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ORIGINAL PAPER

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Analysis of salt-stress-inducible ESTs isolated by PCR-subtraction in salt-tolerant rice

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Abstract To clarify the mechanisms of stress tolerance in rice and to search for rice genes associated with these mechanisms, we analyzed genes induced by a high salinity treatment using the PCR-subtractive hybridization method (PCR-subtraction). Seedlings of the salt-tolerant rice cultivar Dee-geo-woo-gen (DGWG) were either treated with 250 m *M* NaCl for 5 h or left untreated, and PCR-subtraction was then performed using the untreated (control) plants as a driver and the NaCl-treated plants as a tester. We obtained 384 clones of tester-specific cDNAs as salt-inducible candidates. Northern analysis performed with the cDNA fragments showed that 65 clones had been induced by the NaCl treatment. Sequence analysis and database searching indicated that these clones have homology to proteins functional for detoxification, stress response, and signal transduction in plants. Of these clones, 22% coded for unknown proteins and 12% gave no hits. We selected eight clones from each functional category and analyzed their expression pattern in DGWG. For temporal analysis, seedlings were treated with H_2O or 250 m *M* NaCl for 0, 0.5, 1, 2, 5, 10 or 24 h. Different patterns of transcript regulation were found. For the analysis of expression in response to various types of stress and abscisic acid (ABA) treatments, seedlings were treated for 5 h or 10 h with H_2O, dehydration, cold (4°C), heat (40°C), mannitol, ABA, or wounding. All clones were strongly up-regulated by osmotic stress (dehydration and mannitol) and the ABA treatment.

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Introduction

The lack of food in many countries is a serious global concern. The area of irrigable land is being reduced by salinization, and adverse environmental conditions such as drought, high salinity, and low temperature limit crop quality and yield. Gene manipulation is one means that could be used to improve stress tolerance in crops in order to overcome these environmental limitations by reprogramming the expression of endogenous genes, thus increasing yields.

To survive harsh environmental conditions and sudden changes in the weather, plants use highly developed stress responses at both the protein and gene expression levels. Diverse environmental stresses often activate similar cell signaling pathways and cellular responses, thereby causing an up-regulation of antioxidants and an accumulation of osmoprotectants. During water deficit, plants synthesize compatible solutes such as proline (Pro), mannitol, and glycine betaine (Hanson et al. 1994; Garcia et al. 1997; Yoshiba et al. 1997) and late-embryogenesis-abundant (LEA) and abscisic acid (ABA)-responsive element-binding (AREB) proteins. The genes regulating these biosynthetic pathways can be up-regulated or down-regulated (Xu et al. 1996; Uno et al. 2000; Oztur et al. 2002). LEA proteins have been predicted to play various roles, such as the maintenance of proteins or membrane structures, the sequestration of ions, and the binding of water, and operating as molecular chaperones (Wise 2003; Wise and Tunnacliffe 2004). The phytohormone ABA is mediates many physiological processes that enable the plant to activate adaptive responses to adverse environmental conditions. LEA proteins also respond to ABA (Moons et al. 1995; Shen et al.1996).

Various researchers have recently reported genes and expressed sequence tags (ESTs) that are induced by cold, drought, high salinity stress, and ABA in many plant species. These genes and ESTs were detected through the use of cDNA microarray, differential display, and subtraction methods (Kawasaki et al. 2001; Seki et al. 2002; Ueda et al. 2002; de los Reyes et al. 2003; Rabbani et al. 2003; Sahi et al.2003). This sequence information will aid our understanding of genetic mechanisms that control plant stress responses. However, the mechanisms of salt tolerance are not fully understood, even though studies have reported that genes that could be engineered to improve stress tolerance in plants. Gene transfer of the transcription factor DREB (dehydration-responsive element binding) and LEA proteins has resulted in plants with an increased tolerance to several stressors, including drought, high salinity, and low temperature (Xu et al. 1996; Kasuga et al. 1999). Overexpression of compatible solutes also resulted in improved stress tolerance (Kishor et al. 1995; Nanjo et al. 1999; Holmstrom et al. 2000; Taji et al. 2002).

Rice is a major crop and is used as a model for monocot plants. Although it is salt-sensitive, sensitivity differs widely among cultivars and breeding lines, and different expression patterns of stress-inducible genes and proteins between salt-tolerant and salt-sensitive cultivars in rice have been reported (Moons et al. 1995; Kawasaki et al. 2001; Pillai et al. 2004). Sahi et al. (2003) found that even in two salt-tolerant cultivars some genes had different expression levels. Therefore, it is possible that other salt-tolerant cultivars may have novel ESTs and genes. When seedlings of the salt-tolerant cultivar Dee-Geo-Woo-Gen (DGWG) were subjected to treatment with 250 m *M* NaCl for 72 h, 90% survived, whereas seedlings of salt-sensitive IR28 did not (Igarashi and Yoshiba 2001).

