To identify key proteins in the regulation of salt tolerance in the mangrove plant *Bruguiera gymnorhiza*, proteome analysis of samples grown under conditions of salt stress was performed. Comparative two-dimensional electrophoresis revealed that two, three and one protein were differentially expressed in the main root, lateral root and leaf, respectively, in response to salt stress. Among these, three proteins were identified by internal peptide sequence analysis: fructose-1,6-bisphosphate (FBP) aldolase and a novel protein in the main root, and osmotin in the lateral root. These results suggest that FBP aldolase and osmotin play roles in salt tolerance mechanisms common to both glycophytes and mangrove plants. Osmotin was abundant at early time points following salt treatment and seems to play a role in initial osmotic adaptation in lateral roots of *B. gymnorhiza* under salt stress, but does not contribute towards adaptation to prolonged or continuous exposure to salt stress. The amounts of these proteins were not correlated with those of the respective mRNAs, as determined by microarray analysis. A novel salt-responsive protein, not previously detected by expressed sequence tag analysis or transcriptome analysis, was also identified in this proteomic approach, and may provide insight into the salt tolerance mechanism of the mangrove plant. This is the first report of proteome analysis with detailed analysis of main and lateral roots of mangrove plants under salt stress conditions.

**Keywords:** *Bruguiera gymnorhiza* • Mangrove • Proteome • Salt tolerance.

**Abbreviations:** 2-DE, two-dimensional gel electrophoresis; FBP, fructose-1,6-bisphosphate; LC-MS/MS, liquid chromatography–tandem mass spectrometry; OEE, oxygen-evolving enhancer protein.

**Introduction**

Salinity stress is one of the most significant limiting factors in agricultural crop productivity (Boyer 1982). Hence, improving salt tolerance in crops is essential for sustainable food production. This factor has led to research on plant responses to salt stress. Microarray analysis is a powerful tool for comprehensive examination of gene expression and has been applied to monitor the expression profiles of both glycophytes and halophytes under salt stress (Kawasaki et al. 2001, Seki et al. 2002, Tajj et al. 2004, Gong et al. 2005, Kawaura et al. 2006, Walia et al. 2006, Miyama and Hanagata 2007, Miyama and Tada 2008, Yamanaka et al. 2009). However, the amounts of proteins detected are not always correlated to the levels of expression of the respective mRNAs (Gygi et al. 1999, Chen et al. 2002, Foster et al. 2005). Moreover, many proteins undergo post-transcriptional modifications, such as removal of signal peptides, phosphorylation and glycosylation. Therefore, it is necessary to study the salt stress response at the protein level. The proteomics of various plants in response to salinity have been studied (Lee et al. 2004, Abbasi and Komatsu 2004, Kim et al. 2005, Yan et al. 2005, Jiang et al. 2007, Nohzadeh Malakshah et al. 2007, Vincent et al. 2007); however, to date, there has been no report of proteome analysis of halophytes. Sugihara et al. (2000) reported the identification of oxygen-evolving enhancer protein 1 (OEE1) as a salt-responsive protein in leaves of the mangrove plant *Bruguiera gymnorhiza* and the salt treatment-induced expression of the corresponding gene.

Root tissues are thought to play an important role in salt tolerance, because they are directly exposed to salt. The transcription factor gene *AtNAC2* was induced by salt stress, and overexpression of *AtNAC2* in transgenic *Arabidopsis* plants resulted in promotion of lateral root development (He et al. 2005). Accumulation of Na⁺ in the halophyte...
Thellungiella halophila occurred in older leaves, and the least accumulation of Na⁺ was observed in the lateral roots (Vera-Estrella et al. 2005). These results suggest that lateral root development may play an important role in plant salt tolerance.

Here we report the proteome analysis of the main and lateral roots and the leaves in salt-treated mangrove plant B. gymnorhiza, and the identification of salt-responsive proteins. The abundance of the salt-responsive proteins was compared with the salt-induced expression of the corresponding mRNAs, by using previously collected transcriptome data derived from analysis of the same plant materials (Yamanaka et al. 2009).

**Results**

**Two-dimensional gel electrophoresis (2-DE) of proteins from the main and lateral roots and the leaves of B. gymnorhiza**

Four- to five-month-old plants were treated with 500 mM NaCl for different time periods. The leaves showed wilted symptoms 1 h after treatment, but were almost recovered 24 h after treatment.

