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cDNA Microarray Analysis of Rice Anther Genes under Chilling Stress at the Microsporogenesis Stage Revealed Two Genes with DNA Transposon *Castaway* in the 5′-Flanking Region

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Rice is most chilling sensitive at the onset of microspore release. Chilling treatment at this stage causes male sterility. The gene expression profile during the microspore development process under chilling stress was revealed using a microarray that included 8,987 rice cDNAs. As many as 160 cDNAs were up- or down-regulated by chilling during the microspore release stage. RT-PCR analysis of 5 genes confirmed the microarray results. We identified 3 novel genes whose expression levels were remarkably changed by chilling in rice anther. A new cis element that includes a DNA transposon *Castaway* sequence was found in the 5′ upstream region of two genes which were conspicuously down-regulated by chilling temperatures in rice anther.

Key words: callose; chilling stress; microsporogenesis; tetrad; transposon

In the northern part of Japan, rice crop production is occasionally damaged severely by low temperatures in summer. The most chilling sensitive stage of rice was determined to be at the onset of microspore release.[1)] In effect, chilling treatment at this stage causes tapetum hypertrophy and disordered microspore development, and consequently gives rise to a high degree of male sterility.[2)]

Pollen development begins with the division of diploid archespores in the anther, giving rise to microsporocytes and tapetal cells. The tapetum forms a single layer of cells around the anther locule and provides nutrients and enzymes for microspore development. The microsporocytes produce a wall of callose composed primarily of β-1,3-glucan, and undergo meiosis.[3)] At the end of meiosis, tetrads of haploid microspores are freed into the locule by the action of a β-1,3-glucanase (callase) which is secreted by the tapetal cells.[4)] In transgenic tobacco plants in which β-1,3-glucanase is induced prematurely, little fertile pollen is produced.[5)] Therefore, the timing of β-1,3-glucanase activity is essential for the developing microspores. A β-1,3-glucanase gene was isolated from rice anther at the microspore stage,[6)] and the role of gene expression in chilling injury is now under investigation.

Mechanisms of chilling tolerance in plant seedlings have long been studied intensively with a focus on membrane structure and function.[7)] Chilling tolerance was enhanced in transgenic tobacco into which a gene for glycerol-3-phosphate acyl transferases or chloroplastic fatty acid desaturases from *Arabidopsis* was introduced.[8,9)] Another mechanism involves cellular defense against membrane lipid peroxidation caused by a chill-induced increase in the generation of reactive oxygen species such as superoxide, hydrogen peroxide, and hydroxyl radicals.[10)]

So far, there is very little information on the molecular aspects of chilling damage in rice microspore development, and the anther genes which participate in this process have not been identified. Recently, microarray technology has been developed and is a potent tool for the nonexclusive analysis of gene expression.[11)] Several reports using microarray analysis in plants have been published.[12–16)] In this study, we used a cDNA microarray containing 8,987 rice-expressed sequence tag (EST) clones to analyze the gene expression profile in rice anther at its early microspore stage under chilling. 160 ESTs were up- or down-regulated by chilling in rice anther. We identified three novel genes whose expression levels were conspicuously varied by

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Abbreviations: EST, expressed sequence tag; MITE, miniature inverted-repeat transposable element; cv., cultivar; OPDAR, 12-oxo-phytodienoic acid reductase; SAMDC, S-adenosylmethionine decarboxylase; Radc, rice anther down-regulated by chilling; AP, aspartic proteinase; PSS, plant-specific sequence; JA, jasmonate; PA, polyamine

chilling in rice anther. In particular, two genes have miniature inverted-repeat transposable element (MITE) *Castaway* sequences at nearly the same position in the 5′ upstream region. Their molecular characteristics and expression patterns are discussed.

