH JEUA	5545	Lowland rice	2	Thailand
54	TAH BAHN	21695	Lowland rice	3	Thailand
55	NAH KHAWAN	22379	Lowland rice	2	Thailand
56	NAHNG NUAN	3151	Lowland rice	2	Thailand
57	NAM SAGUI 19	3023	Lowland rice	2	Thailand
58	NAM SAGUI 19	15833	Lowland rice	2	Thailand
59	BEU SAW MI	23595	Upland rice	1	Thailand
60	BUN MAH	3031	Lowland rice	1	Thailand
61	PLAH KHAENG	3241	Lowland rice	2	Thailand
62	PLAI NGAHM PRACHIN BURI	20864	Deep water rice	3	Thailand
63	PUANG NGERN	2963	Lowland rice	1	Thailand
64	PUANG TAWNG	18442	Deep water rice	2	Thailand
65	PUANG NAHK	12266	Lowland rice	2	Thailand
66	PUANG HAHNG NAHK	23233	Lowland rice	3	Thailand
67	LOI HAH RUANG	6230	Lowland rice	3	Thailand
68	LAI MAHK	7025	Lowland rice	4	Thailand
69	LOOK DAENG PATTANI	21963	Lowland rice	3	Thailand
70	LOOK DAENG PATTANI	23303	Lowland rice	2	Thailand
71	SANG YOD	15101	Lowland rice	2	Thailand
72	SAHM RUANG	7288	Lowland rice	3	Thailand
73	SOON	22492	Lowland rice	3	Thailand
74	LUANG PRATAHN	6440	Lowland rice	3	Thailand
75	HANTRA 60	16232	Deep water rice	2	Thailand

*The salt tolerant level was reported based on hierarchical clustering with the seedling phenotypes of salt injury score, relative water content, cell membrane stability, Na^+, K^+ and Na^+/K^+ content in leaves under salt stress condition for 9 days. Four clusters were found and ranked 1 as the most salt sensitive and 4 as the most salt tolerant group.

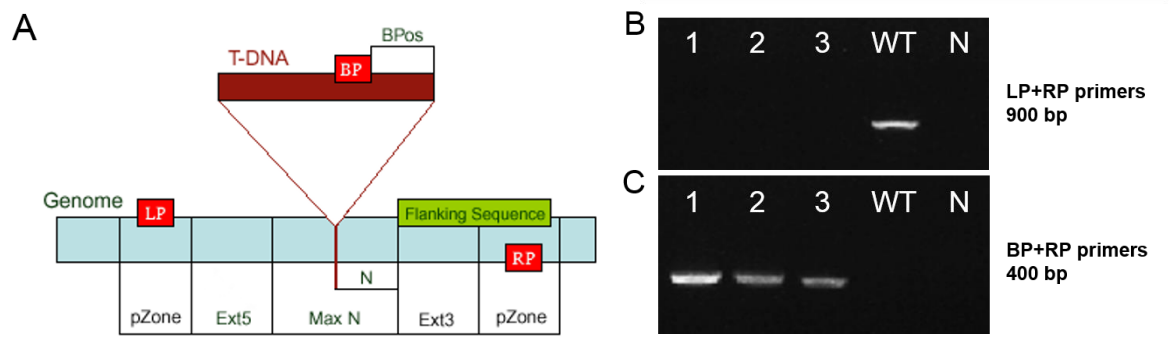


Figure 1 Example of homozygous mutant line screening. Homozygous plants (1, 2 and 3) were screened with the genomic DNA amplification using LP+RP primers and LB+RP primers. The locations of the primers were shown in A. The amplification of LP+RP and LB+RP primers was shown in B and C, respectively. The patterns of the DNA bands were different between wild type (WT) and homozygous plants. N was the negative control sample.

Physiological response comparison of mutant lines and WT

Experimental design

Randomized complete block design (RCBD) with 4 replications and 4 plants per replication was performed to compare physiological responses, determined by fresh weight, dry weight, chl *a*, chl *b* and carotenoid contents, between *Arabidopsis thaliana* ecotype Columbia-0 (Col-0) (WT) and homozygous Arabidopsis mutant lines under normal and salt stress conditions.

