Journal of Experimental Botany, Vol. 55, No. 406, pp. 2213–2218, October 2004
DOI: 10.1093/jxb/erh242 Advance Access publication 10 September, 2004

Journal of Experimental Botany

RESEARCH PAPER

Osmotic stress in barley regulates expression of a different set of genes than salt stress does

Akihiro Ueda[1], Arumugam Kathiresan[2], Mayumi Inada[1], Yukio Narita[1], Toshihide Nakamura[1], Weiming Shi[1,3], Tetsuko Takabe[1] and John Bennett[2,*]

[1] *Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan*

[2] *Plant Breeding, Genetics and Biochemistry Division, International Rice Research Institute, DAPO Box 7777, Metro Manila, Philippines*

[3] *State Key Laboratory of Soil and Sustainable Agriculture, Institute of Soil Science, Chinese Academy of Sciences, Nanjing 210008, People's Republic of China*

Received 2 February 2004; Accepted 7 July 2004

Abstract

Under high salt conditions, plant growth is severely inhibited due to both osmotic and ionic stresses. In an effort to dissect genes and pathways that respond to changes in osmotic potential under salt stress, the expression patterns were compared of 460 non-redundant salt-responsive genes in barley during the initial phase under osmotic versus salt stress using cDNA microarrays with northern blot and real-time RT-PCR analyses. Out of 52 genes that were differentially expressed under osmotic stress, 11, such as the up-regulated genes for pyrroline-5-carboxylate synthetase, betaine aldehyde dehydrogenase 2, plasma membrane protein 3, and the down-regulated genes for water channel 2, heat shock protein 70, and phospholipase C, were regulated in a virtually identical manner under salt stress. These genes were involved in a wide range of metabolic and signalling pathways suggesting that, during the initial phase under salt stress, several of the cellular responses are mediated by changes in osmotic potential.

Key words: Barley, cDNA microarray, gene expression, osmotic stress, salt stress.

Introduction

Water is one of the most essential elements for all living organisms. In plants, transpiration of water is useful in preventing temperature increases. In grass plants in particular, more than 90% of water uptake from the soil is consumed by transpiration during the hot summer season. Therefore, plant growth is severely inhibited under water stress conditions (Yeo *et al*., 1991; Pérez-Alfocea *et al*., 1993). Water stress, caused by drought and/or soil salinity, triggers decreasing cell turgor pressure, and then wilting. To enable water uptake from the soil under water stress conditions, plants synthesize various kinds of osmoprotectants, such as glycinebetaine and proline (Gorham *et al*., 1985; Delauney and Verma, 1993). It is considered that the accumulation of osmoprotectants leads to improving osmotic stress tolerance (Kishor *et al*., 1995). The effectiveness of osmotic adjustment by osmoprotectants was proved using transgenic plants, which accumulated higher concentrations of proline, glycinebetaine, or pinitol (Kishor *et al*., 1995; Sheveleva *et al*., 1997; Takabe *et al*., 1998). On the other hand, it was reported that the regulation of water permeability through water channels also modified the sensitivity to water stress (Aharon *et al*., 2003). Thus, osmotic adjustments by both the accumulation of osmoprotectants and the regulation of water permeability play an important role for tolerance toward water stress.

cDNA microarray has been developed as a tool for comprehensive expression analysis, providing information on gene expression profiling. Many papers using microarray technology have described changes in the transcriptome of model plants, especially *Arabidopsis* and rice, in response to salt stress (Kawasaki *et al*., 2001; Seki *et al*., 2002). Compared with these model plants, barley, a major crop plant, is a moderate salt-tolerant species and thus is a good target with which to study the mechanisms of salt

* To whom correspondence should be addressed. Fax: +63 2 845 0606. E-mail: J.BENNETT@CGIAR.ORG

tolerance in crop plants. However, until now, only one paper has reported using barley cDNA microarrays for transcriptome analysis (Öztürk *et al*., 2002). A customized cDNA microarray was prepared previously using 460 kinds of barley salt-responsive genes obtained by differential display under long-term salt-stress conditions, and the transcriptomes in leaves and roots were investigated during the initial phase of salt stress (A Ueda *et al*., unpublished data).