Materials and Methods

Plant materials and growth conditions. Rice plants (*Oryza sativa* L. cv. Hayayuki) were grown in 200 cm^2 Wagner pots filled with paddy field soil, to which 0.9 g each of nitrogen, phosphorus, and potassium, 0.3 g each of magnesium and calcium, and traces of iron and manganese were added. A growth chamber (12 h light (300 $\mu E/m^2/s$) at 26 °C; 12 h dark at 20 °C, relative humidity 75%) was used. Rice anthers at the early microspore stage, *i.e.*, the uninucleate microspore stage containing tetrads and microspores, were identified as previously described.[17] Chilling treatments were done at 12 °C for 120 h (5 d) from the early microspore stage (50 d after sowing, −10 to −7 cm of auricle distance). Anthers were collected and frozen immediately in liquid nitrogen.

RNA preparation. Total RNA was extracted from frozen samples according to the method of Bekesiova *et al.*[18] Total RNA was extracted from rice anthers and 40 μg was used for each microarray analysis.

Microarray preparation. Microarray analysis was performed basically as previously described.[19] The rice cDNA microarrays were prepared on aluminum-coated and DMSO-optimized glass slides. The sequences used in the construction were generated by PCR. The PCR products were purified by QIAquick 96-column (QIAGEN). DNA solutions were arrayed by robotics, a Generation III ArraySpotter (Amersham Pharmacia).

Fluorescent labeling of probes. Isolated total RNA was reverse-transcribed with Cy5 (Amersham Pharmacia). Reactions were incubated for 2.5 h at 42 °C with 80 μg of total RNA, oligo-(dT)25, random nonamer, control RNA, 1 × SuperScript II reaction buffer, 10 mM DTT, 2 mM dATP, 2 mM dTTP, 2 mM dGTP, 1 mM dCTP, 1 mM Cy5dCTP, and SuperScript II reverse transcriptase. The reactions were denatured at 94 °C for 3 min and the RNA was degraded by incubation with 4 μl of 2.5 N NaOH at 37 °C for 15 min. Following degradation, the mixture was neutralized with 20 μl of 2 M HEPES buffer. The labeled probes were purified using a Qiaquick PCR Purification Kit (QIAGEN) and dried using a vacuum concentrator. The dried probes were resuspended in 9 μl of water and denatured at 95 °C for 4 min, and 6 μl of Oligo A80 (1 mg ml^{-1}) and 45 μl of ExpressHyb (Clontech) were added to the resuspended probe.

Hybridization on microarrays and analysis. Glass slides were incubated with a final volume of 30 μl of probe at 55 °C for 5 h in the dark. After hybridization, the glass slides were washed in 1 × SSC/0.2% SDS for 10 min at 55 °C in the dark, then in 0.1 × SSC/0.2% SDS for 10 min at 55 °C twice in the dark, and finally in 0.1 × SSC for 1 min at room temperature twice. After the final wash, the slides were briefly rinsed with distilled water and air-dried. The hybridized and washed microarrays were scanned using an Array Scanner Generation III (Amersham Pharmacia). ArrayGauge (FujiFilm) was used for image analysis.

RT-PCR analysis of genes. The first strands of the cDNA mixture were generated from 1.0 μg of total RNA and 2.5 μM Oligo d(T)16 primer. Reverse transcription was done for 30 min at 42 °C using ReverTra Ace-α- (Toyobo) according to the manufacturer's instructions. The PCR reaction mixture (10 μl) contained 0.1 μl of reverse transcribed first strands of cDNA in 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 0.01 mg/ml gelatin, 1.5 mM $MgCl_2$, 6 μM each of two primers (1904f: 5′-GTTCCATGTACGAAGCTCCC-3′ and 1904r: 5′-CACAAAGACGGGCGCATCAG-3′ for amplifying *SAMDC1*; 314f3: 5′-AGATATGGCACGTCGGCAGG-3′ and 314r3: 5′-CGTTGGCGCTGTCCTTCATG-3′ for amplifying *OPDAR1*; *Sal*Tf1: 5′-GACGCTGGTGAAGATTGGCC-3′ and *Sal*Tr1: 5′-GGCCATGGGTTCCAGAAATC-3′ for amplifying *OsSal*T; *Act1*f02: 5′-CGCAGTCCAAGAGGGGTATC-3′ and *Act1*r03: 5′-TCCTGGTCATAGTCCAGGGC-3′ for amplifying rice actin 1; 4923f8: 5′-GAGAACACGCTCATCCACAG-3′ and 4923r: 5′-TGCTAGCAGCAGCTTGGCTC-3′ for amplifying *Radc1*), 200 μM each of dNTP mixture, and 0.04 unit/μl of AmpliTaq Gold DNA polymerase (Applied Biosystems). Thirty-five cycles of PCR (94 °C for 0.5 min, 60 °C for 1 min, 72 °C for 2 min) were carried out. PCR products were separated by electrophoresis in 1.5% agarose gels, stained with ethidium bromide and visualized using the BioDoc-It System (UVP).