Growing condition for physiological studies

Homozygous Arabidopsis mutant seeds and WT seeds were sterilized with 7.85% w/w of sodium hyperchlorite and 0.01% of Tween20 for 12 minutes. Then, seeds were washed with sterilized water for 5 times. Then the seeds were placed on MS plates and incubated at 4°C for 48 hours. After that, they were germinated under 35μmol.m^{-1}.s^{-1} light intensity with 16/8 light/dark period at 22°C for 7 days. Then, Arabidopsis seedlings were transferred to grow in the mix of peat moss: perlite: vermiculite at the ratio of 3: 1: 1 and grew in the controlled condition room (closed system) under 70 μmol.m^{-1}.s^{-1} light intensity with 16/8 light/dark period at 23°C for 14 days. Total 44 plants were grown in each tray at the size of 50 x 40 cm^2 and 3 liters of water was supplied at the beginning of the transfer and the level of water was maintained until the end of the experiment.

Twenty-one-day-old seedlings were separated into two groups, one was for normal grown condition and the other was for salt stress grown condition. For normal grown condition, the plants were maintained as mention above, while under the salt stress condition, 3 liters of 250mM NaCl solution was supplied instead of water. The level of water or solution was maintained for 7 days by addition of filtered water. The soil conductivity in normal growing condition was 2 dS.m^{-1}, while in the salt stress condition was 9 dS.m^{-1}.

Collection of physiological parameters

After 7 days of treatment, the above ground tissues were collected and determined as fresh weight per plant. The tissues then were dried under 80°C until no weight change to determine dry weight per plant. Physiological parameters were collected at day 7 after treatment, e.g. fresh weight, dry weight, chl *a*, chl *b* and carotenoids contents from leaf tissue.

For photosynthetic pigment content measurement, the first fully-expanded leaf of each plant sample was collected and weighed. The pigments were extracted with 80% acetone at 4°C for 48 hours in darkness. The absorbance at 470, 646.8 and 663.2 nm was determined using spectrophotometer (Agilent 8453 UV-visible Spectroscopy System). The pigment contents, chlorophyll *a* (chl *a*), chlorophyll *b* (chl *b*) and carotenoids were calculated according to Wellburn (1994) as shown below:

Chlorophyll *a* content = $12.25A_{663.2} - 2.79A_{646.8}$
Chlorophyll *b* content = $21.5A_{646.8} - 5.1A_{663.2}$
Carotenoids content
= [(1000A_{470} – 1.82 Chl *a* – 85.02 Chl *b*)]/ 198

Data analysis

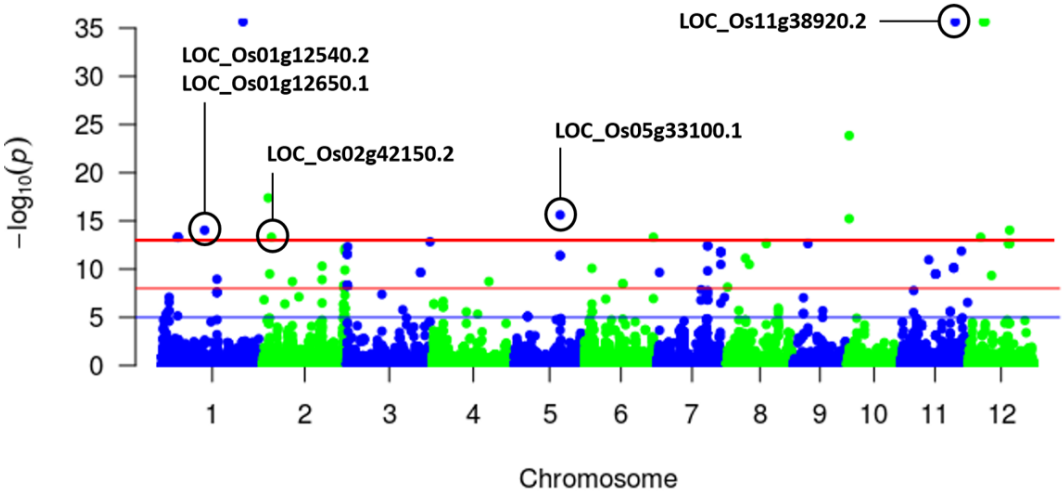
Analysis of variance of each parameter was performed and the means were compared with Duncan's multiple range tests at a significant level of 0.05 using SPSS statistic base software package. The cluster analysis of the SNPs from 10 loci was performed according to Ward's method using JMP10 software.

