Molecular Plant • Volume 4 • Number 1 • Pages 25–41 • January 2011

Root-Specific Transcript Profiling of Contrasting Rice Genotypes in Response to Salinity Stress

Olivier Cotsaftis[a,2], Darren Plett[a,2], Alexander A.T. Johnson[a,b], Harkamal Walia[c,d], Clyde Wilson[e], Abdelbagi M. Ismail[f], Timothy J. Close[c], Mark Tester[a,1] and **Ute Baumann[a]**

a Australian Centre for Plant Functional Genomics, Private Mail Bag 1, Glen Osmond, SA 5064, Australia
b Present address: School of Botany, University of Melbourne, Parkville, Vic 3010, Australia
c Department of Botany and Plant Sciences, University of California, Riverside, CA 92521, USA
d Present address: Department of Agronomy and Horticulture, University of Nebraska, Lincoln, NE 68588, USA
e United States Department of Agriculture Agricultural Research Service, George E. Brown, Jr, Salinity Laboratory, Riverside, CA 92507, USA
f International Rice Research Institute, Manila, The Philippines

ABSTRACT Elevated salinity imposes osmotic and ion toxicity stresses on living cells and requires a multitude of responses in order to enable plant survival. Building on earlier work profiling transcript levels in rice (*Oryza sativa*) shoots of FL478, a salt-tolerant *indica* recombinant inbred line, and IR29, a salt-sensitive cultivar, transcript levels were compared in roots of these two accessions as well as in the roots of two additional salt-tolerant *indica* genotypes, the landrace Pokkali and the recombinant inbred line IR63731. The aim of this study was to compare transcripts in the sensitive and the tolerant lines in order to identify genes likely to be involved in plant salinity tolerance, rather than in responses to salinity *per se*. Transcript profiles of several gene families with known links to salinity tolerance are described (e.g. *HKTs*, *NHXs*). The putative function of a set of genes identified through their salt responsiveness, transcript levels, and/or chromosomal location (*i.e.* underneath QTLs for salinity tolerance) is also discussed. Finally, the parental origin of the *Saltol* region in FL478 is further investigated. Overall, the dataset presented appears to be robust and it seems likely that this system could provide a reliable strategy for the discovery of novel genes involved in salinity tolerance.

Key words: Rice; root; salinity tolerance; microarray; *HKT*; *Saltol*.

INTRODUCTION

Salinity tolerance is a complex physiological trait requiring coordinated, tissue-specific processes throughout the lifecycle of a plant to enable growth on saline soils. Processes conferring salinity tolerance have been extensively reviewed (Tester and Davenport, 2003; Munns et al., 2006; Munns and Tester, 2008) and include: (1) osmotic tolerance to salt; (2) sodium (Na^+) exclusion from the photosynthetic tissue; and (3) tolerance of leaf tissue to Na^+ by means of vacuolar storage of Na^+ and osmotic adjustment of the cytoplasm. Using Affymetrix rice genomic arrays, salinity-induced transcript profiles were documented recently in shoot tissues of IR29, a salt-sensitive *indica* cultivar, and the salt-tolerant *indica* recombinant inbred line (RIL) FL478 (Walia et al., 2005). FL478 was developed from a cross between IR29 and the salt-tolerant *indica* landrace Pokkali and was selected for salinity tolerance equal to or greater than Pokkali (Figure 1).

This previous study revealed a higher number of salinity-induced probe sets, such as those involved in the flavonoid biosynthetic pathway, in the shoots of salt-sensitive IR29 compared with FL478, suggesting that shoot tissues of IR29 were under greater salinity stress than FL478 and that the responses were related more to the salt-induced damage than to enhancing tolerance. Physiological characterization of the two lines supported the suggestion that the shoots were more stressed, a higher overall shoot Na^+ concentration and Na^+:K^+ ratio being measured in IR29 compared with FL478. Single-feature polymorphism analysis of the shoot microarray data yielded the unexpected result that the *Saltol* region in FL478 originated from the salt-sensitive parent, IR29 (Walia et al., 2005). The *Saltol* region is a prominent salinity tolerance quantitative trait locus (QTL) identified as responsible for low shoot Na^+ and low Na^+:K^+ ratio under salt stress (Gregorio et al., 1997; Bonilla et al., 2002).

[1] To whom correspondence should be addressed. E-mail mark.tester@acpfg.com.au, fax +61 8 8303 7102, tel. +61 8 8303 7159.

[2] These authors contributed equally to this paper.

doi: 10.1093/mp/ssq056, Advance Access publication 5 October 2010
Received 10 August 2010; accepted 27 August 2010

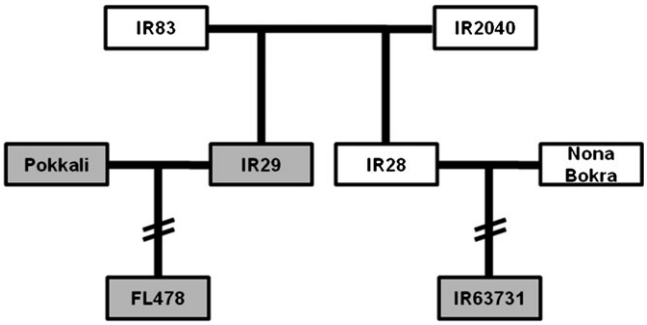

Figure 1. Pedigree of IR29, FL478, Pokkali, and IR63731.
The salt-sensitive *indica* cultivar IR29 was developed at IRRI through crosses between IR83 and IR2040. A single cross of IR29 to the salt-tolerant *indica* landrace Pokkali, followed by seven rounds of pedigree selection (single seed descent) up to the F8 generation, yielded the salt-tolerant recombinant inbred line (RIL) FL478. IR63731 was selected by following the same procedure but with two different parents: the salt-tolerant *indica* landrace Nona Bokra and IR28, a salt-sensitive sibling line to IR29. The four genotypes in shaded boxes were selected for transcript analysis in this study.

The present paper complements the Walia et al. (2005) study by focusing on salinity-induced changes in transcript levels in root tissues of IR29 and FL478. In contrast to shoots, the roots of the tolerant FL478 had more transcripts controlled by salt than the sensitive IR29, perhaps indicating that a greater number of adaptive processes related to salinity tolerance occur in the roots than in the shoots of these cultivars. The FL478-salt-tolerant parent Pokkali is also included in this study as well as an additional salt-tolerant RIL, IR63731, to allow comparison of the salinity tolerance mechanisms of Pokkali and Nona Bokra, currently the two most common source genotypes for salinity tolerance in rice breeding programs (Ismail et al., 2007). IR63731 originates from a cross between IR28, a salt-sensitive sibling line of IR29, and the salt-tolerant *indica* landrace Nona Bokra (Figure 1). A study of the tissue-specific Na^+ accumulation of the four genotypes is described to facilitate interpretation of the root transcript profiles. Also discussed is the response of several gene families known to be important in plant salinity tolerance, as well as some novel genes identified through their response to salt, transcript level, and/or chromosomal position.

RESULTS AND DISCUSSION

Phenotypic Characterization in Saline Conditions

The IR29, FL478, Pokkali, and IR63731 genotypes were evaluated for root and shoot Na^+ and K^+ concentration after 2 d of growth on 50 mM NaCl, followed by 5 d of growth on 70 mM NaCl (Table 1). The root Na^+ concentration was significantly higher in Pokkali compared with the other three genotypes, suggesting that Pokkali is able to sequester Na^+ in root tissues, which may contribute to preventing its transfer to the shoot or may be a consequence of a reduced transfer to the shoot. The Na^+ concentration of FL478 roots was approximately 30% lower than that of Pokkali, and did not differ significantly from IR29.

Table 1. Tissue Na^+ and K^+ Concentrations in Four Rice Accessions.

Tissue	Cultivar	Na^+ concentration (mM)	K^+ concentration (mM)	Na^+:K^+ ($\times 10^3$)
Shoot	IR29	24.1 ± 2.3*	309.0 ± 11.0	78.0 ± 5.4*
	FL478	7.1 ± 0.3	328.2 ± 4.1	21.6 ± 0.8
	Pokkali	6.7 ± 0.7	316.7 ± 10.6	21.2 ± 2.3
	IR63731	3.2 ± 0.4	313.5 ± 8.1	10.2 ± 1.1
Root	IR29	8.5 ± 0.5	48.0 ± 3.1	177.1 ± 4.2
	FL478	8.9 ± 0.9	53.9 ± 5.8	165.1 ± 3.5
	Pokkali	12.4 ± 0.3*	54.9 ± 3.2	225.9 ± 7.6*
	IR63731	9.6 ± 0.6	51.4 ± 4.0	186.8 ± 4.1

Root and shoot tissue samples from salt-stressed IR29, FL478, Pokkali, and IR63731 were harvested and sodium (Na^+) and potassium (K^+) concentrations measured by flame photometry. Concentrations are means ± SEM (n = 5). Statistical significance was calculated using one-way ANOVA and Tukey-Kramer multiple comparison tests. Numbers followed by the * sign are significantly different ($P < 0.05$).

The shoot Na^+ concentration was significantly higher—more than three-fold—in IR29 compared with the other three genotypes, FL478, Pokkali, and IR63731. These results support the finding of Walia et al. (2005) that IR29 is a poor Na^+ excluder that accumulates high levels of Na^+ in leaf tissues under salinity stress. IR63731 had the lowest shoot Na^+ concentration of the four genotypes and also had low root Na^+. A $^{22}Na^+$ radioactive tracer flux experiment could test the hypothesis that IR63731 reduces both net influx into the root and transfer from root to shoot as combined exclusion mechanisms.