Our aim in the investigation reported here was to clarify the mechanisms of stress tolerance in order to search for related novel genes in salt-tolerant rice. We analyzed genes induced by high salinity using the PCR-subtraction method with DGWG plants.

Materials and methods

Plant materials and growth conditions

Seeds of rice (*Oryza sativa* L.) cv. Dee-Geo-Woo-Gen were sterilized and germinated in 1% (v/v) benlate solution for 4 days. They were then sown in pots filled with a mixture of soil and vermiculite (1:1, v/v) and grown under a 14/10-h (light/dark) photoperiod at 27°C for 10 days with daily watering. The seedlings were subsequently gently uprooted and treated as described in the next section.

Stress treatments

We treated seedlings with or without 250 m *M* NaCl for 5 h (for PCR-subtraction), with H_2O or 250 m *M* NaCl for 0, 0.5, 1, 2, 5, 10, or 24 h (for temporal analysis), or with H_2O, dehydration, cold, heat, mannitol, ABA, or wounding for 5 h or 10 h (for analysis under various stress). For the NaCl treatment, mannitol, and ABA treatments, seedlings were transferred to a glass tube with 250 m *M* NaCl, 0.5 *M* mannitol, or 1 m *M* ABA, respectively. For the dehydration treatment, the seedlings were transferred to an empty glass tube. For the cold and heat treatments, the seedlings were incubated at 4°C or 40°C. For the wounding treatment, the leaves were wounded with an iron comb. Seedlings were then frozen in liquid nitrogen and stored at −80°C until RNA extraction.

PCR-subtraction

Total RNA was isolated from whole plants of DGWG seedlings as described by Yoshiba et al. (1995). Poly A^+ RNA fractions from both the tester and driver samples were isolated by two rounds of poly A^+ selection on oligo(dT)-latex beads using the Nucleotrap mRNA Midi and Mini kits (Clontech, Palo Alto, Calif.). Both first-strand cDNAs were synthesized and then digested with *Rsa*I. PCR-subtraction was performed with the control plants as a driver and the NaCl-treated plants as a tester. A driver-specific subtracted cDNA pool was cloned into the pAtlas vector according to the manufacturer's protocol (PCR-Selected cDNA Subtraction kit; Clontech).

Northern blot analysis

Total RNA (10 μg) was separated under denaturing conditions on 1% agarose gels containing formaldehyde, transferred to a nylon membrane (Amersham Bioscience, Piscataway, N.J.), fixed by UV-irradiation, and then hybridized with a labeled DNA fragment of the tester-specific cDNA region using an Alkphos Direct Labeling and Detection System (Amersham Bioscience) or a PCR DIG Probe Synthesis and Detection kit (Roche, Germany).

cDNA sequencing and database analysis

The cDNA sequence was determined using a Thermosequence Primer Cycle Sequencing kit (Amersham Bioscience) and a DNA sequencer (SQ5500E, Hitachi, Japan). All sequences were compared against the DDBJ database and BAC/PAC (bacterial artificial chromosome/bacteriophage P1-derived artificial chromosome) sequences in GenBank with the BLASTN or BLASTX algorisms.

Results

PCR-subtraction

We obtained 384 clones of tester-specific cDNAs as salt-inducible candidates. Of these, Northern analysis showed that 65 were induced by the NaCl treatment (Table 1) and 43 showed homology to known proteins.

Table 1 shows their expression levels in control and salt-stressed plants, cDNA sequence size, homologous genes, BAC/PAC localization, and *E*-values. Some of these clones encode the same protein: nine are homologous to aldehyde dehydrogenase (ALDH) of rice, five to the ATP-dependent Clp protease ATP-binding subunit precursor, three to lysine ketoglutarate reductase/saccharopine dehydrogenase, and two each to CTR-1-like protein kinase, *S*-adenosylmethionine decarboxylase 2, putative sodium-dicarboxylate, and glyoxylase II, respectively. Fourteen have homology to unknown proteins and eight had no matches. We classified these proteins into putative functions as follows: detoxification, stress response, growth and development, signal transduction, others, unknown, and no hits (Table 2).

Expression analysis over time and under various stresses

We selected eight clones (nos. 49, 03, 19, 51, 10, 11, 27, 16) that were homologous to genes accounting for many clones or that were strongly induced in Northern blot analysis (Table 1) and used these for temporal analysis, for analysis under various stresses, or for ABA treatments. For the temporal analysis, the seedlings were treated with H_2O or 250 m *M* NaCl for 0, 0.5, 1, 2, 5, 10, or 24 h (Fig. 1). Different patterns of transcript regulation were found. Clone nos. 49 and 03 were up-regulated after 5 h of NaCl treatment, while signals from the other clones were detected at 0 h or when subjected to the H_2O treatment. The mechanical stress and/or osmotic change caused by uprooting may have caused the up-regulation of these clones. Clone nos. 19, 51, 10, and 27 showed higher signals after 5 h of NaCl treatment than in the preceding 5 h and were down-regulated in H_2O. The up-regulation of clone 11 peaked at between 1 h and 2 h in H_2O and between 0.5 h and 1 h in NaCl. Signals from clone 16 were detected in both H_2O and NaCl, but they were stronger in NaCl, peaking at 5 h.