Results

Identification of chilling-regulated genes using the cDNA microarray

To analyze the gene expression profile during chilling stress in rice anther at the early microspore stage, a microarray containing 8,987 cDNAs of rice EST was used. As the total number of genes in the rice genome is roughly 62,500,[20] about one-seventh was contained in this array. Total RNA was isolated from the rice anthers at the tetrad stage and the middle microspore stage with or without chilling treatment during the tetrad stage, and fluorescence-labeled by reverse transcription before hybridization. The signal intensities from labeled targets derived from chilled and unchilled anthers were compared. On one slide, each cDNA clone was spotted in duplicate and the experiment was repeated twice. For each experiment, χ values were calculated by common

logarithmic transformation of each signal intensity. Subsequently, Z scores were calculated to normalize the sample values to account for variations in RNA labeling, according to the formula $Z = (\chi - \mu)/\sigma$, where μ and σ are the mean and standard deviation of about 4500 χ values which are spotted on half of one slide respectively. Finally, for each EST, two Z scores of duplicate spots on a glass slide were averaged. EST was considered to be chilling-inducible or chilling-repressible if the difference of averaged Z score (unchilled Z score − chilled Z score) was below −1.0 or above 1.0 respectively. Approximately, a difference of averaged Z scores of 1.0 corresponds to a two-fold difference in original signal intensity. As many as 160 ESTs were identified as being potentially responsive to chilling stress in rice anther at the early microspore stage, as they were found reproducibly in 2 experiments. Of these genes, the expression of 38 (24%) was up-regulated and that of 122 (76%) was down-regulated. These 160 ESTs were classified according to their putative functions based on EST descriptions and a protein database search by BLAST algorithm (annotations with similarity scores greater than 40 bits) (Table 1). About 36% of the chilling-responsive ESTs are of unknown function. Most of the chilling-responsive ESTs related to primary metabolism, signal transduction, defense, proteolysis including proteases, and secondary metabolism including 12-oxo-phytodienoic acid reductases (OPDARs) showed down-regulation. In contrast, genes related to translation, such as ribosomal protein, showed up-regulation in chilled anthers (Table 1).

Analysis of rice OPDARs and SAMDCs

Among the 8,987 rice ESTs on the microarray, 5 ESTs are annotated to encode OPDARs and 4 of them were down-regulated by chilling stress in rice anther. There are at least 3 copies of *OPDAR* in the rice genome. The sequence of the most chilling-responsive clone (accession number C26097) was almost identical to the nucleotide sequence of an ORF in AP003525, which is derived from chromosome 6 at 32.7 cM. We designated this gene *OPDAR1* (DDBJ accession No. AB122088). Two chilling-responsive clones (accession numbers C96685 and AU093231) also encoded OPDAR1. One chilling-responsive EST clone (accession number AU057040) and one non chilling-responsive EST clone (accession number D24670) encoded another copy of OPDAR on chromosome 6. These EST clones have up to 88% identities to *OPDAR1*, but the length of the responsive one is about 0.8 kb and that of the non chilling-responsive one is about 0.5 kb. Among the 8,987 rice ESTs on the microarray, 5 ESTs were annotated to encode S-adenosylmethionine decarboxylases (SAMDCs) and 3 of them were up-regulated by chilling stress in rice anther. There are at least 3 copies of *SAMDC* in the rice genome. The sequence of the most chilling-responsive clone (accession number C28220) was almost identical to the nucleotide sequence of an ORF in AP005420 which is derived from chromosome 9. We designated this gene *SAMDC1* (DDBJ accession No. AB122089). One chilling-responsive clone (accession number AU062686) also encoded SAMDC1. One chilling-responsive EST clone (accession number C26568) encoded another copy of SAMDC on chromosome 4. It has up to 89% identity to *SAMDC1*. Two non chilling-responsive EST clones (accession numbers AU100691 and AU056203) encoded yet another copy of SAMDC on chromosome 2, and has up to 85% identity to *SAMDC1*.

