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ORIGINAL PAPER

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Comparative transcriptome analyses of barley and rice under salt stress

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Abstract Although barley and rice belong to the same family Poaceae, they differ in their ability to tolerate salt stress. In an attempt to understand the molecular bases of such differences, we compared changes in transcriptome between barley and rice in response to salt stress using barley cDNA microarrays. At 1 and 24 h after salt stress, many genes were up-regulated in barley, but not in rice. Leaf water potential declined in the first 10 h of stress in both species, but recovered in the period 24–48 h only in barley. In addition, we found that barley partitioned Na^+ to the roots and away from the shoots more efficiently than rice. These differences in physiological responses were correlated with the differences in the steady-state abundance of transcripts for the genes related to adaptive functions. Transcripts for plasma membrane protein 3 and inorganic pyrophosphatase were up-regulated in both species, but only transiently in rice. This indicates that adaptive mechanisms for regulating ion homeostasis are partly conserved in the two species, but it seems that rice cannot sustain cellular ion homeostasis for a long time like barley. These results imply that genetic modification of regulatory controls of early salt-responsive genes might lead to development of the salt tolerance trait in rice.

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Introduction

Salt stress adversely affects crop productivity. Crop plants take up salts from irrigation water, and from soil made saline by evaporation of irrigation water. Because water use efficiency is often higher in salt tolerant plants than in salt susceptible plants (Glenn and Brown 1998), improving salt tolerance in food crops could have a major impact on agriculture by allowing cultivation with lower amounts and lesser quality of water. Salt stress inhibits growth and development of plants by hindering various metabolic activities, cell expansion, and in some cases by triggering programmed cell death (Huh et al. 2002). By reducing the water uptake of roots, salts cause ionic, osmotic and nutrient stresses. Cellular responses to these stresses, and subsequent secondary stresses, such as oxidative stress, add to the complexity of the salt tolerance trait. Molecular studies have revealed that salt tolerance is controlled by interactions between several independently regulated but temporally and spatially coordinated processes (Kawasaki et al. 2001; Ozturk et al. 2002; Seki et al. 2002). At the physiological level, salt tolerant plants exhibit a wide range of mechanisms that include exclusion, compartmentalization and secretion of salts.

In recent years, several transgenic approaches that enable sequestering of salts into the vacuoles or root apoplast have been shown to improve salt tolerance. Transgenic plants overexpressing plasmamembrane- or tonoplast-localized Na^+/H^+ antiporters acquired enhanced salt tolerance (Apse et al. 1999; Shi et al. 2003), whereas mutation in the Na^+ transporter gene (*AtHKT1*) increased sensitivity to salt stress (Maser et al. 2002). These results suggested that Na^+ exclusion from the cytosol is one of the effective mechanisms in reducing salt stress. Certain salt tolerant plant species protect cells from hyperosmotic pressure by accumulating osmoprotectants such as proline and glycinebetaine (betaine; Kavi Kishor et al. 1995; Hayashi et al. 1997; Takabe et al. 1998). Increased syntheses of trehalose and

ononitol in plant cells were also shown to improve stress tolerance (Hölmstrom et al. 1996; Sheveleva et al. 1997). Although the above mechanisms improve salt tolerance under laboratory conditions, their utility has not been tested at the field level. Under field conditions, effective agronomic salt tolerance may require more genetic determinants.

An understanding of how tolerant crop species gradually adapt to salt stress should enable the development of additional salt tolerance traits. In general, however, most of the major cereal crop plants, such as rice and corn, are highly sensitive to salt stress. Hence, despite a wealth of ESTs and genomic sequences in rice, the role of rice genes in salt tolerance remains unknown. Barley is one of the very few crop species that show moderate levels of salt tolerance and is a diploid cereal belonging to Poaceae family. Although the genome size of barley is ~10 times larger than that of rice, comparative genetic analyses revealed extensive conservation of gene content and gene order in the two species (Gale and Devos 1998). Unlike in rice, however, ESTs are the only major genomic resource available in barley (Close et al. 2004). We previously cloned salt-responsive cDNAs from 6-day-old salt-stressed leaf (T. Takabe et al., unpublished) and root (Ueda et al. 2002) tissues of barley using differential display technique. In this study, we arrayed these salt-responsive cDNAs on glass slides and compared the changes in their transcript levels in barley and rice tissues during the initial stages under salt stress. We used Haruna-nijyo (barley) and IR64 (rice) for the present study. Haruna-nijyo, one of the popular cultivars, is widely used for brewing beer, and also for further breeding as a parental line. Salt tolerance in Haruna-nijyo is an inducible feature, acquired during the process of salt stress acclimation. The amount of betaine accumulation, one of the determinants in salt tolerance, is not higher than that of other barley cultivars under non-stress condition, and its accumulation is increased in response to salt stress (Ishitani et al. 1993). This kind of inducible feature is useful to study salt tolerance in barley and improve it in rice. IR64 is also the most widely grown cultivar in the tropics. It shows intermediate salt sensitivity at the seedling stage, between salt sensitive IR29 and salt tolerance Pokkali (Moradi et al. 2003). As a cultivar bred for the irrigated ecosystem, IR64 is vulnerable to the secondary salinity that is developing in irrigated areas. Improvement of salt tolerance in IR64 is therefore a priority, requiring a clear delineation of the similarities and differences in salt responsiveness of gene expression between IR64 and salt-tolerant barley cultivars such as Haruna-nijyo. We also compared the physiological changes in barley and rice tissues under salt stress. The comparative expression analyses provide insights into the differences in sensitivity to salt stress between barley and rice.