The Na^+:K^+ ratios show that IR29 has a markedly higher ratio in the shoot when compared with the three salt-tolerant genotypes. A higher shoot cytosolic Na^+ in IR29 under salinity stress would inhibit enzymatic reactions requiring K^+ as a cofactor, which is a characteristic feature of salt-sensitive genotypes. The salt-tolerant Pokkali had a significantly higher Na^+:K^+ ratio in the root compared with the other genotypes. However, this may be the result of significant sequestration and retention of Na^+ in vacuoles of specialized cell types and may not actually indicate salinity stress in the root tissues of Pokkali.

Analysis of Changes in Transcript Levels under Salt Stress

IR29, FL478, Pokkali, and IR63731 rice plants were cultured in sand tanks for 22 d after germination, at which point a ramped salinity treatment was applied for 7 d with a final salinity level of 7.4 dS m^{-1} (see Figure 1 of Walia et al., 2005, for design of the salinity stress treatment). Control plants remained in normal growth solution for the entire 30-d period. For each cultivar, root RNA isolated from stressed and unstressed plants was analyzed using Affymetrix GeneChip® Rice Genome Arrays. The Affymetrix chip contains 55 428 probe sets representing approximately 49 820 different transcripts (www.affymetrix.com/products_services/arrays/specific/rice.affx#1_1). Statistically

significant differences in transcript levels were identified by applying a moderated *t*-statistic at $p < 0.05$ (Smyth, 2004) and the empirical criterion of greater than two-fold change.

A total of 1957 probe sets (3.5%) were found to be differentially regulated in the root tissue of the four genotypes after 8 d of salt treatment. The largest number of salt-responsive probe sets was observed in FL478 (1067), with 682 of those probe sets being specific to FL478. By comparison, 619 probe sets were differentially regulated in the salt-sensitive genotype IR29. Pokkali, the salt-tolerant parent of FL478, showed 594 differentially regulated probe sets, while 481 probe sets were differentially regulated in the salt-tolerant IR63731. Figure 2A shows the number of salt-responsive transcripts whose levels change uniquely in one genotype, or which change in more than one genotype. When compared to the shoot data published by Walia et al. (2005), the root response of FL478 and IR29 proved to be tissue-specific and weakly correlated to changes in the shoot (Figure 2B and 2C, respectively). This demonstrates the tissue-specific nature of the salinity response in rice and justifies the relevance of this study.

The 1957 probe sets were further grouped according to their salt responsiveness in the different cultivars. For instance, probe sets that were significantly up-regulated in response to salt in cultivars FL478 and Pokkali but down-regulated in the other two genotypes would form a subgroup. Likewise, probe sets significantly down-regulated in IR6371 but from which the transcript levels were stable in the other three genotypes formed another subgroup. These probe set lists can be downloaded in full (Supplemental Table 1).

Finally, in an attempt to identify shared salinity tolerance mechanisms, transcript responses were compared amongst the three closely related or the three salt-tolerant accessions (Figure 3A and 3B, respectively). The salt responsiveness of FL478 was strikingly different from either of its parents as well as that of IR63731. Thus, it is hypothesized that the salinity response of Pokkali and Nona Bokra, the two source genotypes in this study, is quite different and that salinity tolerance in these two lines is a multi-faceted trait that can be achieved by different mechanisms.

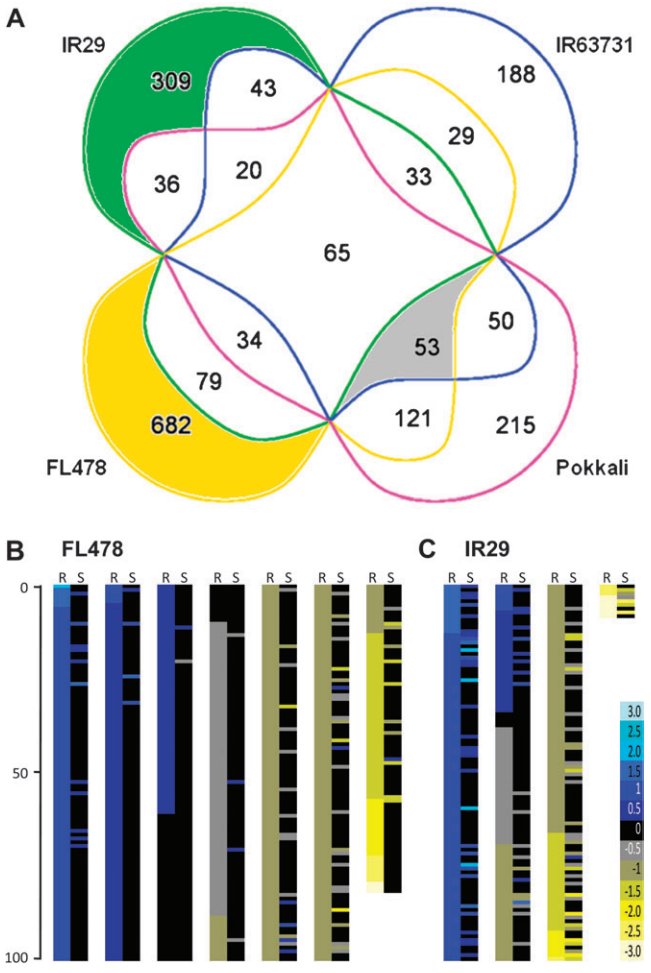

Figure 2. Analysis of Changes in Transcript Levels under Salt Stress.

(A) Four-way Venn diagram showing the number of salt-responsive probe sets that change uniquely in the roots of one genotype (the outermost numbers) or that change in two or more genotypes after 8 d of salinity treatment. IR29 (green), FL478 (yellow), Pokkali (pink), and IR63731 (blue). For example, 682 probes changed uniquely in FL478, 121 changed only in FL478 and Pokkali, 29 in FL478 and IR63731, and 53 in all three salt-tolerant genotypes but not in the salt-sensitive genotype, IR29. The 53 probe sets highlighted in gray that respond significantly in the salt-tolerant genotypes are listed in Table 3.

(B) Comparison of the responsiveness of the 682 FL478-specific probe sets (highlighted in yellow in panel (A)) in roots (R) and shoots (S).

(C) As in (B), but for the 309 probe sets uniquely regulated in IR29 (highlighted in green in panel (A)). The shoot data are from Walia et al. (2005).

Validation of Affymetrix Transcript Levels

To validate the microarray data, quantitative real-time polymerase chain reaction (Q–PCR) was performed on a set of 12 genes selected on the basis of their transcript levels across accessions, salt responsiveness, and/or functional annotation (Figure 4). Genes were particularly scrutinized at the QTLs for Na^+ and K^+ uptake, which confer salinity tolerance in rice (Lin et al., 2004). The primer pairs were designed to amplify fragments within probe sets on the Affymetrix GeneChip® (Table 2). The Q–PCR data were highly correlated with the Affymetrix chip signal intensity (Pearson correlation coefficients ranging from 0.76 to 0.99, with an average of 0.91). The implication of these genes in salinity tolerance is discussed below.

Another source of validation may have come from the recent publication of a microarray-based genome-wide analysis of FL478 and IR29 root transcriptome under salt stress (Senadheera et al., 2009). However, data comparison proved difficult for both technical and biological reasons. First, the authors used the NSF 45k 70-mers microarray platform. Although both the Affymetrix and NSF platforms are valid technologies, major differences in their conceptual design (e.g. short probe sets versus long oligonucleotides, respectively) do not allow direct comparison of the datasets without

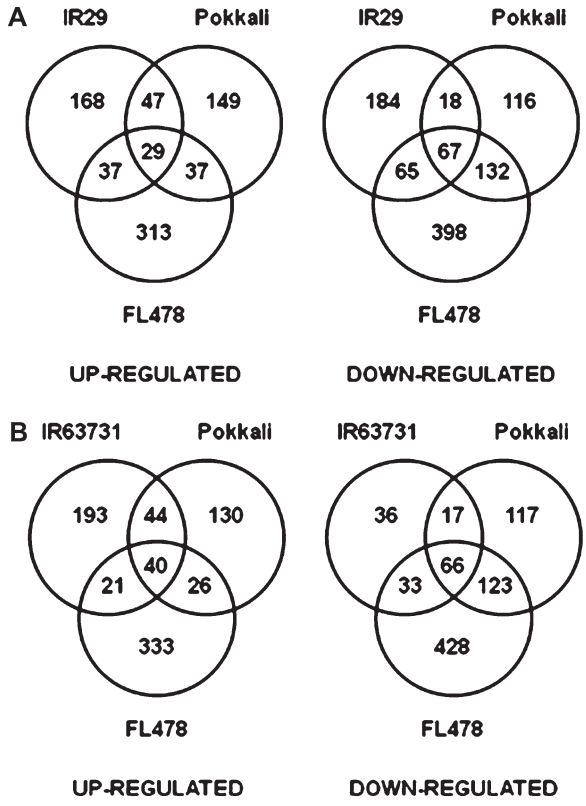

Figure 3. Number of Up- and Down-Regulated Probe Sets.

Three-way Venn diagrams showing the distribution of up- and down-regulated probe sets in the roots of the closely related cultivars (**(A)**: IR29, FL478, and Pokkali) and the three salt-tolerant cultivars (**(B)**: FL478, Pokkali, and IR63731).

normalization (Jung et al., 2008). Second, the differences in the duration and severity of the salinity treatment imposed and, more importantly, in the environmental conditions in which the rice plants were grown are too significant to allow safe comparisons between the two datasets. Senadheera et al. grew plants hydroponically (as opposed to sand tanks in the current experiment) in a growth chamber (as opposed to a glasshouse) with a relative humidity of 40% (versus 40–80%), a low maximum day temperature of 22°C (versus a range of 32–45°C, depending on the day), and a very low irradiance of 100 μmol m^{-2} s^{-1} (versus a maximum of 1104 μmol m^{-2} s^{-1} at midday), which may have been limiting factors for the growth of a tropical plant such as rice. Some genes highlighted by Senadheera et al. for their putative importance for salinity tolerance in the roots of the tolerant line FL478 have a drastically different response to salt in the two experiments. For instance, the putative cation/H^+ exchanger *OsCHX11* (*Os05g31730*) is reported as being up-regulated by a factor of 14.9 in the NSF dataset (Oligo TR063129) but is not significantly responsive to salt when looking at the Affymetrix data (Probe set OsAffx.4492.1.S1_at). Thus, the similarity between the Walia et al. (2005) data and those presented here (i.e. samples coming from the same biological material and analyzed with the same analytical platform) greatly facilitate the discovery of novel genes whose function is to confer salinity tolerance via the root tissue.