Sequence analysis of Radc1

Among the 8,987 rice ESTs on the microarray, 2 ESTs (accession numbers AU172968 and AU174285) with almost identical nucleotide sequences were remarkably down-regulated by chilling stress in rice anther. The sequences of these were almost identical to the nucleotide sequence of an ORF in AC125471 which is derived from chromosome 3. We designated this gene *Radc1* (*rice anther down-regulated by chilling 1*; DDBJ accession No. AB122090). The Radc1 amino acid sequence has an aspartic protease motif which shows 29/48% identity/similarity at the amino acid level with the tobacco chloroplast DNA-binding protein CND41, but lacks the N-terminal Lys-rich helix-turn-helix motif which is essential for DNA binding in CND41 (Fig. 1). In the upstream region of the *Radc1* gene, *Castaway*, a kind of miniature inverted-repeat transposable element (MITE) sequence exists (Fig. 2). *Castaway* also exists in the upstream region of high-salt- and drought-inducible *OsSal*T gene (Fig. 2).[21] *OsSal*T has another MITE sequence, *Stowaway*, in the upstream region (Fig. 2).[22] *Castaway* found in *Radc1* shows 84% sequence identity with *Castaway* found in *OsSal*T (Fig. 2).

Expression analysis of selected genes

To verify and confirm the microarray data, the expression of 5 ESTs, on which we focused for more detailed analysis, was examined by RT-PCR analysis. These 5 ESTs included up- and down-regulated genes and a non-responsive gene in the microarray experiment. For RT-PCR expression analysis, we used 4 sets of fixed oligonucleotide primers that are designed to put an intron between forward and reverse primers in order to distinguish between the amplification from genomic DNA (*SAMDC1*: 0.49 kbp; *OPDAR1*: 0.39 kbp; *OsSal*T: 0.35 kbp; *Act1* (rice actin 1 gene): 0.75 kbp) and that from spliced-out cDNA (*SAMDC1*: 0.39 kbp; *OPDAR1*: 0.27 kbp; *OsSal*T: 0.25 kbp; *Act1*: 0.50 kbp). As shown in Fig. 3, constitutive expression of *Act1* was confirmed by this RT-PCR experiment. As the 4 primer sets did not amplify the fragments which had sizes corresponding to non spliced-out genomic DNA, we ruled out the possibility of genomic DNA contamination in the RNA samples used. We also used a set of fixed oligonucleotide primers (4923f8 and 4923r) to check the expression profile of the *Radc1* gene by the above-

Table 1. Complete List of Genes That Are Significantly Up- or Down-regulated by Chilling Stress in Rice Anther