Materials and methods

Plant materials and growth conditions

Seedlings barley (*Hordeum vulgare* L. cv. Haruna-nijyo) and rice (*Oryza sativa* L. cv. IR64) were hydroponically grown in half-strength Hoagland solution with doubled iron concentration in a growth chamber under 13 h of light (70% humidity, 400 μmol m^{-2} s^{-1}) and 11 h of dark (75% humidity). Temperature was set at 25°C (light period)/22°C (dark period) for barley and 27°C/22°C for rice. Salt stress was applied by transferring seedlings with two leaves in a similar Hoagland solution with 200 mM NaCl for barley and 150 mM NaCl for rice. Salt-stressed plants were harvested after 1 and 24 h of salt stress.

cDNA microarray analysis

Fabrication of barley cDNA microarrays using salt-responsive genes was carried out as described previously (Ueda et al. 2004). DNA was spotted in quadruplicate on aminosilane-coated slides (GAPSII Coated Slide; Corning, Acton, NY, USA) using the GeneTAC G3 arrayer (Genomic solutions, Ann Arbor, MI, USA). Non-plant cDNAs (Ambion, Austin, TX, USA) were spotted as external controls for normalizing the signal intensities between barley and rice arrays. Extraction of total RNA, cDNA labeling using cy3 or cy5 and microarray experiments were carried out as described previously (Ueda et al. 2004). Hybridization was carried out using the automated GeneTAC Hybridization station (Genomic Solutions) for 16 h at 60°C for barley targets and 55°C for rice targets. After washing, the slides were briefly centrifuged, and scanned using the GeneTAC LSIV laser scanner (Genomic Solutions). Scanned images were analyzed using the Integrator Analyzer 3.3 software (Genomic Solutions). All hybridizations were repeated at least three times using RNA extractions from three experimental tissues (biological replications), and expression levels were averaged over three independent experiments. The incorporation efficiencies of cy3 and cy5 in reverse transcription were not significantly different. After global normalization of cy5 and cy3 intensities and transformation of the raw data to $\log_2$ ratios, the statistical significance of expression values for each gene was carried out using the R statistical package (http://www.r-project.org). The significance of differential regulation in expression level between control and salt stress was measured by the ANOVA F-test using SAS software. Significantly regulated genes were highlighted genes up- or down-regulated more than twofold (more than 1 or less than −1 in $\log_2$ ratio value) are listed in Supplementary Table 1, while Table 1 lists highly responsive genes [more than threefold change (more than 1.52 or less than −1.52 in $\log_2$ ratio value)].

Table 1 Genes that are highly or significantly up- or down-regulated in response to salt stress in barley and rice