Roots of young B. gymnorhiza are classified morphologically into two types, main and lateral roots. The Cl⁻ content in salt-treated B. gymnorhiza is regulated differently in leaves and in the two types of roots, with minimum and maximum accumulation occurring in the lateral roots and leaves, respectively (Yamanaka et al. 2009). Root tissues are naturally exposed to saline conditions. Since morphological and physiological differences in these roots may play important roles in salt tolerance, we performed proteome analysis in both types of roots and in leaves from salt-treated B. gymnorhiza. Total proteins in the main and lateral roots and in the leaves of B. gymnorhiza were extracted after 0, 1, 3, 6, 12 and 24 h, and 3, 6 and 12 d of treatment with 500 mM NaCl. Proteins were then separated by 2-DE, and those protein spots showing reproducible changes were selected and their expression patterns were analyzed.

In proteins extracted from the main root, two spots (MR1 and MR2) showed changes in abundance in response to salt stress when compared with the non-stressed condition at 0 h (Figs. 1, 4A, B). Increased expression was observed for spot MR1 at 6–24 h, and maximum expression was observed at 24 h. Spot MR2 appeared after 24 h of stress treatment, reached a maximum after 3 d, and then declined.

![Fig. 1 Time-dependent changes in protein levels of B. gymnorhiza main root under salt stress conditions. Plants were treated with 500 mM NaCl, and proteins in the main roots were extracted and separated by 2-DE. At least three replicates were performed for each sample. MR1 and MR2 represent salt-responsive proteins. Spots STD10 and STD100 represent internal standard spots designated as 10 and 100 intensities, respectively.](https://academic.oup.com/pcp/article-abstract/50/3/439/1842607)
In the lateral root, three spots (LR1, LR2 and LR3) showed induced expression in response to salt stress (Figs. 2, 4C–E). Spots LR1 and LR2 appeared after 1 h treatment, reached a maximum at 3–6 h, and then declined to undetectable levels. The expression pattern of LR3 was quite different from the expression of the other salt-responsive proteins. Increased LR3 expression was observed after 12 h treatment, and remained elevated for at least 6 d.

In proteins extracted from leaves, one protein, spot LE1, was identified as a salt-responsive protein (Figs. 3, 4F). Enhanced synthesis of LE1 was detected after 3 and 6 d of salt treatment.

**Identification of salt-responsive proteins from B. gymnorhiza**

Among these six differentially responsive proteins, three with relatively high abundance (MR1, MR2 and LR1) were subjected to inner sequence analysis. Gel pieces, each containing a salt-responsive protein spot, were excised and peptide sequences were determined using a protein sequencer. Peptide sequences were used in homology searches of the NCBI non-redundant protein database (Table 1).

MR1 was identified as chloroplast-type fructose-1,6-bisphosphate (FBP) aldolase (FBP aldolase), which is involved in the Embden–Meyerhof or glycolytic pathway and reversibly catalyzes the conversion of FBP to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The LR1 peptide sequence was shown to be highly homologous to osmotin (Table 1). Finally, the peptide sequence of MR2 was determined, but no similar sequences were found by database searching (Table 1).

The amino acid sequence of LR2 was not determined. However, its molecular weight and pI were very similar to those of MR2 (Figs. 1, 2). LR3 was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and was identified as either trypsin, high-molecular weight cobalt-containing nitrile hydratase or hypothetical protein (Table 2). LE1 was not analyzed in this study because of its low abundance.

**mRNA expression of salt-responsive proteins**

We previously performed transcriptome analysis of roots from salt-treated *B. gymnorhiza* by using oligo microarray assays (Yamanaka et al. 2009, NCBI GEO accession No. GSE10942).

![Fig. 2](https://example.com/fig2.png)  
**Fig. 2** Time-dependent changes in protein levels of *B. gymnorhiza* lateral root under salt stress conditions. Plants were treated with 500 mM NaCl, and proteins in the lateral roots were extracted and separated by 2-DE. At least three replicates were performed for each sample. LR1, LR2 and LR3 represent salt-responsive proteins. Spots STD50 and STD100 represent internal standard spots designated as 50 and 100 intensities, respectively.
Fig. 3 Time-dependent changes in protein levels of *B. gymnorhiza* leaves under salt stress conditions. Plants were treated with 500 mM NaCl, and proteins in the leaves were extracted and separated by 2-DE. At least three replicates were performed for each sample. LE1 represents a salt-responsive protein. Spots STD10 and STD100 represent internal standard spots designated as 10 and 100 intensities, respectively.