Clone	EST accession		Putative ID	difference of averaged Z scores
translation				
7640	AU070286	AU173957	chloroplast 30S ribosomal protein S7	2.1
8135	AU070441	AU174085	chloroplast 30S ribosomal protein S8	1.2
2302	AU175087	AU175088	protein translation factor SUI1	1.2
7320	AU077773	AU077774	protein translation factor SUI1	1.2
9031	AU161922	AU161923	protein translation factor SUI1	1.0
124	D22238	C96682	elongation factor 1-gamma	−1.0
159	AU102118	C96770	elongation factor 1-gamma	−1.1
5341	AU031252		40S ribosomal protein S15	−1.1
312	AU062501	AU092216	60S acidic ribosomal protein P0	−1.0
668	AU062621	AU108592	60S acidic ribosomal protein P0	−1.0
5464	AU164919	AU031524	60S ribosomal protein L18	−1.1
5501	AU095410	AU031615	60S ribosomal protein L2 (L8)	−1.1
5905	AU164554		60S ribosomal protein L7	−1.0
104	D22132		ribosomal protein L18a	−1.2
3427	C19518	C99258	ribosomal protein S4 type I (rps4)	−2.1
cell structure				
7891	D47590	AU082750	HMG protein	1.1
5402	AU095352	AU031408	pistil extensin like protein	−1.0
125	AU166286		villin 3	−1.0
transport				
6457	D24715	AU173362	*E. coli* cation transport protein ChaC	1.0
7621	AU173952	AU057343	vacuolar sorting receptor	1.1
603	C26456	AU164984	beta prime COP coatomer protein	−1.1
133	C96646	AU166285	cation-chloride co-transporter	−1.0
proteolysis				
651	C26503	AU091279	aspartic proteinase precursor	1.4
8125	D49033	AU101829	AtAPG8c mRNA for autophagy 8c	1.1
4332	C91690	AU029682	cysteine proteinase 1 precursor	1.0
7147	AU055940	AU055941	oryzacystatin-I	1.4
2744	C98695		polyubiquitin	1.0
4923	AU172968	AU166209	Radc1	2.6
8640	AU174285	AU174286	Radc1	3.6
4949	AU065677	AU030354	serine proteinase	1.1
528	C26358	AU100706	SEC61 alpha subunit	−1.4
secondary metabolism				
7405	AU056351		dihydrofolate reductase	1.3
132	C96685	C96686	OPDAR	1.2
314	C26019	AU092237	OPDAR	1.2
339	C26097	AU093231	OPDAR	1.4
7329	AU057040	AU057041	OPDAR	1.0
9071	AU175176	AU176556	shikimate kinase precursor	1.0
689	C26568	AU108589	SAMDC	−1.0
883	AU062686	AU166559	SAMDC	−0.8
1904	C28220	C97560	SAMDC	−1.0
53	AU067858	AU067859	xanthine dehydrogenase	−1.0
storage protein				
8808	AU174343	AU174344	OsSBP for selenium binding protein	1.1
8232	D49189	AU082276	maize Fer1	1.2
energy production				
7813	AU075867	AU075868	ATP synthase C chain	1.6
4506	AU064654	AU094876	ATP synthase delta chain	1.2
5918	D23879	AU031680	cytochrome b5 reductase (NFR)	−1.6
other				
6872	AU070733		cytochrome P450	1.5
7846	AU173996		Cyt-P450 monooxygenase	1.0
6249	AU173240	AU173241	isopenicillin N epimerase	1.1
2365	AU174453		lectin precursor (agglutinin)	1.0
3764	C73711	C99490	male sterility protein 2	1.7
2371	AU174447		Acyl-CoA-binding protein	−1.2
unknown				
348	AU062521	AU166365		1.1
479	C26301	AU166411		−1.1
1218	C26865	AU166775		1.3
1559	AU069076			1.4
1690	C27876	AU100890		1.6
3003	AU078191			1.5
3026	C73175	AU172643		1.0
3068	C98974			1.0
3273	AU172688			−1.2
3297	C19175	AU094360		−1.9
3325	C19310			−1.7
3434	AU162217	C99361		−1.1
3653	C20268			1.2
3673	C20375			1.0
3693	C20472			1.0
3726	C73631	C99434		2.0
3780	AU078130	C99517		1.0
4012	AU164283			1.0
4019	AU064364			1.0
4174	AU058082			−1.3
4217	AU101426	AU172815		4.0
4271	AU162267	AU029519		1.0
4339	C91704	AU029692		1.0
4345	C74445	AU172829		1.2
5694	AU174850			−1.2
5766	AU174902	AU174903		1.4
6204	D24309	AU173209		−1.6
6255	D24399	AU031803		−1.2
6666	AU181075			1.5
6714	AU032158	AU175073		1.1
6921	AU077754	AU077755		1.1
7003	AU078041	AU078042		1.3
7022	AU071077	AU162620		1.4
7059	AU071233			1.9
7323	AU057050			1.5
7369	AU057246	AU057247		2.1
7381	AU057197	AU057198		1.4
7386	AU057208			1.4
7388	AU173946	AU057231		1.3
7586	AU057277			1.1
7611	AU162748	AU057349		1.4
7627	AU057395	AU057396		1.1
7639	AU057483	AU057484		1.3
7748	AU057862			1.0
7757	AU057824	AU057825		1.1
8101	D48962	AU163113		1.5
8133	D49049			1.2
8153	AU101838	AU101839		1.5
8199	AU162105	AU162106		1.8
8244	D49239	AU101865		1.3
8258	D49335	AU101883		1.4
8727	D41415	AU033134		1.0
8814	AU161719	AU161720		1.7
8909	C25216	AU174362		1.0
8934	AU181057			1.1
9000	AU161904			1.2
9005	AU102062	AU102063		1.6
9054	AU097686			1.0