Gene ID	Gene name	Leaf		Root	
		1 h	24 h	1 h	24 h
Highly up-regulated genes in barley					
AU312370	Pyrroline-5-carboxylate synthetase	0.8	***2.5***	0.5	***1.6***
AU312393	Aldehyde dehydrogenase	0.7	***1.8***	0.3	**1.4**
AU312390	Pyrroline-5-carboxylate synthetase	0.7	***1.7***	0.2	**1.4**
AU252354	Phosphoglycerate dehydrogenase	**1.3**	0.2	***1.7***	0.8
AU312397	Tryptophan synthase β-1 chain	0.1	−0.1	***1.7***	**1.0**
AU312412	PMP3	0.3	**1.1**	***1.5***	***2.3***
AU252379	Proline-rich protein	0.9	0.1	0.4	***4.4***
AU252308	Cytochrome P450 CYP99A1	0.5	−0.1	−0.3	***4.2***
AU252311	Cytochrome P450 CYP99A1	0.8	−0.1	−0.4	***4.0***
AU252310	Cytochrome P450 CYP99A1	0.3	−0.1	−0.4	***3.8***
AU252315	Cytochrome P450 CYP99A1	0.6	−0.3	−0.2	***3.7***
AU252400	Proline-rich protein	0.0	−0.2	−0.1	***3.5***
AU252316	Putative cytochrome P450	0.7	−0.2	−0.3	***2.3***
AU312389	Asparagine synthetase (glutamine-hydrolyzing)	0.9	**1.3**	0.8	***1.8***
AU312465	ORF122	**1.0**	−0.1	0.1	***1.6***
AU312469	ORF122	0.7	−0.3	0.0	***1.6***
Highly down-regulated genes in barley					
AU252365	Phosphoenolpyruvate carboxylase	−0.9	***−2.1***	−0.5	0.0
AB073084	Proline transporter	−0.1	***−1.8***	0.8	0.8
AU312461	ATP synthesis beta chain	−0.8	***−1.7***	0.1	0.5
AJ250664	Omega−3FAD	−0.7	***−1.6***	0.2	0.3
AU312477	HSP17.9	−0.6	−0.8	***−1.7***	0.2
AU252355	Cytosolic phosphogluconate dehydrogenase	−0.2	−0.9	***−1.7***	−1.3
AU312410	Water channel 1	−0.3	−0.5	−0.8	***−2.1***
AU312405	Water channel 2	**−1.2**	−0.8	**−1.3**	***−1.7***
Highly down-regulated genes in rice					
AU312392	Transketolase 1 protein positives	***−2.2***	**−1.2**	**−1.0**	**−1.5**
AU312376	Glutamyl-tRNA reductase	***−1.8***	**−1.3**	0.4	−0.9
AU312406	Rubisco SSU	***−1.6***	***−2.0***	0.6	***−1.9***
AU312461	ATP synthesis beta chain	**−1.4**	***−1.8***	0.9	−0.5
AU252367	Glyceraldehyde 3-phosphate dehydrogenase	**−1.1**	***−1.6***	−0.4	−0.7
AU252334	Polyubiquitin	−0.5	***−1.6***	0.0	0.0

All data were transformed to $\log_2$ ratio (salt stress/control). Highly up-/down-regulated genes (threefold changes) are indicated in italics. Significantly regulated genes (twofold changes) are indicated in bold

Northern hybridization

For Northern blot analysis, 10 μg of total RNA was separated on a 1.2% (w/v) agarose gel containing 0.66 M formaldehyde and blotted onto a nylon membrane (Hybond-N; Amersham Bioscience, Uppsala, Sweden). Northern hybridization was performed as described previously (Ueda et al. 2001). Quantification of signals obtained by Northern analysis was performed using CS Analyzer software (version 2.02, ATTO), and expression level was shown as the $\log_2$ ratio (salt stress/control).

Physiological analysis

For Na^+ extraction, leaves and roots were dissected and roots were immediately washed with distilled water to remove the nutrient solution on the root surface. Samples were dried at 80°C for 48 h, and baked to ashes at 500°C for 48 h. Na^+ was extracted in Milli-Q water (Millipore, Bedford, MA, USA) with vigorous shaking for 24 h. After brief centrifugation, the supernatant was filtered using cellulose acetate filters (0.45 μm pore size, Millipore). The Na^+ content was measured using an ion chromatograph system (SHIM-PACK IC-C6; Shimadzu, Kyoto, Japan). The 2nd leaf blades were used for determination of leaf water potential. Dissected blades were immediately set into the measurement chamber and then sealed with parafilm. Prior to measurement, samples were incubated in the chamber for equilibrium of water status for 30 min at 25°C, and leaf water potential was determined using the PotentiaMeter (Decagon, Pullman, MA, USA).

Quantitative RT-PCR

First strand cDNA was synthesized from 5 μg of total RNA by SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Quantitative RT-PCR was performed with the LightCycler using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland). Final magnesium concentration was adjusted to 3 mM in all PCR experiments. Specific primers were designed using LightCycler Probe Design

Software version1.0 (Roche). Specific primers are as follows: α-tubulin forward (5′-ccacttcatgctttcg-3′) and reverse (5′-cgatgcgggagaacac-3′), catalase forward (5′-gctggggccaaactac-3′) and reverse (5′-catcacactgggagagg-3′), Fd-GOGAT forward (5′-agtgaatgctccagcc-3′) and reverse (5′-gtttccttgatgcggg-3′). All PCR products were normalized using the expression value of α-tubulin as an internal standard.