Generally, salt stress causes both osmotic stress and ionic stress. Under salt stress, osmotic stress is triggered by an excess of salt in the soil, and ionic stress is caused by the over-accumulation of salt in the cells. These stresses individually affect the physiological status (Lefèvre *et al*., 2001; Ueda *et al*., 2003). During the initial phase of salt stress, osmotic stress is dominant in the inhibition of plant growth. In this work, in order to distinguish the effects of osmotic stress and ionic stress on gene expression, induced genes were studied in barley leaves and roots during the initial phase of osmotic stress using the barley customized cDNA microarray, previous results on expression profiling under salt stress were compared (A Ueda *et al*., unpublished data), and then the osmotic stress-specific gene expression was identified.

Materials and methods

Plant material and stress treatments

Barley (*Hordeum vulgare* L. cv. Haruna-nijyo) seedlings were grown hydroponically in half-strength Hoagland solution with doubled iron concentration under 13 h light phase (light intensity 400 μmol m^{-2} s^{-1}, 25 °C, humidity 70%)/11 h dark phase (22 °C, humidity 75%). Osmotic stress was applied to 15-d-old seedlings with three leaves by Hoagland solution containing polyethylene glycol (PEG, average molecular weight 6000). The final concentration of PEG (approximately 20% w/v) was adjusted with the PotentiaMeter to be osmotically equivalent to that of 200 mM NaCl. Barley leaves and roots were harvested at 1 h and 24 h after the stress treatments. Stressed leaves and roots were stored at −80 °C until RNA extraction.

Measurement of leaf water potential

Changes in leaf water potential under osmotic stress and salt stress were monitored using the PotentiaMeter (Decagon, Pullman, MA). The 2nd leaf blades were used to determine leaf water potential. Leaf blades were cut 3 cm in length, set into the attached chamber, and then sealed with parafilm. Prior to measurement, the leaves were incubated in the chamber for 30 min to equilibrate the ambient water potential at 25 °C.

Monitoring barley transcriptome

The barley cDNA microarray was prepared using 460 salt-responsive genes obtained by differential display (Ueda *et al*., 2002; T Takabe *et al*., unpublished data), and included non-plant cDNAs (Array Control™, Ambion, Austin, TX) as external standards for normalizing the signal intensities among different slides. The cDNA inserts of 460 clones were amplified by 50 cycles of PCR (94 °C for 1 min, 54 °C for 1 min, and 72 °C for 2 min). PCR was performed in a solution containing 1× PCR buffer, 0.2 mM dNTPs, 250 nM M13 forward and reverse primers, 200 ng of plasmid DNA, and 2.5 U of *Taq* polymerase (Perkin-Elmer Life Science, Boston, MA). After ethanol precipitation, the concentration of PCR products was adjusted to between 150 ng μl^{-1} and 250 ng μl^{-1} in 50% (v/v) dimethylsulphoxide. DNA was spotted in quadruplicate on aminosilane-coated slides (GAPSII Coated Slide; Corning, Acton, NY) using the GeneTAC G3 arrayer (Genomic Solutions, Ann Arbor, MI). Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA). Cy5- (control) and Cy3- (stressed) labelled cDNAs were synthesized by reverse transcribing 75 μg total RNA using MICROMAX Direct Labeling Kit (Perkin-Elmer Life Science). 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. Hybridized slides were washed in 1× SSC, 0.2% SDS at 55 °C for 20 s twice, in 0.1× SSC, 0.2% SDS at 55 °C for 20 s twice, and finally in 0.1× SSC at 25 °C for 20 s twice. Each hybridization was repeated at least three times (technical replicates) using RNA from at least three biological replicates. After hybridization, the slides were scanned using GeneTAC™ LS IV laser scanner and analysed using the Integrator Analyzer 3.3 software (Genomic Solutions). The signal intensities of elements in each slide were normalized globally. The significance of differential regulation was measured statistically by the ANOVA *F*-test, available in the SAS package.

Northern blot analysis and real-time RT-PCR

Differential regulation of selected genes was validated by performing northern blot analysis as described previously (Ueda *et al*., 2001). For real-time RT-PCR analysis, first strand cDNA was synthesized from 5 μg total RNA using SuperScriptIII reverse transcriptase (Invitrogen). Quantitative PCR was performed with LightCycler using the LightCycler-FastStart DNA Master SYBR Green I kit (Roche, Basel, Switzerland). All PCR experiments were performed with 3 mM $MgCl_2$. Specific primers were as follows: Actin forward (5′-AGACCTTCAACACCCCTGCTATGT-3′) and reverse (5′-CCAATCCAGACACTGTACTTCCTT-3′); GAPDH forward (5′-TTTCGGAAGGATCGGGAG-3′) and reverse (5′-ATCAGGTCGACAACACGGTT-3′); phosphogluconate dehydrogenase forward (5′-ATTATCCGGGCAAGGTTTCTT-3′) and reverse (5′-CCATAGAACCTGAAGCTACA-3′). Fold changes were estimated by the expression value of actin as an internal standard and transferred to $\log_2$ ratio (osmotic stress/control). Real-time RT-PCR experiments were repeated using total RNA from three biological replicates.