For the analysis of expression in response to various types of stress and ABA treatment, the seedlings were treated for 5 h and 10 h with H_2O, dehydration, cold (4°C), heat (40°C), mannitol, ABA, or wounding. Figure 2 shows the expression levels of the eight clones following subjection to the various stresses. All of the clones were strongly up-regulated by osmotic stress (dehydration and mannitol) and ABA treatment. Clones 19 and 10 were up-regulated by cold treatment. Clone 16, homologous to a low-temperature-induced gene of *Hordeum*, was not induced by cold but was induced following 5 h of heat treatment. Wounding induced clone nos. 19, 51, 27, and 16, although the expression levels were not high.

Discussion

To compare the profiles of gene expression in salt-tolerant rice (cv. DGWG) seedlings between control and salt-stressed conditions, we used the PCR-subtraction method. In all, 384 putative salt-inducible cDNA fragments were cloned. Northern blot analysis showed that 65 of these clones were induced by the NaCl treatment. Therefore, 83% of clones gave false positives. This result indicates the importance of confirming the expression levels by RNA gel blot analysis using PCR-subtraction. From the nucleotide (or amino-acid) sequence data, it emerged that many of the salt-stress-associated clones strongly matched established genes or EST sequences in rice or other plant species (Table 1). Fourteen clones have not been functionally annotated, and eight show no homology with sequences in the public databases (Table 2). The possible role of these clones in the stress response process needs further analysis and characterization. We classified homologous genes into putative functions as follows.

Detoxification

ALDHs oxidize aldehydes to carboxylic acid and simultaneously reduce NAD and/or NADP. Forty-five genes for ALDH in plants, including eight in rice, have been reported, and two ALDH genes from *Arabidopsis* were shown to be up-regulated by dehydration, high salinity, and cold in a microarray study using 7,000 full-length cDNAs (Seki et al. 2002). Overexpression of *Ath-ALDH3* in transgenic *Arabidopsis* plants improved stress tolerance (Sunkar et al. 2003). In rice, *ALDH2a* was up-regulated following submergence (Nakazono et al. 2000). Rabbani et al. (2003) reported that ALDH was stress-inducible. In this study, many of our clones encoded ALDH: these clones have homology to *ALDH7B6* (http://www.uchsc.edu/sp/sp/alcdbase/pla-aldh.html), which was cloned by induction with incompatible blast fungus in rice (DDBJ accession no. AF323586). Our RNA gel blot analyses confirmed high transcript induction of an ALDH clone under NaCl treatment (Fig. 1). Therefore, *ALDH7B6* is likely to be a good candidate gene for salt tolerance in DGWG.

Glyoxalase II is another important enzyme in cellular detoxification in many organisms (Maiti et al. 1997; Chen et al. 2003). Our results showed that two clones encoded this enzyme. Glyoxalase II is part of the glutathione-related glyoxalase detoxification system and responds to reactive oxygen species. In the antioxidative system, superoxide dismutase and glutathione reductase up-regulates under various environmental stress conditions in plants (Ueda et al. 2002; Mittova et al. 2003; Wang et al. 2003).

Stress response

Lysine ketoglutarate reductase/saccharopine dehydrogenase (LKR/SDH), *S*-adenosylmetionine decarboxylase 2 (SAMDC 2), and *S*-adenosylmethionine synthetase (SAMS) were classified as stress-related

Table 1 Salt-inducible cDNA clones isolated by PCR-subtraction in rice (*Oryza sativa* L.) cv. Dee-Geo-Woo-Gen