Western blot analyses

Leaf total proteins from barley and rice were extracted with ice-cold buffer (50 mM Tris–HCl, 10 mM EDTA, 2 mM 2-mercaptoethanol, 10% glycerol, pH 7.0). Twenty-five micrograms of total protein was separated by 7.5% SDS-PAGE, and then transferred onto nitrocellulose membrane. Polyclonal antibody (1:2000), raised against barley methionine synthase (Narita et al. 2004), was incubated for 4 h. After incubation of second antibody (1:500,000, Peroxidase conjugated goat anti-rabbit IgG, Pierce, Rockfird, IL, USA) and washing, signal was detected with chemiluminescent reagent (SuperSignal West Femto Maximum Sensitivity Substrate, Pierce). Induction level was estimated with three replications.

Results

Physiological responses to salt stress

Because salt stress affects both water relations and ionic balance in plants, we first compared the pattern of Na^+ accumulation in leaf and root tissues of barley and rice during the first 48 h under stress. The Na^+ content increased in both barley and rice immediately after the onset of stress (Fig. 1). However, barley and rice differed in their amount and rate of Na^+ accumulation in roots and leaves. While the Na^+ accumulated more rapidly during the first 12 h and stabilized in barley roots, the relatively smaller increment in Na^+ levels in rice occurred slowly over a period of 24 h. In contrast to the root tissues, the amount and rate of Na^+ accumulation in leaves was higher in rice than in barley (Fig. 1). We also monitored the changes in water potential in barley and rice leaves. To monitor the leaf water potential is useful to know the magnitude of damage by salt stress because osmotic stress dominantly affected water relations in plants during the initial phase of salt stress (Ueda et al. 2004). As shown in Fig. 2, the leaf water potential decreased by about twofold within 10 h of salt stress in both species. This observation indicated that barley and rice were subjected to the same magnitude of osmotic stress during first 10 h, in spite of treatment with different NaCl concentrations. While the leaf water potential in barley was restored to -1.08 MPa by 24 h of stress, the leaf water potential in rice continued to decline to -2.01 MPa at 48 h (Fig. 2). These results indicated that rice and barley differ in their early responses to salt stress after the first 24 h, and such differences could explain their divergence in sensitivity to salt stress.

Gene expression analysis using barley cDNA microarray

Using the differential display technique, we previously isolated 460 non-redundant cDNA clones from barley seedlings that were subjected to salt stress for 6 days, and the predicted functions of these cDNA clones represent a wide range of biological activities (Muramoto et al. 1999; Ueda et al. 2002; Nakamura et al. 2004; T. Takabe et al., unpublished). Because several secondary stresses occur after 6 days of salt stress, here, we studied how the expression of these genes differs in barley and rice during the initial phase under salt stress. We spotted 460 barley cDNA probes on glass slides, and hybridized with homologous targets from barley or heterologous targets from rice tissues under similar stringency conditions. We estimated the efficiency of heterologous hybridization through pair-wise comparisons of gene expression for all the cDNA elements on the array after performing global normalization. The Spearman coefficient (Sp) values for the 460 genes established high correlations between heterologous and homologous probing using non-stressed tissues (Sp value ranged from 0.64 to 0.73). The ratio of total signal intensities between rice and barley ranged from 0.56 to 0.99. These results indicated that many of the barley cDNA sequences are conserved in rice and thus suggested that heterologous probing is

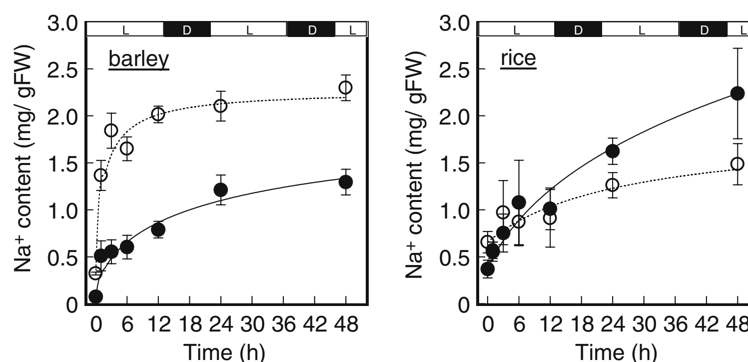

Fig. 1 Na^+ content in barley and rice under salt stress (*closed circle*, leaves; *open circle*, roots). Data represent the average ± SE of four samples. Barley and rice were stressed under 200 and 150 mM NaCl conditions, respectively. *L* and *D* indicated the cycle of light/dark period