RESULTS AND DISCUSSION

Predicted loci from GWAS and identification of orthologous genes in Arabidopsis

Based on the threshold of *p* values from Wald test less than 10^{-13}, 10 loci on chromosome 1, 2, 5, 10, 11 and 12 was predicted to be involved with Na^+ content under salt stress condition. Among these, five of them, LOC_Os02g42150.2, LOC_Os01g12540.2, LOC_Os01g12650.1, LOC_Os05g33100.1, and LOC_Os11g38920.2, were located in the QTL regions previously reported (Figure 2 and Table 2). None of these were consistent with the predicted loci from GWAS performed by Kumar et al (2015). This should be due to the difference in rice populations used for the analyses. This research was done with local Thai rice varietie, while Kumar's was performed with Indian rice varieties. Moreover, the p-value threshold used by Kumar et al (2015) ($p<10^{-4}$) was higher than what we used in this experiment ($p<10^{-13}$). This is due to the number of SNPs obtained from each dataset. In our data, 77,063 SNPs were obtained for the association, while the 6,000 SNP chip was used in Kumar et al's experiment.

Some predicted loci from the GWAS were characterized for their functions. LOC_Os02g42150.2 encodes for OsWAK14, which was previously identified to be a positive regulator for rice blast resistance (Delteil *et al.*, 2016; Cyrol *et al.*, 2016). There has been no report on salt stress response in this gene. LOC_Os01g12540.2 encodes for a GTPase with unknown function. In Arabidopsis, RAB11 GTPases are required for salt tolerance (Asaoka *et al.*, 2013). Therefore, this gene may have the similar function in rice. LOC_Os01g12650.1 encodes for a reticulon domain containing protein. In Tibetan wild barley (*Hordeum spontaneum* L.), the reticulon domain-containing protein (F2DHE3) was up-regulated in the variety that had the higher Na^+ accumulation. This finding was suggested to be involved with salt adaptation in this species (Shen *et al.*, 2016). Moreover, reticulon family protein (RTNLB2) in Arabidopsis was also up-regulated under salt stress condition (Ma and Bohnert, 2007).



No	Rice Locus	Arabidopsis Locus	Gene Product Name
1	LOC_Os01g12540.2	AT5G39960	GTPase of unknown function domain containing protein
2	LOC_Os01g12650.1	AT4G11220	Reticulon domain containing protein
3	LOC_Os02g42150.2	AT1G21230	OsWAK14 - OsWAK receptor-like protein kinase
4	LOC_Os05g33100.1	AT3G23600	Endo-1,3;1,4-beta-D-glucanase precursor
5	LOC_Os11g38920.2	AT1G74520	HVA22

Figure 2 Manhattan plot of the Na^+ content in leaves after 6 days of salt stress. The selected loci were cut off with $P<10^{-13}$. The positions that were consistent with QTL regions were circled and the orthologous genes were listed in the table below.

Table 2 Loci identified by GWAS with Na^+ content in leaf tissues and the p-value threshold used was less than 10^{-13}.

