Note

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Detection of Differences in mRNA Expression Regulated by Salt-Stress in Mangrove Cultured Cells

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Abstract

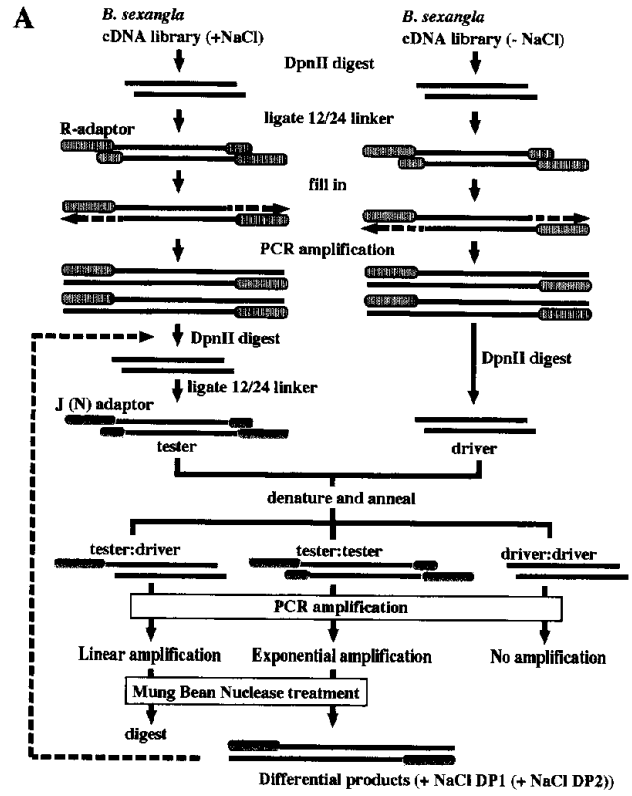
Identifying genes involved in salt-stress responses in mangrove plants is one of the most critical steps in elucidating the salt-resistance mechanisms in these plants. In this study, we screened cDNAs whose mRNA amounts were regulated by salt-stress using cDNA Representational Difference Analysis (cDNA RDA) from suspension-cultured cells of a mangrove, *Bruguiera sexangula*. Four cDNA fragments whose mRNA amounts were up regulated by salt-stress were isolated. Database searches revealed that these cDNAs encode proteins similar to endo-polygalacturonase, alcohol dehydrogenase, and two putative *Arabidopsis* proteins. In addition, eight cDNA fragments whose mRNA amounts were down regulated by salt-stress were also isolated. Further characterization of these cDNAs will likely be important in elucidating the salt-resistance mechanisms in mangrove plants.

Accession numbers: AB072257 (u1), AB072258 (u2), AB072259 (u3), AB072260 (u4), AB072261 (d1), AB072262 (d2), AB072263 (d3), AB072264 (d4) AB072265 (d5), AB072266 (d6), AB072267 (d7), AB072268 (d8)

Keywords: *Bruguiera sexangula*, mangrove, representational difference analysis, salt tolerance

Although the importance of mangrove forests has been recognized for the earth ecosystem, presently the reason why mangrove plants can grow in brackish habitat at molecular level is still unknown. Salt-stress is one of the most serious factors limiting plant growth and productivity (Yancey *et al.*, 1982). To elucidate salt-tolerance mechanisms in higher plants, numerous key factors have been cloned i.e., late-embryogenesis abundant proteins (Xu *et al.*, 1996), P5CS (Kishor *et al.*, 1995), DREB1A (Kasuga *et al.*, 1999), and AtNHX1 (Apse *et al.*, 1999). In contrast, the mechanism that can explain why plants can grow in brackish habitat is still an enigma. We postulated that mangrove plants must have acquired some proteins essential for salt-tolerance mechanisms during evolution. Many reports are available to address the mechanisms of mangrove plants at organ level (Werner and Stelzer, 1990). However, there are few reports about their mechanisms at molecular level because only few model systems are available to analyze these mechanisms. Recently, an efficient culture condition to initiate callus from a mangrove plant, *B. sexangula*, was established. This callus has salt-tolerance up to 200 mM NaCl (Mimura *et al.*, 1997a; Mimura *et al.*, 1997b). In this study, cDNAs whose mRNA amounts were either up or down regulated by salt-stress were screened from mangrove-cultured cells using cDNA RDA (Hubank and Schatz, 1994).

Suspension culture, derived from the mangrove callus, was established (Kura-Hotta *et al.*, 2000). To make the cDNA libraries, this culture was cultivated in amino acid medium (Thompson *et al.*, 1986) in the presence of 0 or 100 mM NaCl. The mangrove cDNA libraries were successfully constructed using ZAP-cDNA synthesis kit (Stratagene). Each cDNA library contained over one million independent clones. With these cDNA libraries, the cDNA RDA was performed as Hubank and Schatz (1994). The schematic diagram of the cDNA RDA in detecting cDNA fragments whose mRNA expressions were up regulated by salt stress was shown in **Fig. 1A**. The adaptor sequences for the RDA were listed in **Fig. 1B**.