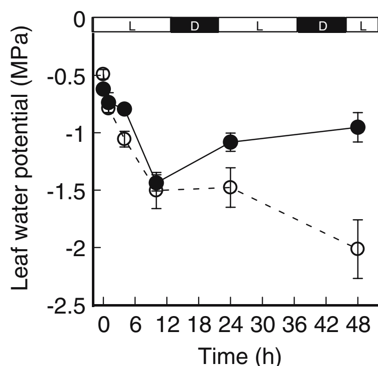

Fig. 2 Changes in leaf water potential in barley (*closed circle*) and rice (*open circle*) under salt stress. Data represent the average ± SE of eight samples. Barley and rice were grown under salt stress conditions as described in Fig. 1. Leaf blades were set into the measurement chamber and kept for equilibrium of water status for 30 min at 25°C before measurement. *L* and *D* indicated the cycle of light/dark period

valid for comparing the expression levels of salt-responsive genes in the two plant species. In Supplementary Table 1, we identify the genes that were significantly regulated at one or at both time points. Significance was estimated statistically by the ANOVA F-test. We found that 67 and 5 genes were up-regulated in barley and rice, respectively. By contrast, the number of highly induced genes was 16 in barley and zero in rice (Table 1). Totals of 30 and 39 genes were significantly down-regulated in barley and rice under salt stress (Supplementary Table 1), whereas 8 and 6 genes were highly down-regulated in barley and rice, respectively (Table 1).

Expression profiling in roots

In barley roots, a total of 49 genes were significantly up-regulated during the initial phase under salt stress (Supplementary Table 1). Out of these, 13 genes showed up-regulation after 1 h. These genes encode signaling elements such as receptor-like protein and serine/threonine protein kinase. After 24 h, the genes involved in the biosynthesis and transport of amino acids, and genes of the cytochrome P450 family were up-regulated in barley roots. In rice roots, however, only five genes showed significant up-regulation. Interestingly, all five genes were up-regulated only at 1 h, and their transcript levels were either restored or down-regulated by 24 h of stress. A vacuolar H^+ translocating inorganic pyrophosphatase that was up-regulated in rice roots at 1 h was induced only at 24 h in barley roots. In barley roots, a total of 14 genes showed down-regulation. While the transcript levels of water channel protein 2, phospholipase C and SalT were down-regulated within 1 h, the mRNA abundance of water channel protein 1 showed significant down-regulation only by 24 h under stress. In rice roots, transcripts of 12 of 13 down-regulated genes were decreased only at 24 h of stress.

Expression profiling in leaves

In general, the number of genes that showed up-regulation was less in leaves than in roots (Supplementary Table 1). Out of 460 genes, 26 showed significant up-regulation during the first 24 h of stress in barley leaves. Genes that are involved in signal transduction, such as calcium-dependent protein kinase, phosphatidylinositol-4-phosphate-5-kinase, pleiotropic drug resistance 5-like ATP binding cassette (PDR5-like ABC) transporter and SET1 [Su(var)3–9, Enhancer-of-zeste, Trithorax1], were up-regulated in barley leaves within 1 h under salt stress. After 24 h, genes encoding BADH (betaine aldehyde dehydrogenase), lipoxygenase, P5CS (Δ^1-pyrroline-5-carboxylate synthetase), methionine synthase and asparagine synthetase were induced in barley leaves, but were never induced in rice leaves. Only 1 of the 460 genes tested showed up-regulation in rice leaves under salt stress (Supplementary Table 1). In contrast to barley leaves where 21 genes showed down-regulation, about 32 genes were down-regulated in rice leaves during the initial phase of salt stress. Several of the genes that were down-regulated in rice leaves are involved in amino acid biosynthesis and in the oxidative pentose phosphate pathway. Interestingly, the expression levels of most of these genes in barley leaves were not affected by salt stress.

Comparison of salt-responsive transcriptomes in barley and rice

Among significantly responsive genes under salt stress, only two up-regulated genes and ten down-regulated genes were commonly observed in both species (Table 2). While expression of PMP3 (plasma membrane protein 3) gene was induced in both plants at 1 h, it was suppressed in rice at 24 h. On the other hand, the expression patterns of six genes were different in barley and rice, either in the timing or in the extent of induction or suppression. Contrasting expression was observed in two genes related to amino acid biosynthesis, tryptophan synthase and methionine synthase.