No.	P-value	Rice chr.	Position of candidate SNPs on rice chromosome	Locus	Gene product name	Orthologous gene in Arabidopsis	Referents for salt tolerance QTL
1	1.42×10^{-14}	2	25349420	LOC_Os02g42150	OsWAK14 - OsWAK receptor-like protein kinase	AT1G21230	Dixit *et al.*, 2015
2	2.44×10^{-14}	1	6900271	LOC_Os01g12540	GTPase of unknown function domain containing protein	AT5G39960	Koyama *et al.*, 2001
3	2.44×10^{-14}	1	6969721	LOC_Os01g12650	Reticulon domain containing protein	AT4G11220	Koyama *et al.*, 2001
4	2.73×10^{-14}	5	19412122	LOC_Os05g33100	Endo-1,3;1,4-beta-D-glucanase precursor	AT3G23600	Dixit *et al.*, 2015
5	4.89×10^{-15}	12	17420950	LOC_Os12g29350	ATP binding protein	AT3G48770	-
6	3.42×10^{-16}	10	1023521	LOC_Os10g02644	Hypothetical protein	-	-
7	2.78×10^{-18}	2	2320436	LOC_Os02g38380	Transposon protein	-	-
8	5.94×10^{-36}	11	23175746	LOC_Os11g38920	HVA22	AT1G74520	Dell'Acqua *et al.*, 2015
9	5.94×10^{-36}	1	34769930	LOC_Os01g60110	E2F-related protein	AT1G09575	-
10	5.94×10^{-36}	12	6912259	LOC_Os12g12560	NADP-dependent oxidoreductase	AT3G03080	-

LOC_Os05g33100.1 encodes endo-1,3;1,4-beta-D-glucanase precursor. Endo-1,4-beta-D- glucanase plays a role in cell wall extensibility. It was reported to be involved in flooding, heavy metal, ozone and salt stress in various species (Gall *et al.*, 2015). The transcription factor OsMPS is induced by salt stress and regulates cell wall biosynthesis genes, including endo-1,4-beta-D- glucanase in rice (Lippold *et al.*, 2009; Schmidt *et al.*, 2013). LOC_Os11g38920.2 is orthologous to HVA22, which is induced by environmental stresses, including salt stress (Brands and Ho, 2002). HVA22 in Arabidopsis is regulated by abscisic acid (ABA) and salt stress (Chen *et al.*, 2002). There is a high potential that this gene in rice is involved in salt stress response.

Five loci that are located in the salt tolerant QTL were selected for further characterization by searching for the orthologous genes in Arabidopsis by using the data from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) (Kahawara *et al.*, 2013). These orthologous genes are AT5G39960, AT4G11220, AT1G21230, AT3G23600 and AT1G74520 (Table 2).

Physiological responses under salt stress in homozygous Arabidopsis mutant lines

The homozygous mutant lines of Arabidopsis were evaluated for salt stress responses with WT. All of the mutant lines and wild type (WT) had the similar fresh weight and dry weight in both conditions (Figure 3). In normal condition, all mutants showed the significant lower levels of all photosynthetic pigment contents than wild type, except the mutant with insertion at AT1G74520 that had the similar level of carotenoids to WT. The lowest amount of chl *a* and chl *b* in normal grown plants was found in AT4G11220 and AT3G23600 mutant lines, which had about a quarter of chlorophyll content of WT. However, the carotenoid contents of these two mutant lines were similar to those of WT (Figure 4). The significantly lower carotenoid content in plants grown under normal condition was found in the AT5G39960 and AT1G21230 mutants, while other mutant lines showed the similar level of carotenoids (Figure 4).

To determine the effects of salt stress, stability index, calculated from the ratio of pigment content in stress grown plants to pigment content in normal grown plants, was used for the comparison (Figure 5). This parameter reveals the ability to maintain photosynthetic pigment levels under salt stress condition, which will reflect the ability of photosynthetic performance under salt stress. It has been shown that the salt tolerant genotypes had the higher ability to maintain chlorophyll content (Sevengor et al. 2011) For chl *a*, stability indices of all mutant lines showed no significant difference from stability index of chl *a* in WT. On the contrary, four mutant lines, AT5G39960, AT4G11220, AT3G23600 and AT1G74520, showed significantly lower stability index of chl *b* contents than that of WT. Although the AT1G21230 mutant had the similar stability indices of chl *a* and chl *b* content to WT, it had the significantly higher stability index of carotenoid. Another mutant line that had the significantly higher stability index of carotenoid than WT was AT5G39960 mutant. AT1G74520 mutant showed the similar stability index of carotenoid to WT, while the other two mutants, AT4G11220 and AT3G23600 had the significant lower carotenoid stability index than WT (Figure 5). These data indicate that mutations in these genes of Arabidopsis affect the stability index of photosynthetic pigments, which implies the importance of these genes in salt tolerance.