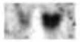
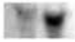
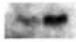
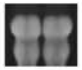
Probe No.	Northern Blot C N	Size (bp)	Accession no.	Annotation				BAC/PAC localizaition		
				Homologous gene	Source	Acc.no. of homologous gene	E-value	Homologous clone	Acc.no. of homologous clone	E-value
1		666	AB178646	putative phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplast precursor	*Oryza sativa*	AP004380	3E-62*	chr 3 BAC clone OSJNBa0017N12	AC092075	7.0E-82
2		475	AB178647	unknown, water-stressed 1(WS1) cDNA	*Sorghum bicolor*	AW680644	2E-17	chr 7 BAC clone OJ1118_G09	AP003874	1.7E-97
3		659	AB178648	ATP-dependent Clp protease ATP-binding subunit precursor	*Oryza sativa*	AY166599	3E-69*	chr 4 BAC clone OJ000315_02	AL731639	1.1E-67
4		459	AB178649	blti2 gene (low temperature-induced gene)	*Hordeum vulgare*	AF069328	3E-23*	chr 6 clone P0655A07	AP003634	1.4E-71
5		689	AB178650	alcohol dehydrogenase 1 protein	*Oryza sativa*	AF172282	3E-59*	chr 11 clone OSJNBa0037P04	AC123521	3.3E-79
6		544	AB178651	putative phytpsulfokine peptide precursor	*Oryza sativa*	AF068333	6E-21*	chr 12 BAC clone OSJNBa0023M16	AL954364	1.7E-54
7		546	AB178652	unknown, heat-stressed seedling cDNA	*Triticum aestivum*	BE496848	2E-20	chr 3 clone OSJNBb0071C04	AC136136	2.7E-65
8		781	AB178653	ERD1 protein (a Clp A, B-like protein)	*Arabidopsis thaliana*	D17582	1E-53*	chr 2 PAC clone P0458B05	AP004777	6.4E-58
9		540	AB178654	No hits found	-	-	-	chr 4 BAC clone OSJNBb0088C09	AL731628	8.8E-95
10		692	AB178655	receptor-like serine/threonine kinase	*Arabidopsis thaliana*	AP002061	1E-15*	chr 3 clone OSJNBa0032O19	AC107315	1.4E-68
11		559	AB178656	CTR1-like protein kinase	*Rosa hybrid cultivar*	AY032953	9E-13*	chr 2 PAC clone P0458B05	AP004777	3.3E-34
12		573	AB178657	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	5E-54*	chr 9 BAC clone OJ1344_B01	AP005570	6.5E-44
13		340	AB178658	unknown, cDNA clone:J023067J12	*Oryza sativa*	AK070796	3E-77	chr 10 clone OSJNAa0011L09	AC122144	2.9E-31
14		261	AB178659	No hits found	-	-	-	chr 3 clone OJ1012B02	AC135205	1.0E-30
15		1029	AB178660	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	0	chr 9 BAC clone OJ1344_B01	AP005570	2.1E-50
16		437	AB178661	blti2 gene (low temperature-induced gene)	*Hordeum vulgare*	AF069328	2E-41*	chr 6 clone P0655A07	AP003634	1.2E-54
17		214	AB178662	No hits found	-	-	-	chr 1 PAC clone P0492F05	AP002902	3.7E-33
18		259	AB178663	transformer-SR ribonucleoprotein	*Nicotiana tabacum*	Y09506	2E-7*	chr 3 clone OJ1012B02	AC135205	4.5E-32
19		296	AB178664	S-adenosylmethionine decarboxylase 2	*Oryza sativa*	AJ251899	5E-94	chr 2 BAC clone OJ1476_F05	AP004063	3.8E-35
20		305	AB178665	unknown, drought stressed leaf cDNA	*Triticum aestivum*	BF478383	2E-24	chr 3 BAC clone OSJNBa0059E14	AC135958	4.6E-49

Table 1 (Contd.)