Validation of microarray data

We confirmed the differential expression of genes identified by microarray analyses by performing Northern blot analyses and quantitative RT-PCR in control and salt-stressed tissues of rice and barley for some representative barley cDNAs that were found to be differentially expressed in cDNA microarrays. Consistent with the microarray hybridization data, the Northern hybridization results revealed that tryptophan synthase, methionine synthase, P5CS, water channel 2 and inorganic pyrophosphatase are differentially regulated in barley and rice under salt stress (Fig. 3). Validation by Northern blot analysis was also done for another four genes in rice

Table 2 Regulation of gene expression in barley and rice under salt stress

Gene ID	Gene name	Barley				Rice			
		Leaf		Root		Leaf		Root	
		1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h
Genes whose expressions are similar in barley and rice									
AU312412	PMP3	0.3	**1.1**	**1.5**	***2.3***	**1.4**	−0.5	**1.3**	0.0
AU312471	F19D11 (At2g46740)	**1.0**	−0.1	0.2	0.2	−0.2	−0.3	**1.2**	−0.2
AU312461	ATP synthesis beta chain	−0.8	*−1.7*	0.1	0.5	**−1.4**	***−1.8***	0.9	−0.5
AU312421	Ribosomal protein L23	**−1.0**	**−1.0**	−0.2	0.1	−0.9	**−1.1**	−0.8	−0.5
AU312419	HSP70	−0.3	−0.9	**−1.3**	−0.6	−0.4	**−1.2**	−0.4	−0.5
AU312414	Dnak like protein	−0.2	**−1.3**	−0.5	−0.4	−0.1	**−1.0**	−0.4	−0.5
AU312407	Hypothetical protein F10M23.190	−0.8	**−1.2**	−0.6	**−1.0**	**−1.3**	−0.8	0.1	**−1.2**
AU312400	Actin protein	−0.6	**−1.2**	−0.2	−0.1	−0.3	**−1.3**	−0.6	−0.7
AU312372	Fd glutamate synthase	−0.8	**−1.0**	0.4	−0.1	**−1.5**	**−1.4**	0.1	−0.8
AU252397	Oxalate oxidase	0.2	−0.3	**−1.0**	0.9	0.4	0.2	0.1	**−1.2**
AU252367	Glyceraldehyde3-phosphate dehydrogenase	**−1.1**	0.0	−0.7	0.1	**−1.1**	*−1.6*	−0.4	−0.7
AU252355	Cytosolic phosphogluconate dehydrogenase	−0.2	−0.9	*−1.7*	**−1.3**	−0.2	−0.5	−0.7	**−1.2**
Genes whose expressions are different in barley and rice									
AU312397	Tryptophan synthase β-1 chain	0.1	−0.1	*1.7*	**1.0**	−0.5	**−1.3**	0.1	**−1.1**
AU312365	PDR5-like ABC transporter	**1.0**	−0.4	−0.1	0.0	−0.1	−0.3	−0.8	**−1.0**
AU252385	Tubby like protein	0.2	0.2	−0.1	**1.2**	−0.5	**−1.2**	0.0	−0.3
AU252300	Inorganic pyrophosphatase	**−1.0**	−0.8	0.1	**1.2**	−0.6	**−1.0**	0.5	−0.5
AU252297	Inorganic pyrophosphatase	−0.7	−0.5	0.4	**1.3**	−0.4	**−1.0**	0.9	−0.2
AB164396	Methionine synthase	0.7	**1.5**	**1.1**	0.9	−0.1	**−1.2**	−0.1	−0.6

All data were transformed to $\log_2$ ratio (salt stress/control). Highly up-/down-regulated genes (threefold changes) are indicated in italics. Significantly regulated genes (twofold changes) are indicated in bold

(Fig. 4). Similar to the results in Fig. 3, expression levels indicated by Northern blot analysis were identical to those obtained by microarray analysis except for weakly expressed genes. We also verified the validity of heterologous hybridization data through quantitative RT-PCR in salt-stressed rice leaves by using primers and probes for three rice homologues. Fold changes in expression levels of α-tubulin, catalase and Fd-GOGAT transcript levels were similar to those observed through microarray analyses (Table 3). In addition, we also examined if divergent

Fig. 3 Northern blot analyses of gene expression in barley and rice for genes selected from the microarray. Genes were chosen to be representative of different regulatory responses among the tissues and species. Total RNAs (10 µg/lane) were blotted onto a nylon membrane and hybridized with barley cDNA as a probe. Hybridization and washing for rice RNA were carried out at 60°C. Fold changes of transcript amount by Northern blot analysis was compared in $\log_2$ ratio with microarray data