Results and discussion

Physiological status of barley under osmotic stress

Under salt or drought stress, plant tissues are severely dehydrated, with a consequent decrease in leaf water potential. Hence, leaf water potential is often used as a parameter of water stress (Ueda *et al*., 2003). To compare the physiological status of barley under osmotic stress and salt stress, the changes in leaf water potential were monitored after both treatments. By 1 h of PEG treatment (approximately 20% w/v), leaf water potential declined from −0.52 to −0.89 MPa (Fig. 1). Although the lowest value was observed at 10 h (−1.45 MPa), leaf water potential was restored to −0.78 MPa after 24 h of stress treatment. The barley plants were also subjected to salt stress (200 mM NaCl) which was the same osmotic potential as the PEG solution. During the first 10 h, a similar decrease in leaf water potential was observed under osmotic and salt stress

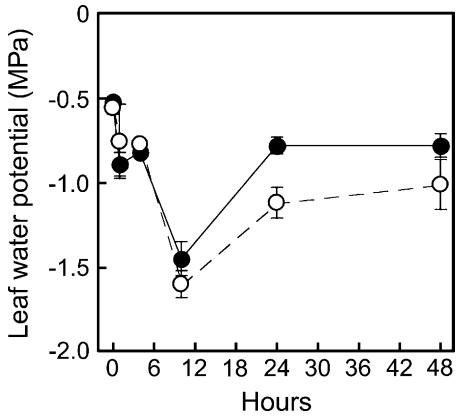

Fig. 1. Changes in leaf water potential under osmotic stress (closed circles) and salt stress (open circles). Values are the means ±SE of 5–8 samples.

conditions (Fig. 1). This indicated that barley plants suffered from the same degree of water stress under either PEG or salt stress during the first 10 h. However, recovery was greater under osmotic stress than under salt stress after 24 h of treatment, suggesting Na^+ and/or Cl^- may exert additional osmotic effects.

Identification of osmotic stress responsive candidates

Four hundred and sixty non-redundant salt-responsive ESTs were arrayed on glass slides and changes in their abundance in response to osmotic stress were monitored. Fifty-two genes showed differential expression under osmotic stress during the first 24 h (Table 1). During the same period, however, 92 genes were differentially expressed under salt stress (A Ueda *et al*., unpublished results). As seen in Fig. 2, transcript levels of 18 of the up-regulated genes under salt stress were also up-regulated under osmotic stress. Sixteen genes also showed down-regulation under both osmotic and salt stress conditions. However, a total of 18 genes showed different expression patterns under osmotic stress than under salt stress (Table 1). Based on the mode of regulation and the stimulus to which they responded, differentially expressed genes were classified into six groups (Table 1).

The up-regulation of 18 genes (Group 1) was observed by both salt and osmotic stress. Of these, the pattern of change in induction was close to identical for 4: PDR (Pleiotropic Drug Resistance) 5-like ABC (ATP Binding Cassette) transporter, lipoxygenase, probable serine/threonine kinase, and putative cytochrome P450. With these genes it seems safe to say that the osmotic component of salt stress is responsible for the effect. With the other 14 of this set, there are enough differences between either the location (leaves versus roots), extent, or timing, between salt and osmotic stress to indicate that the two types of stress are not causing an identical response. The four definite cytochrome P450s, and the two proline-rich protein genes show a much greater induction due to salt than to osmotic stress; with these it seems likely that salt stress includes an osmotic component, but then has a further inductive effect as well. Sixteen genes (Group 2) were suppressed by both salt and osmotic stress. The patterns were close enough to be considered identical between the two stress conditions for six of these: thiamine biosynthetic enzyme, proline transporter, actin protein, Fd-GOGAT, HSP (Heat Shock Protein) 17.9, and phospholipase C. The differences in location and/or timing of the decrease in the other 10 were sufficient to make it uncertain whether the salt effect was due entirely to its osmotic component. With three genes (hypothetical protein, sucrose synthase and alanine aminotransferase in Group 3) and nine genes (HSP70, translation elongation factor eEF-1 and eEF-2, sugar transporter, etc. in Group 4), there was a clear increase and decrease in activity due to osmotic stress at one point or another, but not by salt stress. Group 5 has one gene, GAPDH (glyceraldehyde-3-phosphate dehydrogenase), which is increased in the roots at 24 h by osmotic stress, but not by salt stress. On the other hand, it is suppressed in the roots at 1 h by salt stress, but not by osmotic stress. Obviously the two imposed stress conditions are acting differently on this gene. By contrast, the five genes (ORF122, 235 amino acids long hypothetical, three no homologues) in Group 6 are suppressed in the roots at 1 h due to osmotic stress, but increase in the roots at 24 h due to salt stress.