Fig. 4 Changes in abundance of the differentially regulated proteins in salt-treated *B. gymnorhiza*. Relative expression level of salt-responsive proteins MR1 (A), MR2 (B), LR1 (C), LR2 (D), LR3 (E) and LE1 (F) were quantified from 2-DE images at the time points shown after salt treatment. In A and E, an asterisk indicates a significant difference from the control (values at 0 h) at *P < 0.05* by Student’s *t*-test. *n* = 3.
Fig. 5A shows the change in FBP aldolase gene (Bg01-07_B02) expression in the main roots following salt treatment. The abundance of FBP aldolase mRNA was decreased after 12 h salt treatment, then returned to initial levels by 12 d. The regulation pattern for FBP aldolase mRNA was similar in the lateral root (data not shown). Thus, the abundance of the expressed protein did not correlate with that of the mRNA.

LR1 was shown to be highly homologous to osmotin; however, the osmotin cDNA identical to the identified osmotin-like protein sequence determined in the proteome analysis was not included in the microarray. Therefore, we examined the regulation of all seven osmotin-like gene sequences [CL15Contig2 (Bg03-05_E13, BP943182), CL38Contig1 (Bg04-16_B21, BP947305), Bg03-05_F14 (BP943196), Bg04-03_O05 (BP944909), Bg04-06_I05 (BP945119), Bg04-30_D05 (DB994400) and Bg04-30_K13 (BP949905)] found on the microarray in lateral root under salt stress conditions (Fig. 5B). Among the seven clones, expression of two (Bg04-03_O05 and Bg04-06_I05) remained constant, the expression of one (Bg04-30_D05) doubled, and four (CL15Contig2, CL38Contig1, Bg03-05_F14 and Bg04-30_K13) were synchronously up-regulated 4- to 13-fold following salt treatment. The expression levels observed for the mRNAs of these genes were not correlated with that of LR1 proteins. The regulation pattern for these osmotin genes was similar in the main root (data not shown) although the up-regulation of protein spots corresponding to osmotin in the main root was not observed (Fig. 1).

**Table 1** Identification of salt-responsive protein spots

<table>
<thead>
<tr>
<th>Protein spot</th>
<th>Mol. wt</th>
<th>pl</th>
<th>Inner peptide sequences</th>
<th>Homologous protein (% homology)</th>
<th>Accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>MR1</td>
<td>35</td>
<td>5.7</td>
<td>VAPEVIAEYTV</td>
<td>FBP aldolase (100)</td>
<td>AAK62818.1</td>
</tr>
<tr>
<td>MR2</td>
<td>40</td>
<td>7.5</td>
<td>MQFSLAYGAGL</td>
<td>No hit</td>
<td>–</td>
</tr>
<tr>
<td>LR1</td>
<td>20</td>
<td>7.5</td>
<td>LFXTADIGQXPA</td>
<td>Osmotin-like protein (82)</td>
<td>CAC22342.1</td>
</tr>
</tbody>
</table>

Table 2 Identification of LR3 using LC-MS/MS

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Sequencea</th>
<th>Probability</th>
<th>MP/Cb</th>
<th>gi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypsin</td>
<td>LGHEHIDPFLGNEQFINAAK</td>
<td>100%</td>
<td>58/26%</td>
<td>gi</td>
</tr>
<tr>
<td>High-molecular weight cobalt-containing nitrile hydratase</td>
<td>VVSYEYEEEIPGMMGA</td>
<td>100%</td>
<td>35/17%</td>
<td>gi</td>
</tr>
<tr>
<td>Hypothetical protein</td>
<td>ISGILYEETR</td>
<td>97%</td>
<td>19/11%</td>
<td>gi</td>
</tr>
</tbody>
</table>

*aThe amino acid sequence of the peptide with the highest score identified by MS/MS. bThe matched peptides/the percentage of sequence coverage.