Probe No.	Northern Blot C N	Size (bp)	Accession no.	Annotation				BAC/PAC localizaition		
				Homologous gene	Source	Acc.no. of homologous gene	E-value	Homologous clone	Acc.no. of homologous clone	E-value
21		1042	AB178666	ATP-dependent Clp protease ATP-binding subunit precursor	*Oryza sativa*	AY166599	6E-72*	chr 2 PAC clone P0458B05	AP004777	1.5E-88
22		407	AB178667	lysine ketoglutarate reductase/saccharopine dehydrogenase	*Zea mays*	AF271636	1E-36*	chr 2 BAC clone OJ1311_D08	AP004849	5.4E-61
23		338	AB178668	sucrose transporter (suc4 gene)	*Arabidopsis thaliana*	AJ289166	2E-16	chr 12 clone P0243A04	BX664710	1.5E-43
24		910	AB178669	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	0	chr 9 BAC clone OJ1344_B01	AP005570	1.7E-89
25		608	AB178670	lysine ketoglutarate reductase/saccharopine dehydrogenase	*Zea mays*	AF271636	1E-82*	chr 2 BAC clone OJ1311_D08	AP004849	1.9E-48
26		408	AB178671	ATP-dependent Clp protease ATP-binding subunit precursor	*Oryza sativa*	AY166599	4E-48*	chr 2 PAC clone P0458B05	AP004777	7.4E-66
27		337	AB178672	Ca^{2+} ATPase (gene name SS304, partial cds.)	*Oryza sativa*	D21274	1E-31	chr 3 clone OJ1754_E06	AC104433	2.3E-36
28		383	AB178673	unknown, drought-stressed cDNA clone DSAF116B11A011	*Sorghum bicolor*	CF757295	1E-24	chr 4 BAC clone OSJNBa0014K14	AL606604	1.8E-65
29		208	AB178674	No hits found	-	-	-	chr 10 BAC OSJNBa0076F20	AC025296	1.4E-26
30		252	AB178675	myo-inositol phosphate synthetase (RINO1)	*Oryza sativa*	AB012107	1E-116	chr 3 clone OSJNBb0019D17	AC116604	3.2E-40
31		588	AB178676	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	0	chr 9 BAC clone OJ1344_B01	AP005570	4.3E-44
32		247	AB178677	putative carbonate dehydratase protein	*Arabidopsis thaliana*	AY122986	2E-13*	chr 9 BAC clone OSJNBb0019B14	AP005755	1.1E-21
33		225	AB178678	No hits found	-	-	-	chr 10 BAC OSJNBa0076F20	AC025296	2.6E-30
34		402	AB178679	CTR1-like protein kinase	*Rosa hybrid cultivar*	AY032953	8E-23*	chr 2 PAC clone P0458B05	AP004777	7.6E-59
35		453	AB178680	unknown, cold-stressed seedling subtracted cDNA	*Triticum aestivum*	BQ282574	1E-4	chr 2 BAC clone OJ1003_F05	AP003974	1.0E-09
36		578	AB178681	No hits found	-	-	-	chr 3 BAC clone OSJNBb0031F05	AC133861	9.0E-111
37		706	AB178682	unknown, ABA-treated embryo cDNA clone WHE623E04J07	*Triticum aestivum*	BE517046	7E-55	chr 1 PAC clone P0415A04	AP003345	1.3E-140
38		730	AB178683	S-adenosylmethionine decarboxylase 2	*Oryza sativa*	AJ251899	0	chr 2 BAC clone OJ1476_F05	AP004063	9.3E-144
39		954	AB178684	putative sodium-dicarboxylate cotransporter protein	*Arabidopsis thaliana*	AY133792	2E-30*	chr 8 BAC clone OJ1506_F01.	AP004190	1.8E-134
40		554	AB178685	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	0	chr 9 BAC clone OJ1344_B01	AP005570	4.6E-43
41		279	AB178686	glyoxalase II protein	*Arabidopsis thaliana*	U74610	5E-31	chr 1 BAC clone OSJNBb0063G05	AP003760	9.2E-34
42		590	AB178687	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	0	chr 9 BAC clone OJ1344_B01	AP005570	2.5E-43
43		841	AB178688	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	0	chr9 BAC clone OJ1344_B01	AP005570	2.7E-114

Table 1 (Contd.)

Probe No.	Northern Blot C N	Size (bp)	Accession no.	Annotation				BAC/PAC localizaition		
				Homologous gene	Source	Acc.no. of homologous gene	E-value	Homologous clone	Acc.no. of homologous clone	E-value
44		435	AB178689	lysine ketoglutarate reductase/saccharopine dehydrogenase	*Zea mays*	AF271636	2E-25*	chr 2 BAC clone OJ1311_D08	AP004849	9.0E-74
45		452	AB178690	ATP-dependent Clp protease ATP-binding subunit precursor	*Oryza sativa*	AY085348	0	chr 2 PAC clone P0458B05	AP004777	2.2E-65
46		559	AB178691	Centrin protein	*Oryza sativa*	AY166599	5E-38*	chr 10 clone OSJNBa0094J09	AC078839	1.1E-70
47		571	AB178692	putative beta-alanine-pyruvate aminotransferase	*Arabidopsis thaliana*	AF323586	1E-24*	chr 5 clone P0486C01	AC135924	4.7E-97
48		639	AB178693	S-adenosylmethionine synthetase (SAMS gene)	*Oryza sativa*	AC078839	0	chr 5 clone P0519E07	AC087552	1.5E-111
49		430	AB178694	aldehyde dehydogenase (ALDH)	*Oryza sativa*	AJ296743	0	chr 9 BAC clone OJ1344_B01	AP005570	6.8E-51
50		289	AB178695	No hits found	-	-	-	chr 3 BAC clone OSJNBa0059E14	AC135958	4.9E-46
51		320	AB178696	sucrose transporter 2 (sut2 gene)	*Hordeum vulgare*	AJ272308	8E-10	chr 12 clone P0243A04	P0243A04	8.6E-43
52		310	AB178697	ATP-dependent Clp protease ATP-binding subunit precursor	*Oryza sativa*	AY166599	1E-50*	chr 2 PAC clone P0458B05	AP004777	1.9E-45
53		554	AB178698	unknown, salt-stressed, cDNA clone EBro 04SQ003C24	*Hordeum vulgare*	BM371001	9E-35	chr 5 clone P0676G05	AC087425	1.7E-73
54		592	AB178699	acyl-coA dehydrogenase	*Arabidopsis thaliana*	AY087793	6E-17*	chr 1 PAC clone P0041E11	AP002521	2.0E-119
55		1002	AB178700	unknown, aluminum-stressed cDNA clone WHE3352G12M24	*Triticum aestivum*	BU100397	6E-70	chr 11BAC clone OSJNBa0096I10	AC145349	4.3E-49
56		643	AB178701	No hits found	-	-	-	chr 3 clone OJ1626B05	AC104473	4.5E-98
57		500	AB178702	aldehyde dehydrogenase (ALDH)	*Oryza sativa*	AF323586	4E-20*	chr 9 BAC clone OJ1344_B01	AP005570	3.5E-38
58		334	AB178703	blti2 gene (low temperature-induced gene)	*Hordeum vulgare*	AF069328	1E-31*	chr 6 clone P0655A07	AP003634	5.0E-50
59		260	AB178704	unknown, cDNA partial sequence(C5336712Z)	*Oryza sativa*	AU161063	1E-130	chr 1 PAC clone P0520B06	AP003077	2.4E-48
60		1064	AB178705	putative sodium-dicarboxylate cotransporter protein	*Arabidopsis thaliana*	AY133792	2E-49*	chr 8 BAC clone OJ1506_F01	AP004190	8.0E-135
61		317	AB178706	glyoxalase II protein	*Arabidopsis thaliana*	U74610	3E-16*	chr 1 BAC clone OSJNBb0063G05	AP003760	1.2E-38
62		612	AB178707	unknown, drought stress panicle cDNA clone C0004837	*Oryza sativa*	CA766996	1E-136	chr 3 BAC clone OSJNBb0031F05	AC133861	3.0E-107
63		530	AB178708	Dek1-calpain-like protein	*Oryza sativa*	AY062272	4E-51*	chr 2 clone OJ0003_C09	AP004076	6.5E-88
64		350	AB178709	putative cullin protein	*Olea europaea*	AY059387	5E-07*	chr 3 BAC clone OSJNBb0024J04	AC084296	1.5E-45
65		543	AB178710	putative PGPD14protein (pollen germination related protein)	*Oryza sativa*	AP003453	4E-86*	chr 1 PAC clone P0480C01	AP003453	4.5E-42
		rRNA								