Co-ordinate up-regulation of the genes involved in amino acid biosynthesis, such as those for serine (phosphoglycerate dehydrogenase), methionine (methionine synthase), proline (pyrroline-5-carboxylate synthetase, P5CS), and asparagine (asparagine synthetase); and down-regulation of genes encoding phosphoenolpyruvate carboxylase, Fd-GOGAT, and phosphogluconate dehydrogenase under both osmotic and salt-stress conditions was observed. Although up-regulation differs in significant detail between PEG- and salt-induced stress, this does not completely rule out the possibility that salt stress-induced amino acid syntheses from glycolytic intermediates in plant cells might be mediated by the osmotic component of the saline condition.

Interestingly, however, up-regulation of sucrose synthase and alanine aminotransferase, and down-regulation of proline and sugar transporters were observed only under osmotic stress, not under salt stress. These results suggest that plant cells remobilize the carbon and nitrogen sources differently under these two stress conditions.

It was reported that PMP (Plasma Membrane Protein) 3 mutation causes Na^+ over-accumulation in yeast cells under salt stress (Navarre and Goffeau, 2000) indicating one of its functions should be in preventing damage due to sodium ions. However, with barley it was observed that PMP3 expression was more highly induced by 1 h of osmotic stress than it was by salt stress (Table 2; Fig. 3), although the effects are reversed at 24 h. The gene encoding salT was

Table 1. *Changes in transcriptome of barley leaves and roots under osmotic stress and salt stress*

The mean values were transformed to $\log_2$ ratio. Significant difference in relative level is shown in bold.