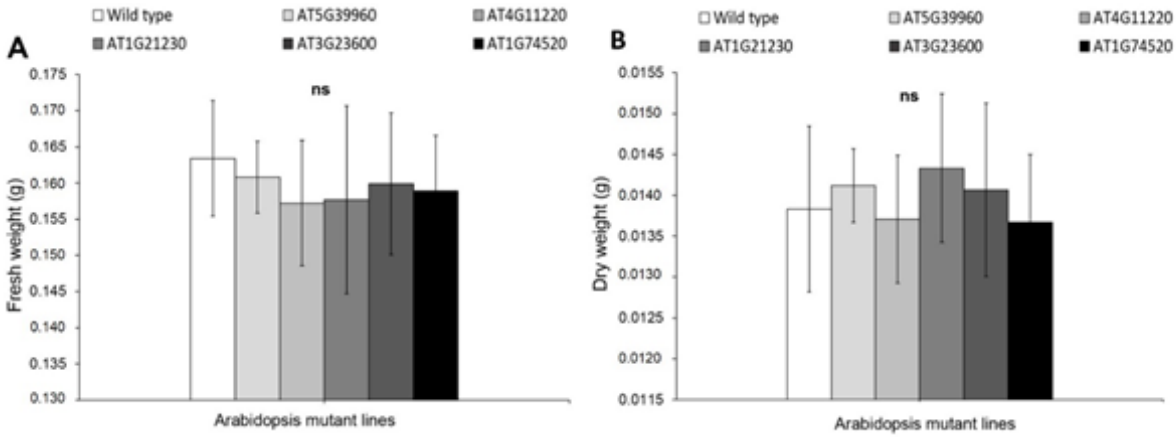


Figure 3 Fresh weight (A) and dry weight (B) of wild type and 5 mutants after grown in salt stress for 7 days

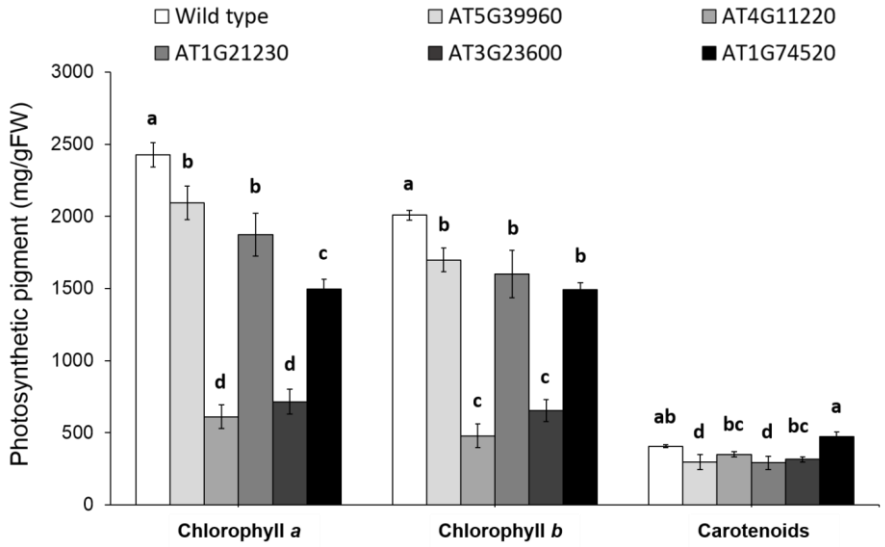


Figure 4 Photosynthetic pigments, chlorophyll *a*, chlorophyll *b*, and carotenoid contents in wild type and 5 mutants under normal conditions.