**Fig. 5** Changes in mRNA levels for FBP aldolase and osmotin genes in salt-treated *B. gymnorhiza*. Relative expression levels for (A) the FBP aldolase (Bg01-07_B02) gene in the main root and (B) osmotin (CL15Contig2, CL38Contig1, Bg03-05_F14, Bg04-03_O05, Bg04-06_I05, Bg04-30_D05, Bg04-30_K13) genes in lateral root, from salt-treated *B. gymnorhiza* were calculated from microarray data (NCBI GEO accession No. GSE10942). In B, open circle, CL15Contig2; filled circle, CL38Contig1; open square, Bg04–30_D05; filled square, Bg04–30_D05; open triangle, Bg04–30_K13, filled triangle, Bg04–06_I05; cross, Bg03–05_F14. n = 1–3.
which reversibly catalyzes the conversion of FBP to glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Enhanced expression of FBP aldolase in salt-treated rice was previously reported (Abbasi and Komatsu 2004), and it was suggested that expression of the protein may play a role in the acclimation of rice seedlings to the anaerobic conditions created by oxidative stress generated in a saline environment. Enhanced expression of FBP aldolase would increase the flow of carbon through the Calvin cycle and lead to an increase in sucrose and amino acid production via increases in carbon flux through glycolysis. These traits would also lead to osmotic production and contribute to stress tolerance. It was reported that the activity of photosynthesis-related enzymes including FBP aldolase was inhibited by salt stress in tobacco; however, genetically engineered tobacco with the ability to synthesize glycinebetaine showed increased salt tolerance and reduced inhibition of the activity of these enzymes, suggesting that glycinebetaine protects the enzymes against salt stress (Yang et al. 2008). These results suggest that FBP aldolase plays an important role in salt tolerance mechanisms common to both glycophyttes and mangrove plants. In the current study, elevated expression of chloroplasts-type FBP aldolase protein was detected in the main root, not in the leaves, in salt-treated mangrove plants. It was reported that enzymes in the glycolytic pathway were incomplete in sycamore amyloplast; however, the pathway is connected to cytosolic metabolism by a hexose-P and an ATP/ADP translocator, and complete starch metabolism is performed (Frehner et al. 1990). This may be the case in root plastids. Konishi et al. (2004) reported that FBP aldolase activity is increased in rice roots treated with gibberellin, and root growth of aldolase antisense transgenic rice was repressed compared with that of control plants, and they suggested that the aldolase activates V-ATPase through physical interaction, resulting in cell elongation including vacuole expansion caused by accumulation of vacuolar solute. It is assumed that similar vacuolar solute accumulation in *B. gymnorhiza* roots may contribute to salt tolerance by adjustment of the osmotic potential under salt stress. In contrast, FBP aldolase mRNA levels in the main root decreased slightly after salt treatment. This result suggests that FBP aldolase is regulated at the translational or post-translational levels. It has been reported that correlation between mRNA levels and protein abundance is usually poor (Gygi et al. 1999, Chen et al. 2002, Foster et al. 2005). Therefore, we plan to produce and characterize transgenic, aldolase-overexpressing plants. Similarly, increased expression levels of OEEs have been reported in leaves of both rice (Abbasi and Komatsu 2004) and *B. gymnorhiza* (Sugihara et al. 2000).

The LR1 protein sequence was highly homologous to osmotin, a protein originally found to accumulate during adaptation of tobacco cells to high osmotic stress, including salt and polyethylene glycol (Singh et al. 1985, Singh et al. 1987). Osmotin accumulation in *B. gymnorhiza* lateral root appeared after 1 h salt treatment, reached a maximum at 3–6 h, and then declined to undetectable levels. Based on this observation, osmotin seems to play a role in the initial osmotic adaptation of *B. gymnorhiza* lateral roots to salt stress. However, because osmotin protein levels are abundant for only an initial, brief period during salt treatment, it is not likely that osmotin contributes to *B. gymnorhiza* adaptation to conditions of prolonged or continuous exposure to salt stress. The Na+ and Cl– ion content in *B. gymnorhiza* roots was observed to increase rapidly by 12 h following salt treatment (Yamanaka et al. 2009). Therefore, increased Na+ and Cl– ion concentrations may play a role in osmotic adaptation of the roots 12 h following salt treatment. It was reported that the tobacco osmotin gene was activated by NaCl treatment and that its osmotin promoter is much more active in root tissues than in shoot tissues (Nelson et al. 1992). The gene for soybean osmotin-like protein, GmOLPa, was constitutively transcribed in soybean root and was induced almost exclusively in the root during 24 h of salt stress (Onishi et al. 2006). Although the osmotin cDNA identical to the identified *B. gymnorhiza* osmotin protein sequence determined in the proteome analysis was not included in the microarray, five similar, osmotin-like genes in the microarray were up-regulated by salt stress. No concordance was detected between changes in levels of gene expression and protein expression. Up-regulation of the osmotin gene in salt-treated *B. gymnorhiza* leaves has been reported previously (Miyama and Tada 2008); however, up-regulation of protein spots corresponding to osmotin in leaf was not observed in this study. Osmotin may be regulated at the translational or post-translational levels, and/or the osmotin gene family may include other copies in the *B. gymnorhiza* genome. To determine the mechanistic function of *B. gymnorhiza* osmotin in salt tolerance, further studies using enzymological and transgenic approaches are necessary.