Accession number	Gene name	Osmotic stress				Salt stress			
		Leaves		Roots		Leaves		Roots	
		1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h
Group 1: Osmotic- and salt-induced candidates									
AU252354	Probable phosphoglycerate dehydrogenase	**1.3**	−0.4	0.7	0.0	**1.3**	0.2	**1.7**	0.8
AU2524307	PDR5-like ABC transporter	**1.0**	0.8	0.7	0.5	**1.3**	0.6	0.5	0.7
AU312385	Lipoxygenase	0.0	**1.9**	0.5	0.4	−0.3	**1.1**	0.2	0.3
AB164396	Methionine synthase	0.9	**1.3**	0.7	0.9	0.7	**1.5**	**1.1**	0.9
AU312370	P5CS	0.1	**1.1**	0.8	0.8	0.8	**2.5**	0.5	**1.6**
AU312412	PMP3	0.1	0.6	**2.6**	**1.2**	0.3	**1.1**	**1.5**	**2.3**
AU252393	Putative growth regulator (axi 1)	0.2	0.1	**1.1**	0.2	0.8	0.1	0.9	**1.2**
AU312378	Probable serine/threonine kinase	0.0	0.5	**1.4**	0.8	0.0	−0.7	**1.4**	0.1
AU252308	Cytochrome P450 CYP99A1	0.1	0.1	0.7	**2.5**	0.5	−0.1	−0.3	**4.2**
AU252311	Cytochrome P450 CYP99A1	0.3	−0.2	0.7	**2.2**	0.8	−0.1	−0.4	**4.0**
AU252310	Cytochrome P450 CYP99A1	0.0	0.0	0.7	**2.2**	0.3	−0.1	−0.4	**3.8**
AU252315	Cytochrome P450 CYP99A1	0.2	−0.4	0.7	**2.1**	0.6	−0.3	0.2	**3.7**
AU252379	Proline-rich protein	0.3	0.0	0.5	**1.6**	0.9	0.1	0.4	**4.4**
AU252400	Proline-rich protein	−0.3	0.3	−0.2	**1.2**	0.0	−0.2	−0.1	**3.5**
AU312389	Asparagine synthetase (glutamine-hydrolysing)	0.3	0.4	0.6	**1.2**	0.9	**1.3**	0.8	**1.8**
AU252316	Putative cytochrome P450	0.0	0.0	0.6	**1.1**	0.7	−0.2	−0.3	**2.3**
AU312411	BADH2 3′-UTR	−0.2	0.9	0.1	**1.0**	−0.2	**1.4**	0.1	0.9
AU312393	Aldehyde dehydrogenase	0.2	0.4	0.1	**1.0**	0.7	**1.8**	0.3	**1.4**
Group 2: Osmotic- and salt-suppressed candidates									
AU312363	Thiamine biosynthetic enzyme	**−1.2**	−0.9	**−1.1**	**−1.2**	**−1.3**	**−1.5**	−0.9	**−1.5**
AU312405	Water channel 2	**−1.2**	**−1.4**	**−1.0**	−0.7	**−1.2**	−0.8	**−1.3**	**−1.7**
AU252365	Phosphoenolpyruvate carboxylase	**−1.2**	−0.9	−0.7	0.1	−0.9	**−2.1**	−0.5	0.0
AU312476	No homologue	**−1.1**	−0.8	−0.4	−0.3	−0.2	**−1.2**	0.2	0.2
AU312414	Dnak like	**−1.0**	**−1.5**	−0.9	−0.9	−0.2	**−1.3**	−0.5	−0.4
AB073084	Proline transporter	−0.5	**−1.5**	0.5	−0.5	−0.1	**−1.8**	0.8	0.8
AU312419	Putative HSP70	−0.7	**−1.5**	−0.8	−0.8	−0.3	−0.9	**−1.3**	−0.6
AU312400	Actin protein	−0.7	**−1.3**	−0.5	−0.4	−0.6	**−1.2**	−0.2	−0.1
AU252353	Fd-GOGAT	−0.9	**−1.2**	−0.1	−0.4	−0.8	**−1.0**	0.4	−0.1
AU312407	Hypothetical protein F10M23.190	**−1.0**	**−1.2**	**−1.3**	**−0.6**	−0.8	**−1.2**	−0.6	**−1.0**
AU312477	HSP17.9	−0.7	**−0.2**	**−2.9**	0.1	−0.6	−0.8	**−1.7**	0.2
AU312362	Phospholipase C	0.0	0.0	**−1.7**	−0.5	0.7	−0.1	**−1.2**	−0.7
AU252355	Cytosolic phosphogluconate dehydrogenase	−0.3	−0.7	**−1.5**	−0.8	−0.2	−0.9	**−1.7**	**−1.3**
AU312401	Subtilisin	−0.9	**−1.0**	**−1.2**	−0.8	**−1.3**	**−1.5**	−0.9	**−1.3**
AU312387	SalT	0.2	−0.1	**−1.1**	−0.4	0.7	−0.2	**−1.0**	−0.9
AU312374	Dihydroorotate dehydrogenase	−0.4	0.7	**−1.1**	−0.4	−0.6	0.0	−0.8	**−1.6**
Group 3: Osmotic-stress induced candidates									
AU312381	Hypothetical protein	−0.1	**1.2**	−0.5	0.8	−0.5	0.2	−0.2	−0.4
AU312418	Sucrose synthase	−0.2	0.1	0.3	**1.5**	−0.2	−0.5	0.3	0.3
AU252360	Alanine aminotransferase	−0.1	−0.5	0.5	**1.1**	0.1	−0.7	0.1	0.8
Group 4: Osmotic-stress suppressed candidates									
AU252330	HSP70	−0.7	**−1.3**	−0.3	−0.9	0.1	−0.6	−0.2	−0.4
AU312386	Translation elongation factor eEF-1 α chain	−0.7	**−1.1**	−0.4	−0.7	−0.4	−0.4	−0.1	0.1
AB073084	Proline transporter 3′-UTR	−0.2	**−1.1**	0.5	−0.4	0.0	−0.8	0.4	0.5
AU312388	Translation elongation factor eEF-2	−0.7	**−1.1**	−0.4	−0.3	−0.5	−0.6	0.0	0.3
AU312403	Hypothetical protein F10M23.360	−0.6	**−1.0**	−0.3	−0.4	−0.5	−0.8	−0.1	−0.5
AU252318	Cytochrome P450	0.2	0.1	**−1.1**	−0.3	0.6	0.1	−0.2	−0.5
AU252395	GcpE protein	0.0	0.1	**−1.1**	0.2	0.6	−0.3	0.8	0.7
AU312359	Probable sugar transporter protein	−0.9	−0.5	−0.6	**−1.2**	−0.8	−0.8	−0.5	−0.8
AU252281	Probable serine/threonine protein kinase NAK	−0.1	−0.6	−0.1	**−1.2**	0.1	−0.5	−0.1	0.0
Group 5: Osmotic-stress induced, salt-stress suppressed candidates									
AU252367	Glyceraldehyde 3-phosphate dehydrogenase	−0.7	0.2	−0.6	**1.1**	**−1.1**	0.0	−0.7	0.1
Group 6: Osmotic-stress suppressed, salt-stress induced candidates									
AU312465	ORF122	0.4	0.2	**−1.7**	0.4	**−1.0**	−0.1	0.1	**1.6**
AU312463	235aa long hypothetical	0.2	0.1	**−1.3**	−0.2	0.8	−0.1	0.1	**1.2**
AU312462	No homologue	0.0	0.3	**−1.2**	0.3	0.6	−0.2	**1.0**	**1.0**
AU312473	No homologue	0.2	0.1	**−1.1**	0.1	0.8	−0.2	0.2	**1.5**
AU312470	No homologue	0.1	0.1	**−1.0**	0.1	0.8	−0.1	−0.1	**1.3**