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[a] C, Control; N, 250 m M NaCl for 5 h

[b] With asterisk, results from BLASTX; without asterisk, results from BLASTN

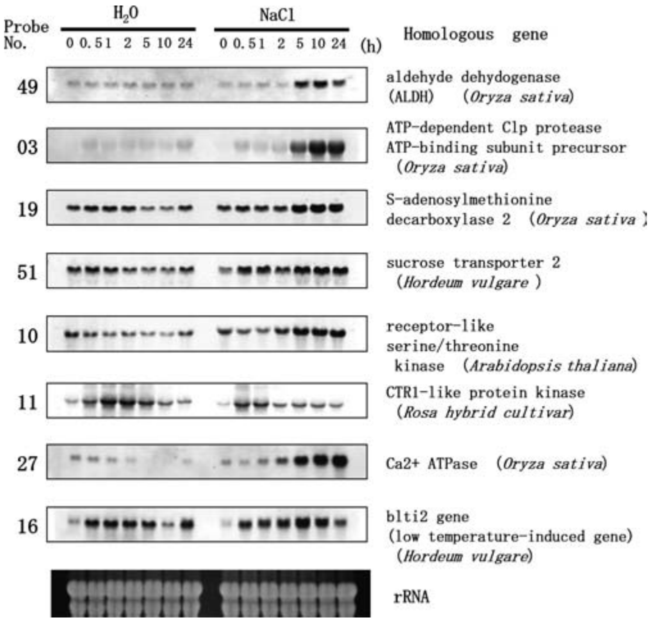

Fig. 1 Northern analysis of selected salt-inducible cDNA clones over time. Each lane was loaded with 10 μg of total RNA extracted from rice cv. Dee-Geo-Woo-Gen seedlings treated with H_2O or 250 m *M* NaCl for 0, 0.5, 1, 2, 5, 10, or 24 h

proteins (Table 2). Lysine is catabolized in plants via saccharopine into glutamate, pipecolic acid, and acetyl Co-A by LKR/SDH. LKR/SDH was up-regulated in rapeseed leaves by osmotic stress (Deleu et al. 1999). Glutamate is a primary precursor for proline and arginine (Galili et al. 2001). Proline is a strong osmolyte in plants, including rice, and arginine is the precursor of the polyamines that act as stress-related compounds (Maiale et al. 2004). SAMDC is involved in polyamine synthesis, while SAMS functions in the biosynthesis of polyamines and ethylene. SAMDC and SAMS have been shown to be induced under cold and salt stress in rice (Lee et al. 1997; Pillai and Akiyama 2004). So it is possible that lysine and polyamine metabolism have important roles in salt tolerance in DGWG.

The sucrose transporter mediates the active transport of sucrose across the plasma membrane. Sucrose is a major transport carbohydrate in plants, and genes encoding the sucrose transporter have been isolated from many plant species. The expressions of these genes change at certain growth points and under stress conditions (Noiraud et al. 2000; Jeong et al. 2002). We detected two kinds of gene-encoding sucrose transporter (*suc4* and *sut2*). Under salt stress, they may regulate sugar transport and adjust the cellar osmotic pressure.