Mining the Datasets for Genes Associated with Salinity Tolerance

Genes Responsive in Salt-Tolerant Lines and Not in IR29

It is suggested that genes that showed no significant response to salinity (or even decreased in abundance) in the salt-sensitive IR29, but that did respond in the three tolerant lines are more likely to be related to tolerance than genes that are responsive in all lines or other subsets of lines. There were relatively few probe sets (53) that identified this pattern of changes in transcript levels (Table 3 and probe sets highlighted in gray in Figure 2A). Notably, transcripts from the stelar Na^+ transporter encoding gene, *OsHKT1;5*, increased in abundance upon salt treatment in FL478, Pokkali, and IR63731 but not in IR29 (Figure 4A). Given the likely role of *OsHKT1;5* in salinity tolerance (as discussed in more detail below), it seems likely that this pattern of transcript responsiveness is, indeed, consistent with a role of these genes in salinity tolerance.

In this list, transcript levels of only two genes were specifically up-regulated by salt in all the tolerant lines but down-regulated in IR29. These transcripts are related to two proteins of unknown function. The first is the protein encoded by *Os11g34460* (probe set Os.52157.1.S1_x_at), which possesses a PAS domain found, for instance, in Erg K^+ channels. Erg proteins have been characterized as voltage-dependent K^+ channels mediating inward rectifying K^+ currents in mammalian cells (Morais Cabral et al., 1998; Schwarz and Bauer, 2004), and may be associated with K^+ homeostasis. In plants, PAS domains have been found to bind ligands and to act as sensors for light and oxygen in signal transduction (Cheng et al., 2003).

The second protein of unknown function is encoded by *Os09g21060* (probe set OsAffx.29954.1.S1_at and OsAffx.29954.1.S1_s_at) and is part of a super family of small cysteine-rich peptides (CRP) recently identified in the plant kingdom (Silverstein et al., 2007). In rice alone, 598 genes encoding CRPs have been identified and the function of most of these genes remains to be identified.

Overall, the 53 probe sets listed in Table 3 represent a pool of genes, some of them novel, which should be further investigated for their potential roles in salinity tolerance. This pool includes genes encoding transcription factors (e.g. TIFY domain containing proteins), an alanine aminotransferase (a gene involved in hypoxia tolerance and nitrogen use efficiency: Miyashita et al., 2007; Shrawat et al., 2008), a myo-inositol oxygenase (discussed below), and a set of heavy metal-associated proteins.

Genes Responsive in IR29 but Expressed Constitutively at Higher Levels in Salt-Tolerant Lines

Taji et al. (2004) demonstrated that the main difference between *Arabidopsis* and *Thellungiella halophila*, a closely related halophyte, is that the latter constitutively expresses genes that are induced in response to salinity in *Arabidopsis*.

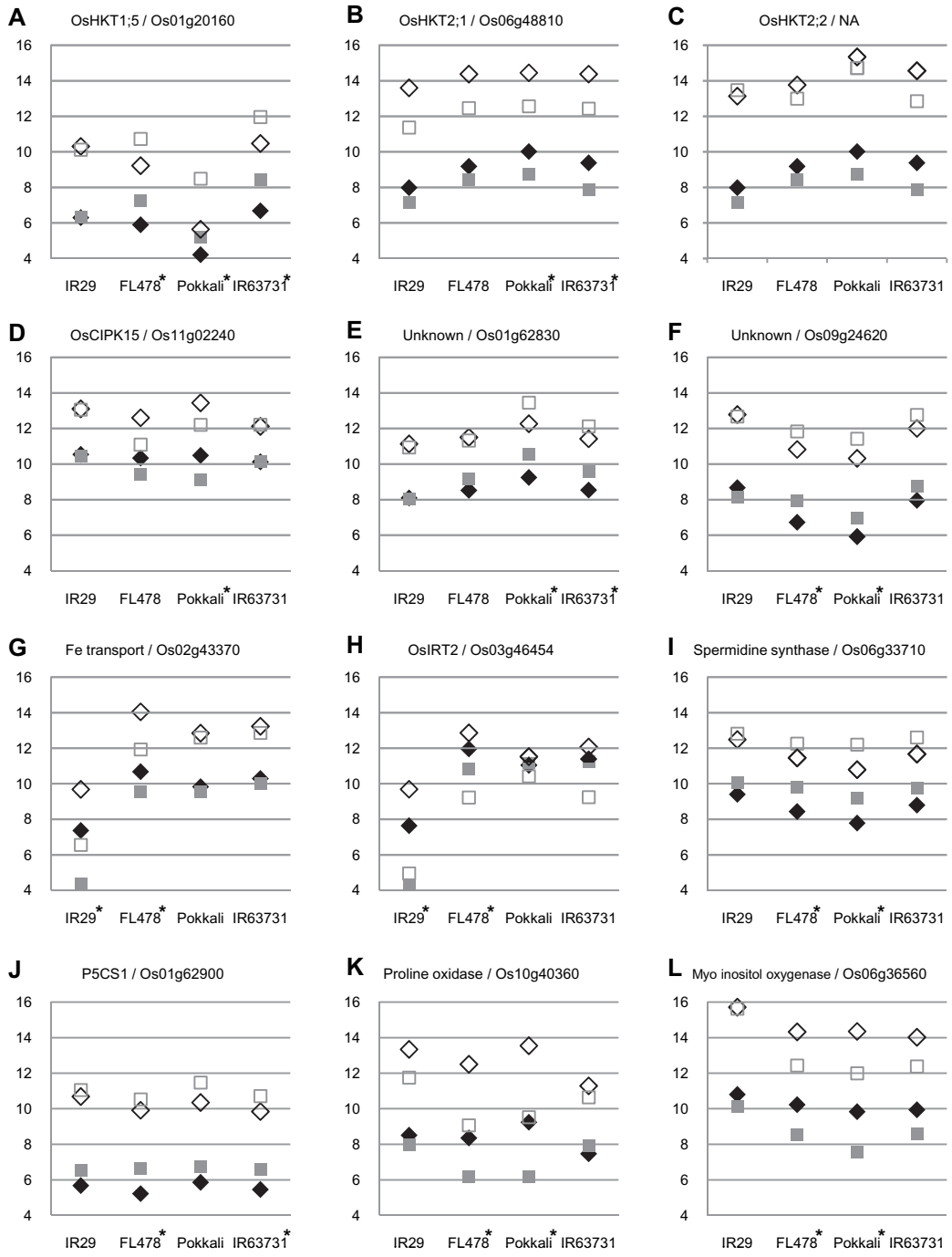

Figure 4. Q–PCR Validation of the Microarray Dataset.

Twelve candidate genes chosen for their functional annotation, hybridization profile, and/or chromosomal location were assayed by quantitative RT–PCR (Q–PCR) of root cDNA from IR29, FL478, Pokkali, and IR63731, in both control and salt-treated conditions. The root cDNAs were synthesized from the same batch of RNA hybridized on the Affymetrix GeneChip®. For each candidate, Affymetrix and Q–PCR values are plotted on the same graph (diamonds, control plants; squares, salt-treated plants; solid symbols, Affymetrix values; hollow symbols, Q–PCR values). Candidates are identified by both their gene annotation and TIGR locus ID, with the exception of *OsHKT2;2*, which does not have a locus ID. The primers used for the Q–PCR are listed in Table 2. The *y*-axis represents either the Affymetrix signal intensity or the normalized mRNA copy number detected by Q–PCR (arbitrary unit, $\log_2$ values). An asterisk (*) next to the cultivar name indicates significant response to salt in Affymetrix data. Pearson correlation coefficients are: **(A)** 0.94, **(B)** 0.85, **(C)** na, **(D)** 0.76, **(E)** 0.93, **(F)** 0.97, **(G)** 0.99, **(H)** 0.86, **(I)** 0.97, **(J)** 0.83, **(K)** 0.95, **(L)** 0.94.

Table 2. Q–PCR Primer List.

Code	Annotation	Probe set ID	Forward primer (5′–3′)	Reverse primer (5′–3′)
A	OsHKT1;5	Os.30563.1.S1_at	ACGACCCCATCAACTACAGCGTCC	TGCTCCACTTCCCTGAGAAGCCAAC
B	OsHKT2;1	Os.2617.2.S1_a_at	GTTAATTTTGTTGTTCTAGC	ATGAGGCTGGAAAGTGTCAG
C	OsHKT2;2	Os.2617.2.S1_a_at	GGGAGCATCTGCCAGGACAA	TTCTTATTTCTACGATTCAAAAGGC
D	OsCIPK15	Os.1310.1.S1_a_at	AAATGAGGCAAGGTTCACTTCA	CAAACTGAAGAACACCATTCCT
E	Unknown	Os.23635.1.S1_at	GACACGCCGATGACAAGACC	GCACAAGCACAGCACCACAA
F	Unknown	Os.22832.1.S1_at	GGGAAATCACCAGCACTACCAC	AGGGCAACAACTGGTCTCTCC
G	Fe transport	OsAffx.2947.1.S1_at	ACAGGTGAAGTGGTACTATGTTGTTG	ACCAGCAATGACACCGTTGT
H	OsIRT2	OsAffx.10755.1.S1_at	GAGGCCGGTAACACCACCAA	ATCCCCTCGAACATCTGGTG
I	Spermidine synthase	Os.4600.1.S1_at	AGTGCAGTACTTCAACAATCCAATG	GCTGCACAATACCATCAAGCA
J	P5CS1	Os.33244.1.S1_at	GATGGGGCTCGCTTTGGTCT	TCCCTTGTCGCCGTTCACTA
K	Proline oxydase	Os.2881.1.S1_at	CGGGTTCCAGGTGAGCAAGT	CATTGCAGCCTTGAACCTCC
L	Myo inositol oxygenase	Os.6345.1.S1_at	CAAGTTGAGATGGTGAAGAAGAG	TGGCTCCCAACAAATTCTGC

Twelve genes (A–L) were selected to validate the micro-array data by quantitative real time–PCR. For each gene, identified by its annotation and Affymetrix probe set ID, the forward and reverse primer sequence is given. The codes in the first column match those of Figure 4.