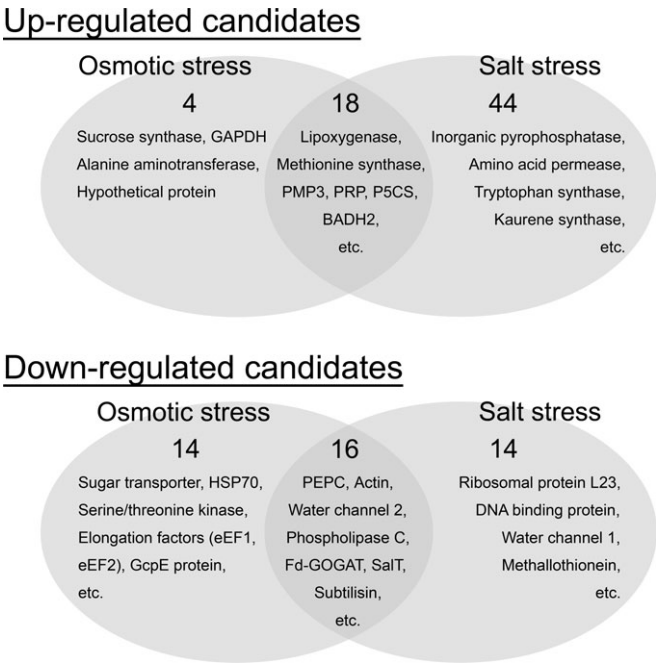

Fig. 2. Changes in transcriptome under osmotic stress and salt stress. The consensus and number of ESTs that are differentially regulated under osmotic stress and salt stress were identified from microarray data.

Table 2. *Quantification of expression levels of GAPDH and phosphogluconate dehydrogenase genes in barley roots under osmotic stress by real-time RT-PCR*

Fold changes were estimated by the expression value of actin as an internal standard, and transferred to $\log_2$ ratio. Significant changes are shown in bold.

Accession number	Gene name	Real-time RT-PCR		Microarray	
		1 h	24 h	1 h	24 h
AU252367	GAPDH	0.0	**1.0**	−0.6	**1.1**
AU252355	Phosphogluconate dehydrogenase	**−1.0**	−0.7	**−1.5**	−0.8

strongly induced by salt stress in rice (Moons *et al*., 1997). In contrast to rice, where expression of salT, is induced by salt, the salT gene was suppressed in barley roots under both stresses.

Validation of microarray experiments

The microarray data were validated by performing northern blot analysis for a few randomly selected genes. As seen in Fig. 3, the mRNA abundance of genes encoding PMP3 and PRP (proline rich proteins) was higher under salt and osmotic stresses, (although the effect was much larger with NaCl than with PEG). The transcript level of phospholipase C in roots was down-regulated by both stress treatments (Fig. 3). These expressions were similarly regulated by osmotic and salt stresses. Up-regulation of hypothetical protein, a candidate in Group 3 in Table 1, was confirmed