B

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R-adaptor   5'-AGCACTCTCCAGCCTCTCACCGCA-3'
                          3'-AGTGGCGTCTAG-5'

J-adaptor   5'-ACCGACGTCGACTATCCATGAACA-3'
                          3'-GTACTTGTCTAG-5'

N-adaptor   5'-AGGCAACTGTGCTATCCGAGGGAA-3'
                          3'-GCTCCCTTCTAG-5'
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Fig. 1 Schematic diagram of cDNA RDA in detecting mangrove cDNAs whose mRNA expressions were up regulated by salt-stress (A) and adaptor sequences used in this analysis (B).

The first PCR was performed to produce amplicons (1 min, 92 ℃ ; 10 s, 66 ℃ ; 3 min, 68 ℃ ; 40 cycles). In order to form testers and drivers, R-adaptor was removed from the amplicons with *Dpn*II and the digested fragments were phenol extracted, ethanol precipitated, and finally purified with gel filtration chromatography (Sephacryl S-400, Amersham Pharmacia Biotech). Forty nanogram of the purified product, derived from +NaCl cDNA library, was ligated to the J-adaptor to form the tester. The second PCR was done to amplify differential products (DP1) in +NaCl cDNA library (1 min, 92 ℃ ; 10 s, 66 ℃ ; 3 min, 68 ℃ ; 10 cycles). In order to remove the background cDNA fragments from DP1, J adaptor was removed from DP1 with *Dpn*II and the digested fragments were purified as mentioned above. After the purification, N-adaptor was ligated to adaptor-free DP1 to form new testers. By using these new testers, final round PCR was performed (1 min, 92 ℃ ; 10 s, 66 ℃ ; 3 min, 68 ℃ ; 20 cycles). These amplified cDNA fragments

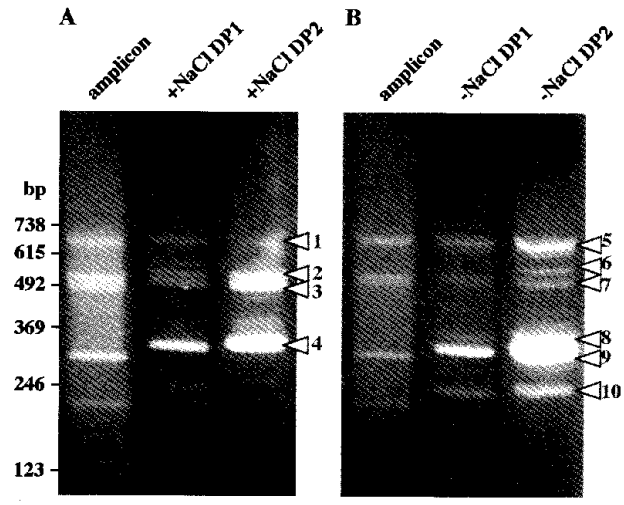

Fig. 2 Detection of cDNA fragments in mangrove cultured cells whose mRNA amounts were up regulated (A) or down regulated (B) in the presence of 100 mM NaCl by cDNA RDA. Major bands in +NaCl and -NaCl DP2 were numbered and cloned into pBluescript SK (*Eco*RV site).

were designated as DP2. Tester-driver annealing conditions used in this study were the same as previous description (Hubank and Schatz, 1994).

Isolation of cDNAs whose mRNA amounts were down regulated by salt-stress was also performed with reverse subtraction. The amplicons and the differential products (+NaCl DP1, 2 and -NaCl DP1, 2) were run on 1.5% agarose gel (**Fig. 2**). Several cDNA fragments were amplified in +NaCl and -NaCl DP2 lanes. These amplified products were then respectively cloned into the *Eco*RV site of pBluescript SK (Stratagene). Based on the sequence analyses of these clones, 20 independent cDNA fragments were obtained.

To confirm the mRNA expressions of these clones in the mangrove-cultured cells, 100 mM NaCl was added to the suspension culture, which was first cultivated under NaCl-free condition. After 0, 3, 6 and 12 h from NaCl addition, cells were respectively collected, and the total RNAs were extracted. Northern blot analyses revealed that mRNAs expression of four clones isolated from +NaCl DP2 (u1, u2, u3 and u4) increased within 3 h (**Fig. 3A**). In contrary, the mRNA expressions isolated from -NaCl DP2 (d1, d2, d3, d4, d5, d6, d7 and d8) gradually decreased by NaCl stress (**Fig. 3B**). These cDNAs were expected to have participated in the mangrove plant salt-tolerance. The mRNAs expressions of the other eight clones did not show any significant difference (data not shown). These clones might have been backgrounds of the subtractions. Database searches with BLAST

program (Altschul *et al.*, 1997) revealed that these cDNA fragments encode proteins similar to endo-polygalacturonase (u1), alcohol dehydrogenase (u2), two putative *Arabidopsis* proteins (u3, u4), lipid transfer protein (d1), glyceraldehydes-3-phosphate dehydrogenase (d2), and pectin methyltransferase inhibitor (d3, d4) (**Table 1**). As for the d5, d6, d7 and d8 clones, no significant similar sequences were found in the databases because the length of their fragments was too short to search for