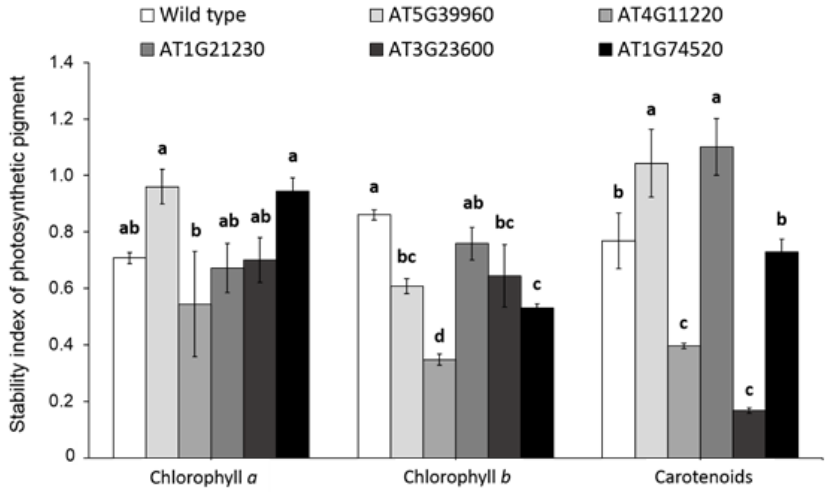


Figure 5 Stability indices of photosynthetic pigments, chlorophyll *a*, chlorophyll *b*, and carotenoid contents in wild type and 5 mutants after salt stress for 7 days

Salt stress causes the reduction of photosynthetic pigments (Munns and Tester, 2008) due to osmotic stress and ionic toxicity (Lakshmi *et al.*, 1996; Misra *et al.*, 1997). Therefore, the stability index of photosynthetic pigments of salt stress is lower than 1. In our experiment, most stability indices were less than 1, except the stability indices of carotenoids in AT1G21230 and AT5G39960 mutants. This may be because the level of carotenoid content in these mutants under normal condition is at the minimal level to be able to survive. Carotenoids are the accessory pigments that only function for light harvest, but also function in the ROS scavenging system to protect the photosynthesis system from photoinhibition (Taiz et al, 2015).

The hierarchy clustering of SNPs in 10 loci and the comparison with salt responsive phenotypes of 75 rice varieties.

When SNPs from 10 identified loci were used for hierarchy clustering, 75 varieties were clustered into 4 groups (Figure 6). IR29, which is the salt susceptible standard is in Group I, while Group II consists of three

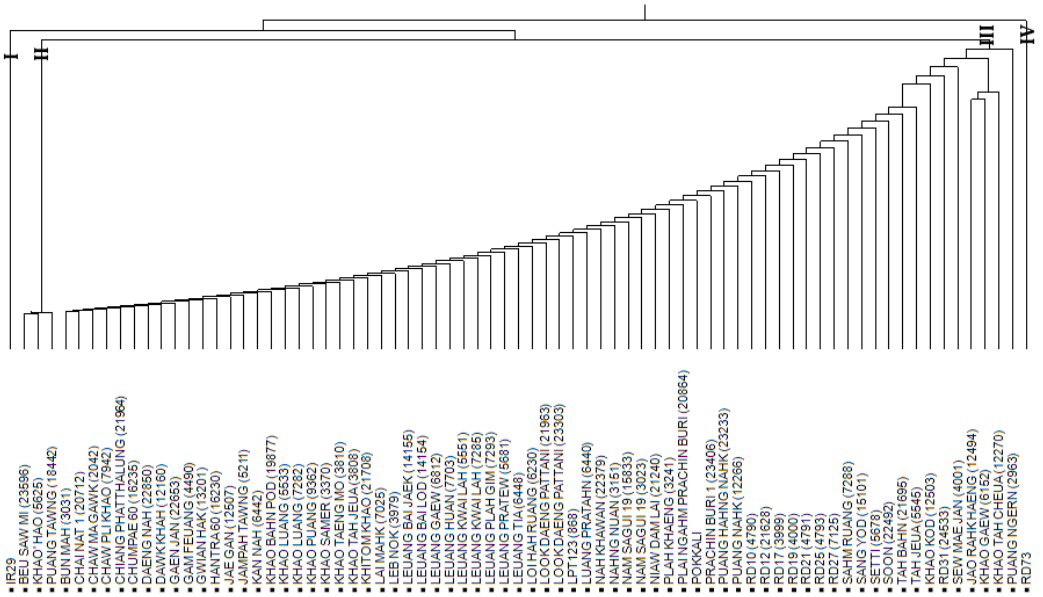


Figure 6 Hierarchy clustering of SNPs from 10 loci identified by GWAS with Na^+ content in leaves at seedling stage under salt stress condition