Table 3. Differentially Regulated Probe Sets in the Salt-Tolerant Genotypes.

Probe set ID	Functional annotation (BlastX)	IR29	FL478	Pokkali	IR63731
Os.11250.1.S1_at	Heat stress transcription factor C-2a	–1.974	–2.073	–3.193	–2.899
OsAffx.12783.1.S1_at	Alanine aminotransferase 2	–1.948	–2.046	–2.926	–2.416
OsAffx.23048.1.S1_x_at	Metal-dependent phosphohydrolase HD domain protein	–1.945	–2.497	–2.291	–2.142
Os.11673.2.S1_x_at	Plus-3 domain containing protein	–1.931	–3.261	–2.819	–2.615
Os.51307.1.S1_at	Zinc finger, C2H2 type	–1.923	–6.505	–5.291	–3.379
Os.11266.1.S1_at	Auxin-induced protein PCNT115	–1.906	–7.775	–3.073	2.869
OsAffx.25038.1.S1_at	Glycosyl hydrolase family 10 protein	–1.885	–2.68	–2.548	–2.13
Os.34841.1.S1_at	Heavy-metal domain containing protein	–1.838	–2.11	–2.441	–2.413
Os.54318.1.S1_at	Cytochrome P450 family protein	–1.828	–4.534	–3.119	–2.517
Os.50883.1.S1_at	Glycosyl hydrolases family 16 protein	–1.772	–2.134	–2.695	–3.045
Os.6125.1.S1_at	Trypanothione-dependent peroxidase	–1.756	–1.898	–2.882	–2.046
OsAffx.15530.1.S1_at	Jasmonate O-methyltransferase	–1.732	–3.186	–2.304	–2.571
Os.23381.1.A1_at	Hypersensitive-induced response protein	–1.725	–2.797	–4.416	–2.32
Os.10235.1.S1_at	Zinc finger, ascorbate oxidase promoter-binding protein	–1.704	–2.351	–2.354	–2.069
Os.32686.1.S1_at	POT family protein	–1.698	–9.259	–2.082	–2.046
OsAffx.19111.1.S1_s_at	Acyl carrier protein	–1.666	–2.259	–2.388	–2.36
Os.18955.1.S1_at	MYB transcription factor	–1.625	–2.902	–3.897	–2.515
Os.12127.2.S1_x_at	Photosystem I chain V precursor	–1.607	–2.227	–2.231	–2.166
Os.56969.1.S1_at	[No hits]	–1.604	–4.36	–2.802	–2.646
Os.6345.1.S1_at	Myo-inositol oxygenase	–1.591	–3.203	–4.769	–2.489
Os.3397.1.S1_at	B-box zinc finger	–1.417	–4.495	–2.547	–2.049
Os.50015.1.S1_at	WRKY transcription factor 28	–1.381	–2.556	–3.046	5.681
Os.31171.1.S1_at	Hypothetical protein	–1.315	–6.016	–7.551	–2.188
OsAffx.10931.4.A1_at	Hypothetical protein	–1.194	–1.508	–1.613	–1.519
Os.52157.1.S1_x_at	Kelch motif, Adagio 3	–1.182	2.697	2.872	2.02
Os.8088.1.S1_at	TIFY motif family	–1.114	–2.357	–2.369	3.115
OsAffx.29954.1.S1_at	Hypothetical protein	–1.114	2.076	2.342	2.394
Os.12012.1.S1_at	TIFY domain containing protein	–1.09	–2.592	–2.76	3.744
Os.10356.1.S1_at	TIFY domain containing protein	–1.084	–3.378	–3.534	3.651
OsAffx.29954.1.S1_s_at	Hypothetical protein	–1.044	2.267	2.628	2.236

Table 3. Continued

Probe set ID	Functional annotation (BlastX)	IR29	FL478	Pokkali	IR63731
Os.4901.1.S1_at	Myb-related transcription factor-LBM2 like	–1.037	–2.032	–2.549	2.55
Os.56080.1.S1_at	VQ motif family protein	–1.023	–2.683	–2.561	2.295
Os.30563.1.S1_at	HKT1;5	1.01	2.577	2.007	3.348
Os.37571.1.S1_at	Plastocyanin-like domain containing protein	1.036	–3.4	–2.302	3.414
Os.55653.1.S1_at	U-box domain containing protein	1.085	–2.388	–2.268	2.729
Os.12032.1.S1_at	Transcription factor WRKY71	1.109	–2.069	–2.051	3.922
Os.28798.1.S1_at	Unknown protein	1.127	–3.441	–2.014	3.011
Os.26411.1.A1_at	Receptor kinase-like protein	1.151	–2.082	–5.026	3.498
Os.5632.1.S1_a_at	Dienlactone hydrolase	1.168	2.912	2.058	2.178
Os.9067.1.S1_at	Cytochrome P450	1.207	–2.031	–2.765	2.458
Os.32964.1.S1_at	POT family protein	1.283	2.552	3.069	2.532
Os.12767.1.S1_a_at	Thiamine biosynthetic enzyme	1.453	2.952	2.802	3.424
Os.37255.1.A1_at	Early nodulin	1.522	3.024	3.4	3.962
Os.53154.1.S1_at	Unknown protein	1.541	2.671	2.161	2.511
Os.21801.1.S1_at	Glutamine amidotransferase related protein	1.55	–2.506	–3.721	2.08
Os.49746.1.S1_at	Myb-like DNA-binding domain, SHAQKYF class family protein	1.615	2.962	3.925	2.234
Os.22312.3.A1_a_at	Universal stress protein family protein	1.66	2.082	2.939	2.958
Os.11897.1.S1_at	Unknown protein	1.687	2.902	2.463	2.517
Os.6864.1.S1_at	Heavy metal-associated domain containing protein	1.722	2.097	3.347	2.117
Os.53714.1.S1_at	Copper amine oxidase	1.728	2.176	2.137	2.287
Os.10388.1.S1_at	Unknown protein	1.798	4.405	3	3.012
Os.28964.1.S1_at	Cysteine proteinase	1.818	2.094	2.335	2.165
Os.21260.1.S1_at	Expressed protein	1.956	3.663	4.569	3.175

List of probe sets whose corresponding transcripts showed significant changes in expression level upon exposure to salt in the salt-tolerant genotypes but that do not have a significant change in the salt-sensitive genotype (IR29). From left to right are given the Affymetrix probe set ID, the functional annotation (obtained by BlastX against nr and SwissProt), and the fold change in expression per genotype (Mean Control – Mean Salt; see Supplemental Table 1 for signal intensity values). Probe sets are ordered based on fold changes in IR29 upon salinization (most down-regulated to most up-regulated).

Based on this observation, the rice dataset was mined for probe sets that were constitutively expressed in the three tolerant lines at a higher level than in IR29 and that were significantly responsive in IR29 only; 13 such probe sets were identified (Table 4). Amongst them, the probe set Os.48060.1.A1_at, related to *OsTIP2;1* (*Os02g44080*), a gene encoding a tonoplast-bound aquaporin, was down-regulated approximately 15-fold (down to background level) upon salt stress in IR29 whereas its expression in the three tolerant lines remained stable at a level eight times higher than in IR29 control conditions. Similarly, expression of the *Arabidopsis* homolog of *OsTIP2;1*, *AtTIP2;1*, is decreased to background level following salt stress (Boursiac et al., 2005). In the salt-sensitive *japonica* rice Nipponbare, *OsTIP2;1* expression was not detected in 4-day-old and 4-week-old roots of plant grown in non-stressed conditions (Li et al., 2008). However, OsTIP2;1 proteins were detected in 15-day-old roots of cultivar Akitakomachi, another Japanese elite cultivar (Sakurai et al., 2008). *OsTIP2;1* was shown to be root-specific and mainly expressed in root epidermis, suggesting a putative role in the promotion of water movement in the root stelar region and, on the basis of the transcript profiles found in this study, possibly a function in salinity tolerance.

Interestingly, only one of the 13 probe sets was significantly up-regulated by salt stress in IR29 (Table 4). This probe set (Os.49093.1.S1_at) is related to a transcript encoding a high-affinity nitrate transporter (*Os02g02170*). It has been shown in poplar that salinity can inhibit nitrate transport and thus up-regulation of nitrogen uptake transporters could improve tolerance to salt stress (Ehlting et al., 2007). With transcript levels upon salt stress still around four-fold higher in the three tolerant lines than in IR29, *Os02g02170* is also a good candidate from which the function should be further investigated in rice.

Genes Expressed Constitutively in All Lines but at Higher Levels in Salt-Tolerant Lines

Salinity tolerance may be related more to the fine tuning of a particular set of genes rather than to the significant up- or down-regulation of genes by salt stress (such as the SOS pathway genes described later on). For this reason, the dataset was mined for probe sets that were constitutively expressed in each genotype but whose transcript levels were significantly different in IR29 compared with the three tolerant lines (Table 5). Of the 64 probe sets identified, 54 were related to transcripts being more highly expressed in IR29 and 10 to transcripts being more

Table 4. Subset of Differentially Regulated Probe Sets in IR29.