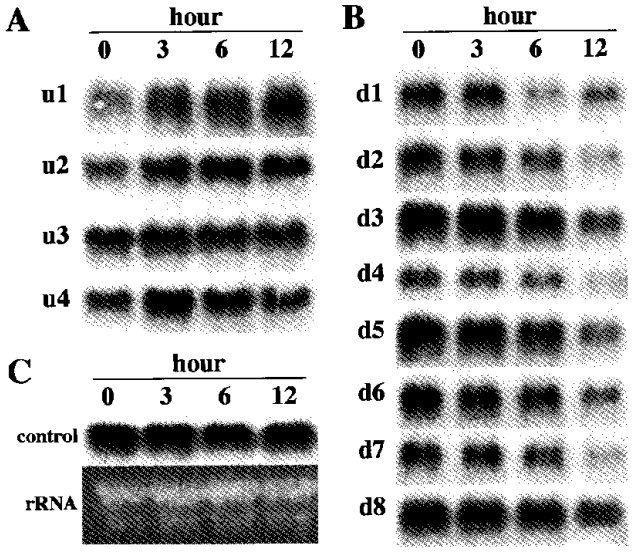

Fig. 3 Effect of NaCl on the mRNA expressions in mangrove cultured cells. The amount of total RNA in each lane was adjusted to 25 μg. mRNAs corresponding to u1-u4 were up regulated (A) and mRNAs corresponding to d1-d8 were down regulated (B). Ethidium bromide stained rRNAs were indicated as a loading control (C). ^{32}P labeled cDNA fragments (u1-u4, d1-d8) were used as probes. Unidentified cDNA, whose mRNA expression was not regulated by salt-stress, was also used as a control probe (C).

Table 1 Proteins homology of the mangrove putative proteins encoded in the screened cDNA fragments.

cDNA number	Band number*	Accession number	Similar protein (Source, Accession number)	Homology (%)
u1	1	AB072257	unknown protein (*Arabidopsis thaliana*, gp:AB008264_7)	Identities: 54/70 (77%) Positives: 63/70 (89%)
u2	2	AB072258	endo-polygalacturonase-like protein (*Arabidopsis thaliana*, pir:T46135)	Identities: 25/57 (43%) Positives: 33/57 (57%)
u3	3	AB072259	unknown protein (*Arabidopsis thaliana*, pir:T05388)	Identities: 65/86 (75%) Positives: 74/86 (85%)
u4	4	AB072260	alcohol dehydrogenase (*Paeonia suffruicosa*, gp:AF009054_1)	Identities: 39/56 (69%) Positives: 46/56 (81%)
d1	5	AB072261	lipid transfer protein (*Avicennia marina*, gp:AF331710_1)	Identities: 35/69 (50%) Positives: 45/69 (64%)
d2	6	AB072262	glyceraldehyde-3-phosphate dehydrogenase (*Nicotiana tabacum*, gp:NTA133422_1)	Identities: 81/97 (83%) Positives: 86/97 (88%)
d3	7	AB072263	pectin methyltransferase inhibitor (*Actinidia chinensis*, prf:2616317A)	Identities: 34/96 (35%) Positives: 44/96 (45%)
d4	8	AB072264	pectin methyltransferase inhibitor (*Actinidia chinensis*, prf:2616317A)	Identities: 37/80 (46%) Positives: 46/80 (58%)
d5	9	AB072265	not hit**	-
d6	9	AB072266	not hit**	-
d7	9	AB072267	not hit**	-
d8	10	AB072268	not hit**	-

mRNAs corresponding to u1-u4 were up regulated and d1-d8 were down regulated by salt-stress in the mangrove-cultured cells. * cDNA fragments were cloned from the bands indicated in **Fig. 2.** ** Similar proteins to the encoded proteins in d5, 6, 7, and 8 were not found in the databases.

their homology.

In this study, twelve cDNA fragments whose mRNA expressions were apparently regulated by salt-stress were successfully obtained by cDNA RDA. This result suggested that the cDNA RDA method was useful in detecting differences in mRNA expression level. Future analyses on these cDNA fragments will contribute to our understanding the molecular level of salt-tolerance mechanisms in mangrove plants.

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