MR2 peptide sequences were not homologous to any sequences in the NCBI non-redundant protein database. Thus, MR2 represents the identification of a novel salt-responsive protein by a proteomic approach, an expressed protein which had not been detected by expressed sequence tag analysis (Miyama et al. 2006). The amino acid sequence of LR2 was not analyzed in this study, but, based on its molecular weight and pi it was postulated to be the same protein as MR2. It is possible that MR2/LR2 may play a major role in the salt tolerance mechanism in the mangrove plant. Future experiments in our laboratory include the isolation and characterization of MR2/LR2 cDNA, and identification of LR3 and LE1.
Materials and Methods

Plant materials
Cultivation of B. gymnorhiza and stress treatment with 500 mM NaCl were performed as previously described (Miyama and Tada 2008). The main and lateral roots and the leaves were collected from each set of three trees at 0, 1, 3, 6, 12 and 24 h, and 3, 6 and 12 d of NaCl treatment, comprising a total of 27 trees. To harvest root samples, treated plants were first pulled from the pots, and then the roots were washed briefly in water, wiped with paper and separated from the rest of the plant. Sampled materials were frozen with liquid nitrogen and stored at –80°C.

Protein extraction
Total protein was extracted from frozen roots and leaves using a ReadyPrep Protein Extraction Kit (Bio Rad, Tokyo, Japan) and then treated with a ReadyPrep 2-D Cleanup Kit (Bio Rad, Tokyo, Japan). Protein concentrations were determined by Bradford assay (Bio Rad).

Two-dimensional electrophoresis
A 60 µg aliquot of total protein extract was used for 2-DE. For the first dimension, 75 mm pH gradient agarGEL strips (ATTO, Tokyo, Japan) with a linear gradient (pH 3–10) were used, and electrophoresis was carried out at a constant voltage of 300 V for 210 min. For the second dimension, 5–20% SDS–PAGE (90×82×1 mm) (ATTO, Tokyo, Japan) with a linear gradient (pH 3–10) were used, and electrophoresis was carried out at a constant voltage of 320 mA for 90 min. Gels were stained with SYPRO Ruby Protein Gel Stain (Molecular Probes, Eugene, OR, USA). Gel images were scanned and spot intensity was quantified in comparison with internal standard spots using ChemiStage (KURABO, Tokyo, Japan). At least three replications were performed for each sample.

Protein identification
Protein spots that showed a response to salt stress were excised from stained gels, in-gel digested according to the method of Rosenfeld et al. (1992), separated using an Alliance HPLC system (Waters Corporation, Milford, MA, USA), and analyzed using the Proscie 494 cLC Protein Sequencing System (Applied Biosystems, Foster City, CA, USA). Amino acid data were searched against the NCBI non-redundant protein database.

In the case of LR3, the protein spot was excised from stained gels, in-gel digested, and analyzed by LC-MS/MS using LTQ Orbitrap XL (Thermo Fisher Scientific Inc., Waltham, MA, USA). Using the Mascot search engine (www.matrixscience.com), production data were searched against the NCBI non-redundant protein database.

Expression analysis at the mRNA level
We previously performed transcriptome analysis of roots from salt-treated B. gymnorhiza using an oligo microarray (Yamanaka et al. 2009, NCBI GEO accession No.GSE10942), in which the same salt-treated main and lateral root and leaf samples used in this study were used for analysis. The mRNA levels of those genes exhibiting salt-responsive proteins were analyzed using the microarray data.

Funding

References


(Received November 17, 2008; Accepted January 4, 2009)