Probe set ID	Functional annotation (BlastX)	IR29	FL478	Pokkali	IR63731
Os.48060.1.A1_at	OsTIP2;1	–14.866	1.023	–1.171	–1.144
Os.47127.1.A1_at	[No hits]	–10.106	1.005	1.618	1.191
Os.20714.1.S1_at	Hypothetical protein	–5.397	1.296	1.018	1.037
Os.10709.1.S1_at *	Hypothetical protein	–3.826	1.031	1.043	1.075
OsAffx.4313.1.S1_at	[No hits]	–3.716	–1.027	–1.035	–1.084
Os.22619.1.S1_at	Hypothetical protein	–3.022	1.130	1.198	1.318
Os.17370.2.S1_x_at *	HERC2-like protein	–2.979	–1.032	1.087	–1.073
Os.5945.1.S1_at *	Unknown protein	–2.969	–1.063	–1.091	–1.098
OsAffx.3995.1.S1_at	Serine/threonine-protein kinase receptor precursor	–2.703	–1.063	–1.147	–1.222
Os.18037.1.S1_at	Pentameric polyubiquitin-like protein	–2.302	1.126	–1.704	1.109
Os.50472.2.S1_x_at	Unknown protein	–2.283	1.186	1.070	1.140
Os.50472.2.S1_s_at	Unknown protein	–2.252	1.268	1.199	1.185
Os.49093.1.S1_at	High-affinity nitrate transporter	2.192	1.376	1.438	1.824

***** The overall lower expression in IR29 may be at least partially resulting from SFP. List of probe sets whose corresponding transcripts showed significant changes in expression level upon exposure to salt in the salt-sensitive IR29 and where expression in the tolerant lines were higher and not salt-responsive. From left to right are given the Affymetrix probe set ID, the functional annotation (obtained by BlastX against nr and SwissProt), and the fold change in expression per genotype (Mean Control – Mean Salt; see supplemental GEO submission for intensity values). Probe sets are ordered based on fold changes in IR29 upon salinization (most down-regulated to most up-regulated).

Table 5. Subset of Constitutively Expressed Probe Sets.

Probe set ID	Functional annotation (BlastX)	IR29	FL478	Pokkali	IR63731
Os.19105.1.S1_at	F-box domain and kelch repeat containing protein	4.39	2.64	2.62	2.80
OsAffx.19171.1.S1_s_at	Serine/threonine protein kinase	6.27	2.75	2.69	2.83
Os.46345.1.A1_s_at	ATPase, AAA family domain containing protein	6.71	2.80	2.78	2.73
Os.53407.1.S1_at	unnamed protein	5.50	2.89	2.76	2.80
Os.46330.1.S1_at	Putative phospholipid-transporting ATPase 2	4.73	3.06	3.09	3.07
Os.6334.1.S1_at	Isochorismate synthase 2, chloroplastic	5.15	3.10	3.29	3.07
Os.52587.1.S1_at	Protein of unknown function	6.50	3.14	3.23	3.55
Os.50209.1.S1_at	MATE efflux family protein, putative, expressed	5.20	3.34	3.01	3.05
OsAffx.30194.1.S1_x_at	Wall-associated receptor kinase	4.81	3.35	3.54	3.19
Os.46345.1.A1_at	ATPase, AAA family domain containing protein	7.87	3.51	3.35	3.27
OsAffx.24070.1.S1_at	P-loop containing Nucleoside Triphosphate Hydrolases	5.20	3.66	3.62	4.22
OsAffx.17817.1.S1_at	hypothetical protein	6.64	3.71	3.83	3.59
Os.52637.1.S1_x_at	Staphylococcal nuclease homolog	5.78	3.73	3.42	3.37
Os.54084.1.S1_at	Unknown	6.04	3.79	4.03	5.14
OsAffx.23567.1.S1_at	Tyrosine kinase	4.88	3.80	3.62	3.46
Os.27766.1.A1_at	Plant protein of unknown function	5.76	3.86	3.65	3.82
OsAffx.6326.1.S1_at	Serine/threonine-specific receptor protein kinase-like	5.36	3.88	3.94	3.95
OsAffx.26008.1.S1_at	P-loop containing Nucleoside Triphosphate Hydrolases	5.37	3.92	3.84	3.74
OsAffx.4937.1.S1_s_at	Putative receptor-like protein kinase	4.67	3.92	3.55	3.76
Os.51968.1.S1_a_at	P-loop containing Nucleoside Triphosphate Hydrolases	5.94	4.12	7.13	3.86
OsAffx.25067.1.S1_at	Disease resistance protein RPS2	7.01	4.12	3.84	3.89
Os.9770.1.S1_at	Unknown	5.85	4.15	4.14	4.05
Os.46616.1.S1_at	Putative pathogen-related protein Rir1b	9.29	4.21	3.82	5.97
Os.27288.1.S1_at	Trypsin-like serine protease	6.09	4.32	4.18	4.08
Os.40049.1.S1_at	Unknown	5.93	4.33	4.42	4.26
OsAffx.7301.1.S1_at	LRR domain; possibly receptor-like protein kinase	6.03	4.33	4.72	4.34

Table 5. Continued

Probe set ID	Functional annotation (BlastX)	IR29	FL478	Pokkali	IR63731
Os.17316.1.S1_at	Flavanone 3-hydroxylase	5.08	4.38	4.19	4.24
Os.11239.1.S1_at	Calmodulin binding protein-like	5.72	4.42	3.47	4.12
Os.16140.1.S1_at	Unknown	6.29	4.44	4.10	4.15
Os.26902.1.S1_at	Trypsin-like serine protease	8.95	4.73	5.21	4.22
Os.49258.1.S1_at	Unknown	6.38	4.83	4.62	4.71
Os.50616.1.S2_at	Transposon Ty3-I Gag-Pol polyprotein	3.43	5.02	5.27	5.06
OsAffx.19388.1.S1_s_at	Likely transposon	7.96	5.05	5.49	5.04
Os.15125.1.S1_at	Unknown	6.90	5.11	5.36	3.68
OsAffx.22888.1.S1_at	Hypothetical protein	6.27	5.19	4.53	4.43
OsAffx.8030.1.S1_at	Hypothetical protein	6.49	5.34	5.19	5.18
Os.54939.1.S1_at	Plant protein of unknown function	6.14	5.34	5.16	4.96
Os.51488.1.S1_at	[No hits]	8.41	5.44	5.19	5.19
Os.46393.1.S1_x_at	Expressed protein	8.22	5.47	4.84	6.20
Os.9781.1.S1_at	Esterase, lipase family	6.48	5.53	5.51	5.44
Os.49736.1.S1_x_at	Zinc finger (C3HC4-type RING finger)-like	4.82	5.57	5.87	5.43
Os.51437.1.S1_at	[No hits]	8.36	6.27	6.19	4.13
Os.5051.1.S1_at	Telomere length regulation protein TEL2 homolog	7.38	6.38	6.10	6.48
Os.47326.1.S1_at	Protein of unknown function	8.17	6.42	6.38	6.40
Os.15125.1.S1_x_at	Protein of unknown function	7.89	6.43	6.56	5.59
Os.11464.1.S1_at	Oxidoreductase, aldo/keto reductase family	6.03	6.68	6.62	6.80
Os.31151.2.S1_at	Unknown	6.04	6.72	6.84	6.70
Os.22245.1.S1_at	RNA polymerase III RPC4 domain containing	8.22	6.96	7.07	7.25
Os.19611.1.S1_a_at	PsbP family protein	8.42	7.16	6.00	7.31
Os.4641.2.S1_at	Putative dolichyl-phosphate beta-glucosyltransferase	7.81	7.34	7.36	7.23
Os.53403.1.S1_s_at	ETHYLENE INSENSITIVE 3-like protein	5.62	7.42	7.71	6.93
Os.22872.1.S1_at	Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases	9.46	7.54	7.51	8.11
Os.35543.1.S1_at	Kinesin heavy chain isolog, putative, expressed	6.88	7.92	7.52	7.82
Os.11084.2.S1_x_at	Histone acetyltransferase subunit NuA4	8.57	7.93	7.97	7.94
Os.47600.2.S1_x_at	Amino acid transporter protein	7.27	8.02	8.25	8.00
Os.41397.1.S1_x_at	Hypothetical protein	9.16	8.45	9.79	8.43
Os.17424.1.S1_at	Hypothetical protein	9.42	8.75	8.48	8.72
Os.3754.1.S1_at	GDSL esterase/lipase	6.08	8.82	7.98	7.19
Os.17424.1.S1_x_at	Hypothetical protein	9.45	8.83	8.51	8.80
Os.7086.1.S1_at	Glycosyl hydrolases family 17	7.28	8.87	7.89	8.50
Os.26587.1.A1_at	Hypothetical protein	10.45	9.35	9.25	9.36
Os.8799.1.S1_at	NADH dehydrogenase	9.09	9.61	9.99	9.73
Os.32471.1.S1_at	Peroxidase	11.84	10.98	11.04	11.31
Os.22592.1.S1_a_at	Histone H3.3	12.49	11.72	11.69	11.65

List of probe sets whose corresponding transcripts were not significantly regulated upon exposure to salt across all accessions, but were expressed at different levels in IR29 compared to the three tolerant lines ($p < 0.05$). From left to right are given the Affymetrix probe set ID, the functional annotation (obtained by BlastX against nr and SwissProt), and the control intensity values per genotype ($\log_2$ values; see supplemental GEO submission for salt intensity values). Probe sets are ordered based on FL478 values (least to most expressed).

highly expressed in the tolerant lines. About one-third of the first subsets were expressed at low to very low levels in the tolerant lines (i.e. $\log_2$ transcription values < 4; see Table 5) and may represent a pool of genes with adverse effects on salinity tolerance. Potentially, these genes were unknowingly selected for during the breeding of IR29, or at least they were not selected against, as IR29 was not bred for salinity tolerance.