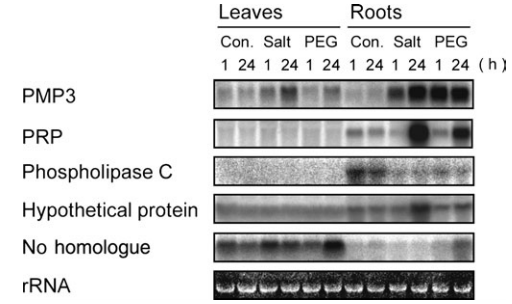

Fig. 3. Validation of microarray data by northern blot analysis [PMP3 (AU312412) and PRP (AU252379) in Group 1, phospholipase C (AU312362) in Group 2, hypothetical protein (AU312381) in Group 3, no homologue (AU312462) in Group 6]. ESTs that showed differential regulation under osmotic stress were hybridized to RNA gel blot containing 10 μg of total RNA per lane. Ethidium bromide staining of rRNA is shown at the bottom. Hybridization and washing were carried out at 65 °C. Salt stress was applied to barley plants by 200 mM NaCl solution. Osmotic stress was given by PEG solution adjusted to the same osmotic potential as that of the 200 mM NaCl solution.

by northern blot analysis. On the other hand, expression of the no homologue protein gene was up-regulated under salt stress, but not osmotic stress. In addition, the microarray data were also evaluated by real-time RT-PCR (Table 2). The expression levels of GAPDH (one of the up-regulated candidates) and phosphogluconate dehydrogenase (one of the down-regulated candidates) were examined by real-time RT-PCR in osmotically stressed-barley roots. The results for real-time RT-PCR analyses showed that fold changes of both GAPDH and phosphogluconate dehydrogenase were also identical to the microarray data.

Information on the transcriptome in barley under drought stress was reported by Öztürk *et al*. (2002). Drought stress and PEG-mediated osmotic stress cause dehydration from plant tissues, but they are essentially different type of stresses. Notwithstanding, some of the typical stress responsive genes, such as P5CS, lipoxygenase, and aldehyde dehydrogenase, were up-regulated in both studies. Interestingly, they reported the up-regulation of a kind of water channel gene (WCP-IV) in barley roots after 10 h of drought stress (Öztürk *et al*., 2002). This study's array carried two genes for water channels in barley, and neither gene was up-regulated under either osmotic or salt stress. Water permeability is one of the important determinants for stress tolerance in plants (Aharon *et al*., 2003), therefore, further study including the analysis of tissue-specific expression should provide useful information.

Crosstalk between salt and osmotic signals under salt stress

Among the 62 genes that showed up-regulation under salt stress (A Ueda *et al*., unpublished results), 18 were up-regulated by osmotic and salt stress conditions (Fig. 2). Sixteen of the 30 salt-repressed genes showed down-regulation under both the stress treatments. However, even among these groups, there were many differences in

location, or timing, or extent of the induction; and only four of the up-regulated and six of the down-regulated genes had a truly similar pattern under both stress conditions. The others, and especially the genes listed in Groups 3, 4, 5, and 6, were subject to differential regulation due possibly to ionic and/or other secondary stresses caused by salt. It is likely that common regulatory networks and/or signalling intermediates govern the expression of genes that are regulated equally by both the stress stimulus (Shinozaki and Yamaguchi-Shinozaki, 1998). The magnitude of expression of such genes was similar under both the stress conditions with the exception of genes encoding cytochrome P450 and PRP. This result suggests that these genes are regulated by the two stress cues. Further understanding of such interactions should become feasible with the availability of regulatory sequences of genes. Nevertheless, the diversity in biological functions of these commonly regulated genes suggests that several of the downstream responses of salt and osmotic stresses may also be shared.

The present study revealed 18 genes (Groups 3–6) that are differentially regulated by only osmotic stress, but not salt stress. This indicates that the changes in expression levels expected from the osmotic component of salt stress, may have been suppressed by other salt-mediated signals. Such differences in transcript profile might reflect the adaptive values of biochemical pathways under different stress conditions. For example, osmotic stress triggered up-regulation of P5CS and down-regulation of the proline transporter in leaf tissues under osmotic stress (Table 1; Groups 1 and 4). The transcript for the proline transporter gene was abundant in the root tip region, especially the root cap and cortex cells (Ueda *et al*., 2001); and proline made in leaves may be translocated to the root tip region. Coordinate regulation was observed in expressions of sucrose synthase and sugar transporter genes in root tissues. Up-regulation of sucrose synthase and down-regulation of the sugar transporter were triggered by osmotic but not salt stress (Table 1; Groups 3 and 4), hence, this would be a good target to dissect further signalling controls for the differentiation of osmotic stress from ionic stress.