Growth and development

Five clones encoded the ATP-dependent Clp protease ATP-binding subunit precursor of rice, and one clone encoded ERD1 of *Arabidopsis*. A cDNA for ERD1 similar to the ATPase subunit of Clp protease was induced by water stress and senescence (Nakashima et al. 1997). Phytosulfokine has been identified as a plant growth factor, and it might aid heat stress tolerance (Yamakawa et al. 1999; Yang et al. 1999). These proteins may suppress the regulation of growth during salt stress in DGWG.

Signal transduction

Several of the clones isolated in this study have a homology to genes that are considered to be related to signal transduction, such as CTR-like protein kinase, receptor-like serine/threonine kinase, and Ca^{2+} ATPase. Plant receptor-like kinase plays a fundamental role in various cellular processes, including hormone signaling, self-incompatibility, regulation of plant development, and plant-pathogen interaction. *CTR1* encodes a serine/threonine protein kinase that acts downstream of the ethylene receptor and may be part of the MAP kinase cascade (Bleecker and Kende 2000). Ca^{2+} ATPase is a key regulator of Ca^{2+} ion efflux in all eukaryotes. In cells of soybean, the expression of this plasma membrane pump was highly and rapidly induced by salt stress and a fungal elicitor (Chung et al. 2000).

Others, unknown, and no hits

Clones homologous to proteins whose functions in plant salt stress responses are unclear were classified as "others". Clones homologous to genes and ESTs whose encoded proteins were unclear were classified as "unknown". Twelve percent of the clones encoded "others", and 22% encoded unknown proteins. Most unknown clones have homology to cDNAs induced by stresses such as salt, drought, cold, and ABA. Twelve percent had no hits.

Expression analysis of over time and under various stresses

Eight clones were analyzed for their temporal transcription response. Six of these were up-regulated immediately following NaCl treatment, and two were up-regulated 5 h following initiation of the treatment (Fig. 1). The early-response genes may functional in signal transduction or the early-stress response. The genes that responded later may play a role in stress adaptation or recovery.

We also analyzed the expression of these eight clones under various stress conditions (Fig. 2). Many salt-regulated genes respond to other stresses (Seki et al. 2002; Rabbani et al. 2003). All of the clones were strongly up-regulated by drought and mannitol in addition to salt. Under high salt conditions, the osmotic homeostasis in cells is disturbed, and water deficiency occurs. However, as salt stress also induces ionic stress, this result may be

Table 2 Putative functional categories and the number of salt-inducible cDNA clones isolated by PCR-subtraction in rice cv. Dee-Geo-Woo-Gen that have homology to these genes

Functional category	Homologous gene (source)	Number of clones
Detoxification	Aldehyde dehydrogenase (ALDH) (*O. sativa*)	9
	Glyoxalase II protein (*A. thaliana*)	2
	Alcohol dehydrogenase 1 protein (*O. sativa*)	1
Stress response	Lysine ketoglutarate reductase/saccharopinedehydrogenase (*Z. mays*)	3
	S-adenosylmethionine decarboxylase 2 (*O. sativa*)	2
	S-adenosylmethionine synthetase (SAMS gene) (*O. sativa*)	1
	Sucrose transporter (suc4 gene) (*A. thaliana*)	1
	Sucrose transporter 2 (sut2 gene) (*H. vulgare*)	1
Growth and development	ATP-dependent Clp protease ATP-binding subunit precursor (*O. sativa*)	5
	Putative sodium-dicarboxylate cotransporter protein (*A. thaliana*)	2
	Putative phytosulfokine peptide precursor (*O. sativa*)	1
	ERD1 protein (a Clp A, B-like protein) (*A. thaliana*)	1
	Acyl-coA dehydrogenase (*A. thaliana*)	1
	Putative beta-alanine-pyruvate aminotransferase (*A. thaliana*)	1
Signal transduction	CTR1-like protein kinase (*R. hybrid cultivar*)	2
	Receptor-like serine/threonine kinase (*A. thaliana*)	1
	Ca2+ ATPase (gene name SS304, partial cds.) (*O. sativa*)	1
Others	Putative phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplast precursor (*O. sativa*)	1
	Centrin protein (*O. sativa*)	1
	Transformer-SR ribonucleoprotein (*N. tabacum*)	1
	Putative carbonate dehydratase protein (*A. thaliana*)	1
	Myo-inositol phosphate synthetase (RINO1) (*O.sativa*)	1
	Putative cullin protein (*O. europaea*)	1
	Putative PGPD14protein (pollen germination related protein) (*O. sativa*)	1
	Dek1-calpain-like protein (*O. sativa*)	1
Unknown	Blti2 gene (low temperature-induced gene) (*H. vulgare*)	3
	Cold-stressed seedling subtracted cDNA (*T. aestivum*)	1
	Salt-stressed, cDNA clone EBro04SQ003C24 (*H. vulgare*)	1
	Drought stressed leaf cDNA (*T. aestivum*)	1
	Drought-stressed cDNA clone DSAF116B11A011(*S.bicolor*)	1
	Drought stress panicle cDNA clone C0004837(*O. sativa*)	1
	Water-stressed 1(WS1) cDNA (*S.bicolor*)	1
	ABA-treated embryo cDNA clone WHE623E04J07 (*T. aestivum*)	1
	Heat-stressed seedling cDNA (*T. aestivum*)	1
	Aluminum-stressed cDNA clone WHE3352G12M24(*T. aestivum*)	1
	cDNA clone:J023067J12 (*O. sativa*)	1
	cDNA partial sequence(C5336712Z) (*O. sativa*)	1
No hits	No hits found	8