In the second subset of probe sets with a lower constitutive expression in IR29, two probe sets stand out, as they have been previously associated with abiotic stress tolerance. Os.49736.1.S1_x_at is related to a transcript encoding a C3HC4-type RING finger protein and was recently named OsRHC19 by Ma et al. (2009). OsRHC19 was found to be localized to the endoplasmatic reticulum and its expression was

induced by ABA, salt (200 mM), drought, and H_2O_2, but not cold stress in the rice cultivar Zhonghua 11. If this gene is indeed crucial to several stress response pathways, it may mean that IR29 is less capable of tolerating abiotic stresses other than salinity stress. The second is Os.53403.1.S1_s_at, which is related to a transcript for an ethylene-insensitive 3 (EIN3)-like protein. EIN3 proteins are transcription factors involved in the ethylene signaling pathway and activate ethylene-regulated genes. In *Arabidopsis*, it has been demonstrated that *ein-3* mutants show reduced salt tolerance at high salt concentrations (Cao et al., 2008).

Overall, the genes linked to the probe sets listed in Table 5 represent a pool of putative genes whose function may be related to plant salinity tolerance.

Candidate Genes Associated with Transport of Na^+ and Other Cations

Control of Na^+ Influx into the Root

The rice *OsHKT2;1* gene encodes a high-affinity Na^+ transporter that mediates high-affinity Na^+ influx into roots under K^+-starved conditions (Horie et al., 2007). In this experiment, this gene was down-regulated by salt across the four accessions studied (Figure 4B), confirming the observation by Horie et al. (2007) that OsHKT2;1-mediated Na^+ influx does not cause Na^+ toxicity due to its rapid down-regulation under saline conditions.

Interestingly, the Pokkali landrace possesses two isoforms of this gene, *OsHKT2;1* and *OsHKT2;2*, which code for two distinct proteins sharing 91% sequence similarity (Horie et al., 2001). (Note that *OsHKT2;2* is absent from the *japonica* cultivar Nipponbare genome.) Due to this high similarity, transcript levels of both genes are captured by the same Affymetrix probe set; however, two pairs of Q–PCR primers were designed that allowed independent measurement of the transcript levels of the two genes (Table 2). *OsHKT2;2* was expressed in all the genotypes analyzed (Figure 4C), meaning that the four lines all share a copy of this gene. It was recently demonstrated that *Os*HKT2;2 can mediate Na^+ transport over a wide range of Na^+ concentrations (Yao et al., 2010). With a moderate down-regulation of this gene in all three tolerant lines but a stable expression in IR29, more Na^+ could enter IR29 compared to the other accessions, which, in turn, could partially explain why IR29 has a higher shoot Na^+ concentration. Finally, higher transcript levels of *OsHKT2;2* were found in Pokkali upon addition of salt than in the other lines, which may relate to the higher root Na^+ concentration observed in this accession.

The SOS Pathway and Associated Genes Involved in Na^+ Transport

The 'salt overly sensitive' (*SOS*) genes form a well known salinity response signaling pathway in plants that is involved in cellular Na^+ detoxification and potentially in the extrusion of excess Na^+ from root epidermal cells to the external medium (Liu et al., 2000; Quintero et al., 2002; Martinez-Atienza et al., 2007). SOS3, a calcineurin B-like (CBL) protein that responds to the initial cytosolic release of calcium associated with abiotic stresses, activates SOS2, a calcineurin B-like protein-interacting protein kinase (CIPK), which, in turn, phosphorylates SOS1, a Na^+/H^+ antiporter localized in the plasma membrane, which mediates Na^+ efflux from the cell. The rice genome contains 10 *CBL*s and 30 *CIPK*s (Kolukisaoglu et al., 2004); however, none of these genes, including *OsCBL4* and *OsCIPK24* (the rice SOS3 and SOS2 orthologs, respectively), was significantly responsive to salt in the current experiment (Supplemental Table 2). Nonetheless, it was found that the transcript levels of the FL478 *OsCBL9* gene were low in both control and salt-treated conditions compared with the levels found in the three other genotypes and that *OsCIPK6* transcript levels were lower in IR29 than in the three tolerant lines.

A recent study demonstrated that 12 out of 20 rice *CIPKs* analyzed were induced by salt (*OsCIPK07*, *08*, *09*, *10*, *11*, *15*, *16*, *17*, *21*, *22*, *29*, and *30*) and that overexpression of *OsCIPK15* in transgenic rice resulted in increased salinity tolerance (Xiang et al., 2007). This difference with our own data may arise because Xiang et al. analyzed leaf material whereas in the present study, the transcripts in roots is examined. To confirm the microarray data, *OsCIPK15* transcript levels were measured using Q–PCR (Figure 4D) and it was found that this gene was down-regulated by salt in two of the salt-tolerant cultivars (FL478 and Pokkali) while transcript levels in IR29 and IR63731 remained unchanged between control and saline conditions. These contrasting results between the current work and that of Xiang et al. suggest that *OsCIPK15* may play different roles in the root and shoot of the rice plant, but the fact that the current work was done in *indica* and not *japonica* rice should also be considered.

An examination was also made of transcript levels of seven vacuolar H^+-translocating pyrophosphatases and 28 members of the large cation/H^+ antiporter gene family, including *SOS1*, *NHX1*, and the 17 *CHX* genes identified by Sze et al. (2004). Only minor differences were observed in the transcript levels of these genes among the four rice lines in both control and saline conditions (Supplemental Table 2). Fukuda et al. (2004) reported an up-regulation of *OsNHX1* in rice roots upon salt treatment, but this was under experimental conditions that were very different from those used in the current study, making comparisons difficult. Fukuda et al. (2004) used 7-day-old rice seedlings from the sensitive *japonica* cultivar Nipponbare, and the up-regulation was monitored 5 and 24 h after the addition of 200 mM NaCl, whereas the *indica* plants in the present study grew at a maximum of 70–80 mM NaCl over a much longer period of time (7 d). However, in accordance with the results of the current work, Fukuda et al. (2004) did not observe any up-regulation at lower NaCl concentrations (50 and 100 mM).

Candidate Genes for Root Na^+ Concentration

Root Na^+ concentration is a complex trait that results primarily from the combination of influx into the root, efflux from the root, Na^+ transfer to and from the xylem sap, and Na^+ storage in the vacuole of certain cell types. To further investigate the

elevated Na^+ concentration observed in Pokkali roots, transcript levels were scrutinized of all the genes listed under two rice QTLs: one for root Na^+ amount on chromosome 1, the other for root Na^+ concentration on chromosome 9 (Lin et al., 2004). The salinity response of *Os01g62830* on chromosome 1 was of particular interest as it was similar to the one of *OsHKT1;5* in each accession. *Os01g62830* is not listed in Table 3 as it was not up-regulated upon salt treatment as much in FL478 as it was in Pokkali and IR63731. In fact, Q–PCR analysis suggested that *Os01g62830* was not up-regulated in FL478 upon salinization (Figure 4E). No further information could be found in public databases on *Os01g62830*, but the elevated transcript levels in Pokkali upon salt treatment compared with the other accessions makes this gene a plausible candidate to explain the elevated Na^+ concentration found in the roots of this tolerant landrace. Similarly, the *Os09g24620* gene of unknown function on chromosome 9 is up-regulated in all three tolerant lines, but not in IR29 (Figure 4F). Further analysis is required to determine the exact function of these two genes.

OsHKT1;5 Controls Root-to-Shoot Na^+ Transfer

OsHKT1;5 has been linked to *SKC1*, a major QTL on chromosome 1 for shoot K^+ content (Ren et al., 2005). Interestingly, *SKC1* co-localizes with *Saltol*, the prominent salinity tolerance QTL mentioned above, which is responsible for both low shoot Na^+ and low Na^+:K^+ ratio under salt stress. Genetic and physiological studies in wheat and barley have since shown that *HKT1;5* is part of a dual gene system (with *HKT1;4*) that controls exclusion of Na^+ from leaf blades in cereals (Davenport et al., 2005; James et al., 2006; Byrt et al., 2007), as originally elucidated in *Arabidopsis* for the orthologs, *AtHKT1;1* (Sunarpi et al., 2005; Davenport et al., 2007). *HKT1;5* encodes a Na^+ transporter that retrieves Na^+ from the xylem sap back into xylem parenchyma cells in the root, thus controlling root-to-shoot Na^+ transfer. However, no correlation was found between shoot Na^+ concentration and *OsHKT1;5* transcript levels across the genotypes studied in the current work. Even though IR29 has a significantly higher shoot Na^+ concentration than the three other salt-tolerant accessions, the transcript level of IR29 *OsHKT1;5* was higher than for Pokkali *OsHKT1;5* (Figure 4A).

Transport rates can be affected by allelic variation (Mäser et al., 2002; Sauer et al., 2004; Ren et al., 2005). To examine this possibility, the Pokkali and IR29 *OsHKT1;5* alleles were partially sequenced and a nucleotide polymorphism was identified that revealed a *Sal*I restriction site in the Pokkali allele. This restriction site is linked to a Leu-to-Val substitution in position 395 of the protein (IR29 and Pokkali, respectively). After reverse transcriptase PCR amplification and digestion of a 310-bp fragment surrounding the *Sal*I site, IR29 showed a different restriction profile from the three other salt-tolerant lines (Figure 5). Moreover, it was recently demonstrated that the recombinant inbred line FL478 contains a small (<1-Mb) segment from its salt-tolerant parent Pokkali in the *Saltol* region (Kim et al., 2009). These data suggest that FL478 inherited its *OsHKT1;5* allele from Pokkali, and not IR29, as previously described (Walia et al., 2005).