In this study, genes were identified that are regulated by osmotic stress under salt stress by comparing the expression profiles of 460 salt-responsive EST genes using microarrays. These results demonstrate that a number of genes that are regulated under salt stress are mediated by osmotic stress caused by excessive salts. A dissection of osmotic stress-mediated responses under salt stress might accelerate the genetic improvement of salt tolerance in target environments.

Acknowledgements

This research was supported by a Grant 'Research for the future' to TT and a Research Fellowship of JSPS for Young Scientist to AU. We are greatful to Dr AT Jagendorf (Cornell University) for critical reading of the manuscript and valuable discussions.

References

Aharon R, Shahak Y, Wininger S, Bendov R, Kapulnik Y, Galili G. 2003. Overexpression of a plasma membrane aquaporin in transgenic tobacco improves plant vigor under favorable growth conditions but not under drought or salt stress. *The Plant Cell* **15,** 439–447.

Delauney AJ, Verma DP. 1985. Proline biosynthesis and osmoregulation in plants. *The Plant Journal* **4,** 215–223.

Gorham J, Jones RGW, McDonnell E. 1985. Some mechanisms of salt tolerance in crop plants. *Plant and Soil* **89,** 15–40.

Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ. 2001. Gene expression profiles during the initial phase of salt stress in rice. *The Plant Cell* **13,** 889–905.

Kishor P, Hong Z, Miao GH, Hu C, Verma DP. 1995. Overexpression of (delta)-pyrroline-5-carboxylate synthetase increases proline production and confers osmotolerance in transgenic plants. *Plant Physiology* **108,** 1387–1394.

Lefèvre I, Gratia E, Lutts S. 2001. Discrimination between the ionic and osmotic components of salt stress in relation to free polyamine level in rice (*Oryza sativa*). *Plant Science* **161,** 943–952.

Moons A, Prinsen E, Bauw G, Van Montagu M. 1997. Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *The Plant Cell* **9,** 2243–2259.

Navarre C, Goffeau A. 2000. Membrane hyperpolarization and salt sensitivity induced by deletion of PMP3, a highly conserved small protein of yeast plasma membrane. *The European Molecular Biology Organization Journal* **19,** 2515–2524.

Öztürk ZN, Talamé V, Deyholos M, Michalowski CB, Galbraith DW, Gozukirmizi N, Tuberosa R, Bohnert HJ. 2002. Monitoring large-scale changes in transcript abundance in drought- and salt-stressed barley. *Plant Molecular Biology* **48,** 551–573.

Pérez-Alfocea F, Estañ MT, Caro M, Guerrier G. 1993. Osmotic adjustment in *Lycopersicon esculentum* and *L. pennellii* under NaCl and polyethylene glycol 6000 iso-osmotic stresses. *Physiologia Plantarum* **25,** 493–498.

Seki M, Narusaka M, Ishida J, *et al*. 2002. Monitoring the expression profiles of 7000 *Arabidopsis* genes under drought, cold, and high-salinity stresses using a full-length cDNA microarray. *The Plant Journal* **31,** 279–292.

Sheveleva E, Chmara W, Bohnert HJ, Jensen RG. 1997. Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiology* **115,** 1211–1219.

Shinozaki K, Yamaguchi-Shinozaki K. 1998. Molecular responses to water stress in *Arabidopsis thaliana*. *Journal of Plant Research* **111,** 345–351.

Takabe T, Nakamura T, Nomura M, Hayashi Y, Ishitani M, Muramoto Y, Tanaka A, Takabe T. 1998. Glycinebetaine and the genetic engineering of salinity tolerance in plants. In: Satoh K, Murata N, ed. *Stress responses of photosynthesis organisms*. Amsterdam: Elsevier Science, 115–131.

Ueda A, Kanechi M, Uno Y, Inagaki N. 2003. Photosynthetic limitations of a halophyte sea aster (*Aster tripolium* L.) under water stress and NaCl stress. *Journal of Plant Research* **116,** 65–70.

Ueda A, Shi W, Nakamura T, Takabe T. 2002. Analysis of salt-inducible genes in barley roots by differential display. *Journal of Plant Research* **115,** 119–130.

Ueda A, Shi W, Sanmiya K, Shono M, Takabe T. 2001. Functional analysis of salt-inducible proline transporter of barley roots. *Plant and Cell Physiology* **42,** 1282–1289.

Yeo AR, Lee KS, Izard P, Boursier PJ, Flowers TJ. 1991. Short- and long-term effects of salinity on leaf growth in rice (*Oryza sativa* L.). *Journal of Experimental Botany* **25,** 881–889.