showing that these clones were up-regulated by the osmotic stress due to high salt treatment. Although cold also induces osmotic stress, only two clones were up-regulated by the 4°C treatment. Rabbani et al. (2003), using a microarray analysis of rice clones, found that 56 of 57 salt-inducible genes were also induced by drought and 22 of these were induced by cold stress. We found that ABA also strongly up-regulated all eight clones. In plants, ABA-dependent and independent signal pathways function in stress responses (Ishitani et al. 1997; Shinozaki and Yamaguchi-Shinozaki 2000; Xiong et al. 2002). Our results suggest that the genes encoding these clones are regulated by ABA-dependent signal pathways under salt stress in DGWG.

Salt tolerance of DGWG

Igarashi et al. (1997) found a positive correlation between the accumulation of proline and salt tolerance in DGWG and that the expression of the *P5CS* gene was higher in DGWG than in salt-sensitive IR28. P5CS (Δ^1-pyrroline-5-carboxylate synthetase) is an enzyme involved in the biosynthesis of proline. In this study, we were unable to obtain cDNA for *P5CS*. However, we did find a possible regulation of lysine metabolism and sucrose accumulation. These functions may help the plant in adjusting to osmotic homeostasis. The salt-inducible cDNAs obtained in this study have close homology to 25 established genes (Table 2). Many of these genes are related to detoxification and stress responses in plants and may contribute to the stress tolerance of DGWG. As mentioned above, some of these genes have already been characterized as stress-inducible genes. In rice, it has been reported that genes for ALDH, ADH, SAMDC, the sucrose transporter, and receptor-like serine/threonine kinase are regulated by salt, drought, cold or ABA treatment. However, the stress regulation of other genes, such as LKR/SDH, acyl-coA dehydrogenase, phytosulfokine, CTR-1-like protein kinase, and Ca^{2+} ATPase has not been reported in rice. We suggest that these genes play a role in the stress

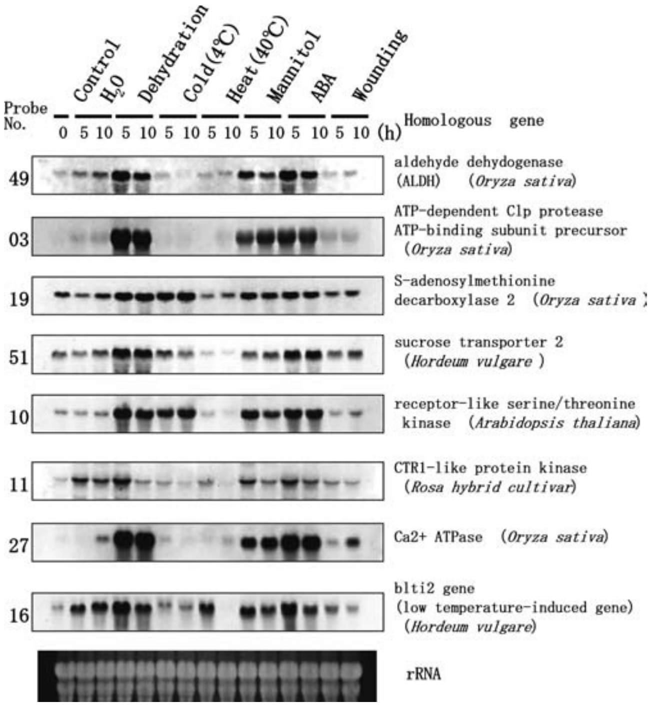

Fig. 2 Northern analysis of selected salt-inducible cDNA clones under various types of stress. Each lane was loaded with 10 μg of total RNA extracted from rice cv. Dee-Geo-Woo-Gen seedlings treated with H_2O, dehydration, cold (4°C), heat (40°C), 0.5 *M* mannitol, 1 *M* ABA, or wounding for 5 h or 10 h

response in rice. Further analysis of these genes may help clarify the mechanisms of stress tolerance and identify novel stress-inducible promoters. We also have shown the rice chromosome BAC/PAC localization of cDNA clones (Table 1). This information will be useful as reference markers for salt-responsive QTLs in rice.

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