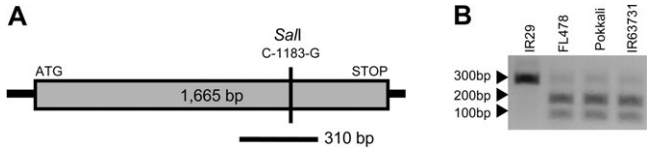

Figure 5. Heredity of the *Saltol* Locus in FL478.
A schematic representation of the full-length 1665-bp cDNA of the *OsHKT1;5* gene is pictured **(A)**. The vertical bar represents the position of the nucleotide polymorphism site at position 1183, which induces a *Sal*I restriction site in the Pokkali allele (IR29 has a cytosine, Pokkali a guanine). After amplification and digestion of a 310-bp region surrounding the restriction site (horizontal bar), digestion products were loaded on a gel **(B)**. The digestion profiles indicate that IR29 has a different allele from FL478, Pokkali, and IR63731 (i.e. no digestion) and that FL478 inherited its *HKT1;5* allele from Pokkali, and not IR29.

The Pokkali and IR29 *OsHKT1;5* alleles were then fully sequenced and the Pokkali allele was found to be identical to the Nona Bokra allele cloned by Ren et al. (2005). The IR29 allele encoded for three amino acid differences to Pokkali to give a protein identical to that found in Nipponbare. Ren et al. (2005) reported that when the Nona Bokra allele was expressed in *Xenopus* oocytes, larger Na^+ currents were produced than in oocytes expressing another allele cloned from the salt-sensitive *japonica* cultivar Koshihikari. Coincidently, both the Koshihikari and IR29 OsHKT1;5 share a Leu residue in position 395 of the protein. Although it is unclear how currents were normalized for the amount of protein present in the oocyte plasma membrane and whether this Leu-to-Val substitution is sufficient to induce a change in Na^+ currents, the results are consistent with a more efficient Na^+ transporter being encoded by the Nona Bokra/Pokkali allele than by the Koshihikari allele. Thus, it may be that the higher *OsHKT1;5* transcript levels observed in IR29 do not result in higher Na^+ sap retrieval because IR29 possesses a putative weaker allele of this gene. The fact that of the three salt-tolerant lines sharing the same allele, IR63731 had the highest transcript levels after salt treatment and had the lowest shoot Na^+ concentration would further support this proposition.

Metal Transport and Salinity Stress

Several heavy metal-associated proteins were identified that were significantly down-regulated in the salt-tolerant lines in response to the salinity treatment (Table 3). Other studies have reported similar depressive effects of salinity on the transcript levels of metal transporter genes. Maathuis (2006) analyzed a variety of transcriptomic experiments and documented seven plant metal transporters (*IRT1*, *ZIP8*, *ZIP10*, *COPT2*, *YSL5*, *Nramp1*, and *MTP1*) that show up to a 16-fold reduction in transcript levels during salt stress, although one gene involved in Zn^{2+} efflux (*HMA1*) was up-regulated. Salinity stress has also been shown to strongly inhibit the release of Fe^{3+}-binding phytosiderophores in barley, leading to greatly diminished Fe

uptake efficiency, and this may be linked to an overall reduction in the photosynthetic metabolites necessary for phytosiderophore production during salinity stress (Yousfi et al., 2007). Studies in *Medicago ciliaris* show that salinity greatly diminishes root acidification under Fe deficiency, apparently due to reduced root proton pump activity, thus reducing Fe uptake (Rabhi et al., 2007). Taken together, these studies suggest that salinity has a negative impact on metal uptake processes in plants and this may be reflected in the number of iron transport-related genes down-regulated during salinity stress that have been identified in this study and by others.

Interestingly, transcript levels of two metal transport-related transcripts—an iron transport-related protein (Figure 4G) and the Fe^{2+} transporter OsIRT2 (Figure 4H)—were much lower under control conditions in IR29 compared with the three salt-tolerant lines. These differences in transcript levels became more pronounced under saline conditions—IR29 significantly down-regulated the two transcripts whereas the salt-tolerant lines displayed relatively minor or no down-regulation. Low basal levels of transcripts of key iron transporters such as OsIRT2, which plays a major role in absorbing Fe^{2+} under submerged growth conditions (Ishimaru et al., 2006), suggest that the salt-tolerant lines FL478, Pokkali, and IR63731 may be inherently more able to tolerate Fe-deficiency conditions than IR29. Furthermore, the observed down-regulation of Fe transporters in response to salinity indicates that IR29 may be vulnerable to Fe deficiency under saline conditions. Experimental testing of these proposals may reveal interesting interactions between salinity and Fe-deficiency tolerance mechanisms.

Candidate Genes Associated with Compatible Solutes

Increased Spermidine Synthase Activity in Salt-Tolerant Lines

Polyamines such as putrescine, spermidine, and spermine are thought to play critical roles in a wide variety of plant stress responses including osmotic shock (Tiburcio et al., 1986), chilling (Shen et al., 2000), and air pollution (Wellburn and Wellburn, 1996). Salt stress, in particular, is known to increase polyamine levels in rice shoot (Krishnamurthy and Bhagwat, 1989) and a few polyamine biosynthesis genes were shown to be up-regulated by salt in maize leaves (Rodriguez-Kessler et al., 2006). However, little is known about the influence of salt stress on polyamines synthesis-related transcript levels in root tissue. In this study, all three of the salt-tolerant lines showed a significantly larger up-regulation of the spermidine synthase 3 gene compared with IR29 (Figure 4I). Although transcript levels upon salt treatment were similar across the four accessions, the three tolerant lines clearly showed a different response to the stress, possibly as these lines increase spermidine production in the roots under salt stress, whereas IR29 does not. Accordingly, a marked increase in free spermidine was found in the roots of the salt-tolerant cucumber line Changchun mici in contrast to the sensitive line Jinchun No. 2 (Duan et al., 2008). An increased level of spermidine in roots has also been associated with decreased shoot Na^+ levels and a concomitant increase in shoot K^+ (Krishnamurthy and Bhagwat, 1989; Rodriguez-Kessler et al., 2006). Furthermore, Zhao et al. (2007) demonstrated that exogenous applications of spermidine significantly blocked inward Na^+ and K^+ currents in epidermal and cortical cells of barley roots, suggesting that polyamines protect plants from saline conditions by blocking Na^+ influx into roots and preventing K^+ loss from the shoot. The lower Na^+ and slightly higher K^+ concentrations found in the shoot of FL478, Pokkali, and IR63731 could be attributed to increased spermidine production under salt stress.

Increased Transcripts of Genes Involved in Compatible Solutes in Salt-Tolerant Lines

The Δ^1-pyrroline-5-carboxylate synthetase 1 (P5CS1) protein is the rate-limiting enzyme in the biosynthesis of proline in plants (Zhang et al., 1995). In the current experiment, although microarray measurements of transcript levels across the four genotypes were similar upon salt treatment, Q–PCR data indicated that the three salt-tolerant lines up-regulate transcript levels for this enzyme more significantly than IR29 (1.2–2.3-fold for the salt-tolerant lines compared with 0.7-fold for IR29) (Figure 4J). This may be a strategy employed by salt-tolerant lines to increase the production of proline, an amino acid that is often considered to be a compatible solute (Delauney and Verma, 1993; Igarashi et al., 1997; Hien et al., 2003). It is notable that this response is less important in the salt-sensitive IR29. Similarly, transgenic lines that overexpress *P5CS1* and produce more proline are more salt-tolerant (Zhu et al., 1998; Su and Wu, 2004).

Salt-Tolerant Lines Reduce Transcript Levels of Compatible Solute Catabolic Pathway Genes

It is becoming evident that control of catabolic enzymes can be a crucial strategy employed by stress-tolerant plants. Recently, Less and Galili (2008) used a bioinformatic approach to show that the transcriptional and post-transcriptional control of catabolic enzymes was more crucial to plant abiotic stress responses than was the regulation of biosynthetic enzymes. The proline pathway was a notable exception to this rule, with biosynthesis of proline being an important and well documented stress response in plants (see above). However, decreasing the degradation of proline would logically be an additional (or an alternative) way of increasing the proline content of stressed plant cells.

Proline oxidase is the primary enzyme responsible for the degradation of proline. A net down-regulation of this gene was observed in FL478 and Pokkali but not in IR29 (Figure 4K). Similarly, in *Arabidopsis*, antisense suppression of proline dehydrogenase (oxidase) led to accumulation of proline and improved salinity tolerance of the transgenic lines (Nanjo et al., 1999). Kant et al. (2006) established that an important difference between *Arabidopsis thaliana* and its close relative, *Thellungiella halophila*, in response to salt stress was the endogenous level of proline found in the plants. The authors

postulated that *Thellungiella* maintained a higher level of proline due to a decreased level of the enzymes that catabolize proline. This suggests that the response seen in FL478 and Pokkali but not in IR29 is likely to contribute to salinity tolerance.

Interestingly, the salt-tolerant line IR63731 did not down-regulate the proline oxidase gene (Figure 4K). This points once more to the difference in salinity responses between the two source genotypes, Pokkali and Nona Bokra. Perhaps since IR63731 has excellent Na^+ exclusion, it is able to avoid the need to accumulate compatible solutes such as proline, or it may not have been sufficiently challenged by the salt stress in the current experiment to require transcriptional regulation of this mechanism.