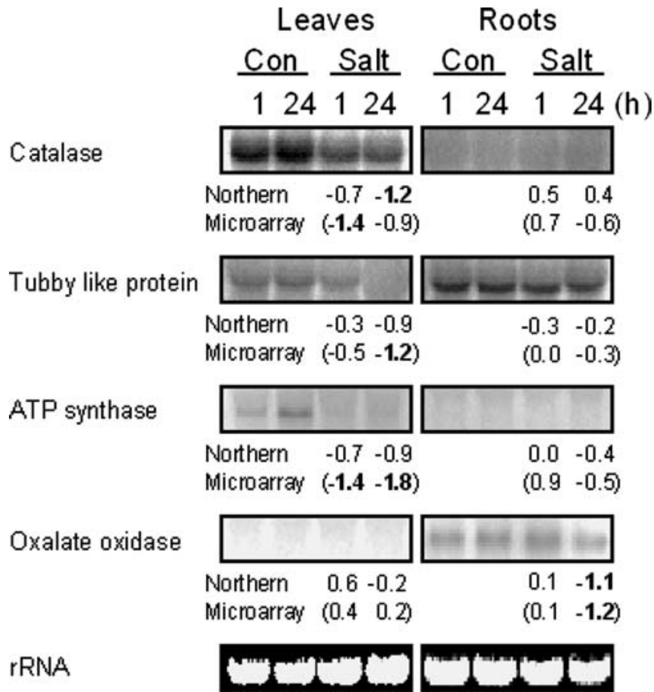

Fig. 4 Northern blot analyses of gene expression in rice selected from microarray data. Genes were chosen to be representative of differential regulation among the tissues and species. Total RNAs (10 μg/lane) were blotted onto a nylon membrane and hybridized with barley cDNA as a probe. Hybridization and washing for rice RNA were carried out at 60°C. Fold changes of transcript amount by Northern blot analysis were compared in $\log_2$ ratio with microarray data

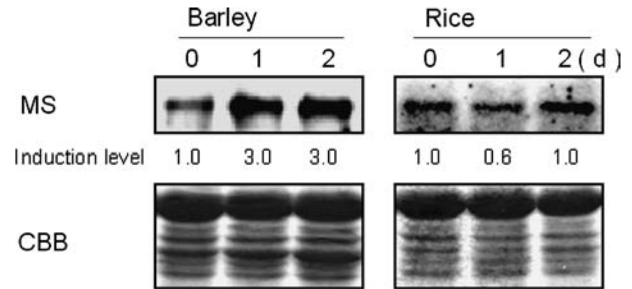

Fig. 5 Western blot analysis of methionine synthase in barley and rice leaves under salt stress. Total proteins were extracted at 0 (control), 1 and 2 days of salt stress treatment. Twenty-five micrograms of leaf total protein was used for Western blot analysis. Induction level was calculated with the MS protein amount of 0 day as a standard. Coomassie brilliant blue (CBB)-stained images are shown as a loading control for SDS-PAGE

responses observed for barley and rice at mRNA levels reflected changes in protein levels using antibodies for barley methionine synthase (Fig. 5). Methionine synthase protein could be detectable in both plants with anti-barley methionine synthase antibody because of very high homology of the protein in barley and rice (>90%). Under non-stressed condition, methionine synthase protein constitutively exists in barley and rice (Fig. 5, 0 d). Western blot analyses revealed that as in the case of transcripts, methionine synthase protein is increased in barley leaves but not in rice leaves under salt stress.

Table 3 Comparison of quantitative RT-PCR and microarray analysis in measuring gene expression levels in salt-stressed rice leaves

	Quantitative RT-PCR		Microarray data	
	1 h	24 h	1 h	24 h
α-Tubulin	0.1	−0.1	0.0	−0.3
Catalase	**−1.3**	−0.9	**−1.4**	−0.9
Fd-GOGAT	−0.6	−0.9	**−1.1**	**−1.2**

Fold changes are shown as $\log_2$ value of the ratio of transcript abundances in stressed and control tissues, and represent average differences in three independent experiments. Significantly regulated genes (twofold changes) are indicated in bold

Discussion

Comparative transcriptome analyses of rice and barley tissues in this study show that the initial responses of the two species to salt stress are highly divergent. Despite the conservation of gene content among various plant species, only a few reports are available on heterologous probing of expression microarrays (Negishi et al. 2002; van Zyl et al. 2002; Horvath et al. 2003; Taji et al. 2004). An important factor that influences the kinetics of heterologous hybridization and hence the utility of heterologous arrays is the degree to which nucleotide sequences are conserved between the two species. In this study, we determined the efficiency of heterologous hybridization by computing Spearman correlation values for each probe–target combination. We observed high correlation values between homologous and heterologous probing. This is to be expected given a high degree of nucleotide conservation between barley and rice genes (Dubcovsky et al. 2001). Negishi et al. (2002) also reported on barley–rice heterologous transcriptome analysis under Fe-deficiency condition, using barley mRNA and rice cDNA array. Northern, quantitative-PCR and western blot analyses validated the differential expression of genes identified from heterologous microarray hybridization.