varieties, BEU SAW MI, KHAO' HAO, and PUANG TAWNG. BEU SAW MI and KHAO' HAO were classified in the most salt sensitive group according to the phenotype studies as shown in Table1, while PUANG TAWNG was classified in the second most salt susceptible group. The rest of the rice varieties in this study, except RD 73, was in Group III, including Pokkali, the salt tolerant standard cultivar. RD 73, which is the latest salt tolerant varieties developed by breeding program in Rice Department of Thailand is in Group IV.

REFFERENCES

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P (2003) Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science 301: 653–657.

Asaoka R, Uemura T, Nishida S, Fujiwara T, Ueda T, Nakano A (2016) New insights into the role of Arabidopsis RABA1 GTPases in salinity stress tolerance. Plant Signal Behav 11: e1149676.

Browning BL, Browning SR (2016) Genotype imputation with millions of reference samples. Am J Hum Genet 98: 116–126.

Cayrol B, Delteil A, Gobbato E, Kroj T, Morelb JB (2016) Three wall-associated kinases required for rice basal immunity form protein complexes in the plasma membrane. Plant Signal Behav 11: e1149676.

Chabi K, Kefela T, Sagbadja H, Ahoton L, Saidou A, Moussa L, Baptistea L, Kotconia S, Gachomo E (2015) A simple and efficient genomic DNA extraction protocol for large scale genetic analyses of plant biological systems Plant Gene 1: 43–45.

Chen CN, Chu C, Zentella R, Pan Z, Tuan-Hua David (2002) AtHVA22 gene family in Arabidopsis:

phylogenetic relationship, ABA and stress regulation, and tissue-specific expression. Plant Mol Biol 49: 633–644.

Davenport R, James RA, Zakrisson-Plogander A, Tester M, Munns R (2005) Control of sodium transport in durum wheat. Plant Physiol 137: 807–818.

Dell'Acqua DM, Gatti DM, Pea G, Cattonaro F, Coppens F, Magris G, Hlaing AL, Aung H, Nelissen H, Baute J, *et al.* (2015) Genetic properties of the MAGIC maize population: a new platform for high definition QTL mapping in *Zea mays*. Genome 16: 1–23.

Delteil A, Gobbato E, Cayrol B, Estevan J, Michel-Romiti C, Dievart A, Kroj T, Morel JB. 2016. Several wall-associated kinases participate positively and negatively in basal defense against rice blast fungus. BMC Plant Biol. 16:17.

Dixit S, Biswal AK, Min A, Henry A, Oane RH, Raorane ML, Longkumer T, Pabuayon IM, Mutte IM, Vardarajan AR, *et al.* (2015) Action of multiple intra-QTL genes concerted around a co-localized transcription factor underpins a large effect QTL. Sci Rep 5: 15183.

Gall HL, Philippe F, Domon JM, Gillet F, Pelloux J, Rayon C (2015) Cell wall metabolism in response to abiotic stress. Plants 4: 112–166.

Jouyban Z (2012) The effects of salt stress on plant growth. Technical Journal of Engineering and Applied Sciences 2: 7–10.

Kawahara Y, Bastide M, Hamilton JP, Kanamori H, McCombie WR, Ouyang S, Schwartz DC, Tanaka T, Wu J, Zhou S, Childs KL, *et al.* (2013) Improvement of the *Oryza sativa* Nipponbare reference genome using next generation sequence and optical map data. Rice 6: 4.

Koyama ML, Levesley A, Koebner MD, Flowers TJ, Yeo AR (2001) Quantitative trait loci for component physiological traits determining salt tolerance in rice. Plant Physiol 125: 406–422.

Kumar V, Singh A, Amitha MSV, Krishnamurthy SL, Parida SK, Jain S, Tiwari KK, Kumar P, Rao AR, Sharma SK (2015) Genome-wide association mapping of salinity tolerance in rice (*Oryza sativa*). DNA Res 22: 133–145.