Myo-Inositol Oxygenase Down-Regulated in Salt-Tolerant Lines

In vivo myo-inositol levels have been correlated with the activity of myo-inositol oxygenase, a catabolic enzyme that catalyzes the oxidation of free myo-inositol to D-glucuronate (Lorence et al., 2004). The functional role of myo-inositol appears to be related to osmotic balance, and possibly to the transport of Na^+ from root to shoot, as was shown in the common ice plant *Mesembryanthemum crystallinum* (Nelson et al., 1999; Chauhan et al., 2000). In this experiment, this gene was significantly down-regulated in FL478, Pokkali, and IR63731 upon salt treatment, whereas transcript levels in IR29 remained stable (Figure 4L). Again, this indicates that salt-tolerant lines decrease the transcript levels of genes encoding enzymes that degrade compatible solutes, as a mechanism to reduce the effects of the imposed stress. Similarly, increased production of myo-inositol through overexpression of the L-*myo*-inositol 1-phosphate synthase (MIPS) gene in transgenic rice provided the plants with increased salinity tolerance (Das-Chatterjee et al., 2006). In *Arabidopsis* roots, exogenously applied myo-inositol decreases K^+ efflux induced by free radicals, possibly by decreasing currents through outwardly rectifying K^+ channels and nonspecific cation channels (Cuin and Shabala, 2007).

More recently, it was found that the tonoplast INT1, the primary transporter for myo-inositol *in planta*, is responsible for regulating the transport of vacuolar myo-inositol into the cytoplasm (Schneider et al., 2008). Two other *Arabidopsis* INT transporters, INT2 and INT4, mediate myo-inositol movement across the plasma membrane (Schneider et al., 2006, 2007), and thus may work in concert with INT1 to efficiently mobilize myo-inositol and maintain osmotic balance within cells or, potentially, aid in stress signaling. Interestingly, the INT2 gene in rice (probe set Os.27403.1.A1_at) is not responsive to salt but its transcript levels were found at much higher levels in FL478 roots than in the three other cultivars, where transcript levels are low. Potentially, this would lead to FL478 being less challenged by the osmotic component of the salinity stress—a factor that may be involved in the improved salinity tolerance seen in FL478 compared with Pokkali.

Conclusion

An analysis of transcript levels in response to salt exposure in the roots of rice plants is presented, providing a useful complement to the earlier work in shoots of the same set of plants. A comparison of transcript levels amongst the genotypes studied and of changes in these levels in the sensitive and the tolerant lines can provide insights into processes that are adaptive (i.e. related to salinity tolerance), rather than simply being responses to salinity that may or may not be involved in tolerance.

The large differences observed between the different salt-tolerant lines indicate the diversity of approaches employed by plants to address the challenge of salt stress. Nonetheless, several root-specific genes with similar transcript profiles observed across the three salt-tolerant lines present a set of candidate genes whose function in relation to plant salinity tolerance should be further investigated, for example: the *Os11g34460* PAS motif gene; the *OsTIP2;1* aquaporin; the *Os02g02170* high-affinity nitrate transporter gene; and the *Os01g62830* gene of unknown function under the root Na^+ amount QTL on chromosome 1.

This comparative transcriptomic approach provides a valuable indication of a range of candidate genes useful for further study. It is also noteworthy that the modest levels of salt applied for longer periods of time in this experiment increase the likelihood that these genes may be of functional relevance to situations in the field.

METHODS

Plant Material and Salinity Assay

Seeds of rice genotypes IR29 (IRGC 30412), FL478 (GID 1192884), Pokkali (IRGC 108921), and IR63731 (GID 89496) were received from IRRI in The Philippines. Dehusked seeds were surface-sterilized in 1% sodium hypochlorite and rinsed several times before being allowed to germinate in deep cell culture dishes in a growth chamber (28/24°C day/night; 12-h photoperiod; 80% humidity). After 5 d, seedlings were transplanted to a 10-L hydroponic tank filled with nutrient solution (5 mM NH_4NO_3; 5 mM KNO_3; 2 mM $Ca(NO_3)_2$; 2 mM $MgSO_4$; 0.1 mM KH_2PO_4; 0.05 mM NaFe(III)EDTA; 50 μM H_3BO_3; 5 μM $MnCl_2$; 5 μM $ZnSO_4$; 0.5 μM $CuSO_4$; and 0.1 μM Na_2MoO_3; pH 5.5). The solution was changed after 7 d, and a mild salt stress applied in two increments (50 mM NaCl and 0.75 mM $CaCl_2$ for 2 d, followed by 70 mM NaCl and 1 mM $CaCl_2$ for 5 more days). $CaCl_2$ was added to the solution to maintain a constant Ca^{2+} activity of 1 mM throughout the experiment. Roots and shoot tissues were harvested 19 d after germination and Na^+ and K^+ content measured with a flame photometer (Sherwood Scientific Ltd, Cambridge, UK).

Plant Material, Affymetrix GeneChip® Hybridization, and Microarray Annotation

The plant material used for the microarrays was prepared from the four genotypes as described in Walia et al. (2005). RNA

preparation, cDNA and cRNA synthesis, and Affymetrix GeneChip® hybridization were also performed as described in Walia et al. (2005). Three biological replicates per treatment and per genotype were analyzed, with the exception of IR29 control roots, which had two replicates only. Functional annotation and physical location of the genes represented by the probe sets in the *japonica* genome (TIGR assembly version 5) were obtained from the Affymetrix website (www.affymetrix.com/support/technical/annotationfilesmain.affx; 18/03/2008). In addition, all probe set sequences were blasted against the NCBI non-redundant database (blastx E-value; cutoff e^{-15}, April 2006).

Statistical Analysis of the Microarray Data

Signal intensity values for each probe were read from the Cel-files using the Bioconductor packages implemented in R (www.bioconductor.org). The data were normalized using the Robust Multichip Average method of background correction, quantile normalization, and summarization of cell signal intensities (Irizarry et al., 2003). Statistical analysis was performed using the limma package (linear models for microarray data) (Smyth, 2004). Genes that had different transcript levels between the control and salt-treated roots of each variety were identified at $P < 0.05$ with an empirical Bayes' *t*-test using false discovery rate (FDR) for multiple testing correction (Benjamini and Hochberg, 1995) and by using the empirical criterion of more than two-fold change.

Similarly, genes candidates showing constitutive expression differences in non-treated roots of the four cultivars were identified at $P < 0.05$. Single feature polymorphisms (SFPs), in other words sequence differences in mRNAs between cultivars, can result in reduced hybridization to individual probes representing an mRNA on the microarray, thus leading to lower signal intensities. We therefore inspected the probe intensities of all candidates for SFPs after background subtraction and quantile normalization of the data at the probe level. For this purpose, we used both the root and the shoot microarray data (Walia et al., 2005). Only those candidates showing no indication of SFPs and hence true expression differences were considered.

Identification of Genes Potentially Located under QTLs Associated with Salinity Tolerance

For each probe set, the physical location was obtained of the represented gene in the *japonica* genome from the Affymetrix annotation file mentioned above. Since *indica* varieties were being used in this study, it was important to obtain the physical location of the probe sets in the *indica* genome. For this purpose, the assembled *indica* genome sequence was retrieved from Gramene (ftp://ftp.gramene.org/pub/gramene/CURRENT_RELEASE/; version Jan_2005.48) and a BLASTN search performed of the Affymetrix probe set sequences against the *indica* genome in house. Some genes are represented by more than one probe set on the chip. In this case, all probe sets representing a gene potentially located under a QTL were considered. The sequences for the markers bordering the QTLs that affected the root phenotype on chromosome 1, 4, 7, and 9 described by Lin et al. (2004) were retrieved from the Gramene database. The physical positions of the marker sequences were determined in both the *indica* and *japonica* genomes, by performing in-house BLASTN searches and using the Gramene website database tools, respectively.

Quantitative Real-Time PCR

Leftover purified RNAs from the microarray experiment were treated with rDNase I using a DNA-*free* kit (Ambion) before RNA integrity was checked on a 1.2% agarose gel (w/v). cDNA synthesis was performed on 1 μg of RNA with a 19-mer polyT primer and a SuperScript III reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR (Q–PCR) was carried out essentially as outlined in Burton et al. (2004) with the following modifications. Three replicates of each of the seven standard concentrations were included with every Q–PCR experiment. Three replicate PCRs for each of the cDNAs were included in every run containing the following: 2 μL of cDNA solution, the diluted standard or water, 5 μL IQ SYBR Green PCR reagent (Bio-Rad Laboratories), 1.2 μL of each of the forward and reverse primers at 4 μM, 0.3 μL 10× SYBR Green in water and 0.3 μL water (final volume 10 μL). Reactions were performed in a RG6000 Rotor-Gene Real Time Thermal Cycler (Corbett Research); 3 min at 95°C followed by 45 cycles of 1 s at 95°C, 1 s at 55°C, 30 s at 72°C, and 15 s at the optimal acquisition temperature for each specific gene product. Five control genes (GaPDh, Actin, Tubulin, PpIase, and ElF1) were assessed and the best three were chosen for the calculation of the normalization factor (Actin, Tubulin, and ElF1). Q–PCR normalization was carried out as detailed in Vandesompele et al. (2002) and Burton et al. (2004). Primer sequences for GaPDh, Actin, and Tubulin can be found in Kim et al. (2003). PpIase primers are: Forward: AATCACTTCGCATCGGACAT; Reverse: GCAAATCCTCGGCAGTAGAC. ElF1 primers are: Forward: ATCTGGGAAATCATCGGTTCTG; Reverse: AGATCGTCCACAATGGTCATCA.

Accession Numbers

Sequence data from this article can be found in the EMBL/GenBank data library under accession numbers EF373553 and HQ162137 for the Pokkali and IR29 alleles, respectively, of *OsHKT1;5*. All microarray data from this work are available from the NCBI website (GEO ID GSE14403).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

This work was supported by the Australian Research Council (to M.T.).

ACKNOWLEDGMENTS

We wish to warmly thank Dr Neil Shirley for his help with Q–PCR. No conflict of interest declared.

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