The differential regulation of a relatively larger number of genes in barley roots than in rice roots after 1 h under salt stress suggests that barley root cells are more responsive to salt stress signals. Moreover the divergent transcript profiles between rice and barley reflect fundamental differences in their biological responses to salt stress. For example, transcripts of P5CS (proline synthesis) and BADH2 (betaine synthesis) are up-regulated in barley but not in rice (Table 1, Supplementary Table 1). These changes suggest that barley might gain an advantage from the early production of

two osmoprotectants, proline and betaine. Furthermore, the down-regulation of water channel 1 and 2 genes was observed only in barley. Water channel 1 is equivalent to the gene encoding plasma membrane-localized water channel HvPIP2;1 (Katsuhara et al. 2002). Down-regulation of these water channels in barley could have led to a reduction in water permeability across the plasma membranes. Thus, coordinated regulation of early synthesis of osmoprotectants with regulation of water permeability could be responsible for the reversal of the initial decline in leaf water potential under salt stress in barley (Fig. 2). This reversal was not seen in rice. PMP3 and inorganic pyrophosphatase were up-regulated under salt stress. However, these expressions were only transient up-regulations in rice (Table 2). In budding yeast, disruption of PMP3 gene leads to a salt-hypersensitive phenotype because of excess accumulation of Na^+ (Navarre and Goffeau 2000; Inada et al. 2005). In our previous report, plant PMP3 can restore sensitivity to salt stress in pmp3-deficient yeast and overexpression of plant PMP3 gene conferred salt tolerance in yeast (Inada et al. 2005). This suggested that PMP3 protein has an important role in maintaining cellular ion homeostasis in plants. Inorganic pyrophosphatase also plays a role in the determination of salt tolerance (Gaxiola et al. 2001). Unlike in barley roots, however, the transcript levels of PMP3 and inorganic pyrophosphatase returned to pre-stress levels in rice roots by 24 h under stress, indicating that rice roots may not be able to sustain these mechanisms of cellular ion homeostasis. These results indicate that coordinated regulation of genes involved in osmolyte synthesis and ion homeostasis could have enabled early adaptation of barley to salt stress, and may cause difference in Na^+ accumulation pattern in roots between barley and rice during initial 24 h of salt stress. The signals responsible for such early responses in barley, however, could not be established in this study. Nevertheless, the up-regulation of transcripts of ABA inducible protein, kaurene synthase and *axi1* after 24 h of salt stress in barley roots (Supplementary Table 1) is indicative of the role of hormones as secondary messengers in at least some of the early salt responses. Previously, some papers reported expression profilings in rice (Kawasaki et al. 2001) and barley (Ozturk et al. 2002) under salt stress. In comparison with our results, some genes were similarly regulated by salt stress, such as up-regulation of P5CS and down-regulations of metallothionein and HSP70. Similar to our results, induction of LTI6B expression, a homolog of PMP3 gene, was transiently found in the transcriptome of a salt-tolerant rice, Pokkali, but not in a salt-sensitive rice IR29 (Kawasaki et al. 2001). Therefore, strong expression of PMP3 gene under salt stress might be a feature in barley, but not in rice.

Our expression data suggest that fundamental processes, such as photorespiration, glycolysis and ATP synthesis, are suppressed under salt stress in both the species. For example, the down-regulation of Fd glutamate synthase and Fd-GOGAT in leaves suggests that biosynthesis of glutamate, and the respiratory N cycle might be impaired by salt stress. The down-regulation of transketolase, fructose bisphosphatase, cytosolic phosphogluconate dehydrogenase and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) in rice but not in barley indicates that shunting of glycolysis through the pentose phosphate pathway is affected in rice. Since the pentose phosphate pathway is a major source of cellular NADPH without ATP consumption, down-regulation of the pentose shunt might affect active sequestering of salts in rice cells as judged by their slower accumulation of sodium salt when compared to barley tissues. Nevertheless, the coordinated down-regulation of PEP carboxylase and GAPDH, and up-regulation of phosphoglycerate dehydrogenase, asparagine synthetase, methionine synthetase, tryptophan synthetase, P5CS and amino acid permease in barley indicates that pre-stress level glycolytic intermediates are used for synthesis of amino acids under salt stress. These results imply a significant role for carbon and nitrogen partitioning in adapting to salt stress in barley.

To understand the differences in salt stress responses between barley and rice, we compared the changes in sodium content and leaf water potential during the early phase under salt stress. The diploid nature and a high degree of nucleotide sequence conservation between rice and barley enabled us to perform heterologous hybridization and directly compare the salt responses in the two species. Our results showed that barley and rice regulate different sets of genes during the early phase under stress, reflecting their divergence in biological responses. These differentially regulated genes in barley and rice could serve as 'candidate genes' for further improvement of salt tolerance in rice.

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