Lakshmi A, Ramanjulu S, Veeranjaneyulu K, Sudhakar C (1996) Effect of NaCl on photosynthesis parameters in two cultivars of mulberry. Photosynthetica 32: 285–289.

Li H, Durbin R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 25: 1754-60.

Lippold F, Sanchez DH, Musialak M, Schlereth A, Scheible WR, Hincha DK, Udvardi MK (2009) *AtMyb41* regulates transcriptional and metabolic responses to osmotic stress in Arabidopsis. Plant Physiol 149: 1761–1772.

Luan S, Lan W, Lee C (2009) Potassium nutrition, sodium toxicity and calcium signaling connections through the CBL-CIPK network. Curr Opin Plant Biol 12: 339–346.

Ma S, Bohnert HJ (2007) Integration of *Arabidopsis thaliana* stress-related transcript profiles, promoter structures, and cell-specific expression. Genome Biol 8: R49.

McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo M. A. (2010). The genome analysis toolkit: A MapReduce framework for analyzing next-generation DNA sequencing data. Genome Research, 20: 1297-1303.

Misra AN, Sahu SM, Misra M, Singh P, Meera I, Das N, Kar M, Shau P (1997) Sodium chloride induced changes in leaf growth, and pigment and protein contents in two rice cultivars. Biol Plant 39: 257–262.

Moradi F, Ismail AM (2007) Responses of photosynthesis, chlorophyll fluorescence and ROS-scavenging stems to salt stress during seedling and reproductive stages in rice. Ann Bot 99: 1161–1173.

Munns R, Tester M (2008) Mechanisms of salinity tolerance. Annu Rev Plant Biol 59: 651–681.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15: 473–497.

Patterson N, Price AL, Reich D (2006) Population structure and eigen analysis. PLoS Genetics 2: 2074–2093.

Quintero JM, Fournier JM, Benlloch M (2007) Na^+ accumulation in shoot is related to water transport in K^+-starved sunflower plants but not in plants with a normal K^+ status. J Plant Physiol 164: 60–67.

Rubio F, Gassmann W, Schroeder JI (1995) Sodium-driven potassium uptake by the plant potassium transporter HKT1 and mutations conferring salt tolerance. Science 270: 1660–1663.

Schmidt R, Schippers JHM, Mieulet D, Obata T, Fernie AR, Guiderdoni E, Mueller-Roeber B (2013) MULTIPASS, a rice R2R3-type MYB transcription factor, regulates adaptive growth by integrating multiple hormonal pathways.Plant J 76: 258–273.

Sevengor S, Yasar F, Kusvuran S, Ellialtioglu S (2011) The effect of salt stress on growth, chlorophyll content, lipid peroxidation and

antioxidative enzymes of pumpkin seedling. Afr J Agric Res 6: 4920–492.

Shen Q, Fu L, Dai F, Jiang L, Zhang G, Wu D (2016) Multi-omics analysis reveals molecular mechanisms of shoot adaption to salt stress in Tibetan wild barley. Genomics 17: 889–903.

Siringam K, Juntawong N, Cha-um S, Kirdmanee C (2011) Salt stress induced ion accumulation, ion homeostasis, membrane injury and sugar contents in salt-sensitive rice (*Oryza sativa* L. spp. *indica*) roots under iso-osmotic conditions. Afr J Biotechnol 10: 1340–1346.

Taiz L, Zeiger E, Moller IM, Murphy A (2015) Plant Physiology and Development. The sixth edition. Sinauer Associates.

Wellburn AR (1994) The spectral determination of chlorophyll *a* and *b*, as well as total carotenoid, using various solvents with spectrophotometers of different resolution. J Plant Physiol 144: 307–313.

Zeng L, Shannon MC, Lesch SM (2001) Timing of salinity stress affects rice growth and yield components. Agric Water Manag 48: 191–206.

Zhou X, Stephens M (2012). Genome-wide efficient mixed-model analysis for association studies. Nature Genetics. 44: 821-824.

Zhu JK, Hasegawa PM, Bressan RA, Bohnert PHJ (1997) Molecular aspects of osmotic stress in plants. CRC Crit Rev Plant Sci 16: 253–277.