Continued on next page

Table 1. (continued)

Clone	EST accession		Putative ID	difference of averaged Z scores
primary metabolism				
7438	AU056550		3-ketoacyl-CoA thiolase-like protein	1.9
3092	AU064119	AU101254	acyl-CoA oxidase	1.1
7555	AU076256	AU076257	adenosine monophosphate binding protein 3	1.0
3787	AU064238	AU172746	adenosylhomocysteinase	1.4
163	AU166291	AU166292	alpha-amylase isozyme 3D precursor	1.3
826	AU068387	AU166535	alpha-amylase isozyme 3D precursor	1.2
3730	C73655	AU108182	beta-ketoacyl synthase	1.4
8098	D48949	AU097625	bifunctional nuclease	1.1
6219	D24338	AU173217	β-ketoacyl reductase	1.0
7281	AU056257	AU056258	carbonic anhydrase	2.3
5419	AU095385	AU095386	cysteine synthase	1.6
5474	AU164935	AU164936	cysteine synthase	1.9
469	C93441	AU166412	hexokinase 1	1.6
2519	C71989	AU101080	invertase	1.5
2559	AU101108	AU101109	lipase	1.0
1425	AU108817	C97061	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	1.0
8085	D48732	AU032835	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase	2.1
8589	D40492	AU174256	oxalate oxidase	1.2
2791	C72731	AU165528	plant short chain alcohol dehydrogenase	1.0
8304	D39365	AU163158	sucrose synthase	1.9
8428	AU174192		sucrose synthase	1.7
5214	AU101521	AU101522	3-hydroxy-3-methylglutaryl-CoA reductase	−1.0
31	C25787	AU102080	glutamine synthetase shoot isozyme	−1.1
414	C26203	AU100677	glycine decarboxylase complex H-protein	−1.1
3444	C99346	AU101318	*Homo sapiens* h-bcs1 (BCS1) mitochondrial protein	−1.5
5695	AU174855	AU174854	nonspecific lipid-transfer protein 2 precursor (LTP 2)	−1.2
signal transduction				
4154	C74259	AU091362	CaM-like protein	2.0
7219	AU056086	AU056087	CBL-interacting protein kinase 1 (CIPK1)	1.0
6586	D25079		finger protein WZF1	1.1
4082	C74114	AU091318	NAC-like protein	1.2
2459	C71770	C98483	RING finger-like protein	1.2
4278	AU162272	AU029581	WD domains, G-beta repeats	1.0
3298	C19151	AU091905	homeobox 1 protein OSH1	−1.2
4105	C74163	AU101400	RING-box protein	−1.2
transcription				
7594	AU057316	AU057317	*Arabidopsis thaliana* scarecrow-like 1 (SCL1)	1.1
4389	C91785	AU172851	Dof zinc finger protein	1.1
7463	AU162697	AU056522	OsNAC6 protein	1.1
641	AU166454	AU166455	the largest subunit of RNA polymerase II	1.1
defense				
2693	C72401	AU172487	*Arabidopsis thaliana* ERD15 protein	1.0
121	D22210	C96640	chitinase	1.0
8614	D40768	AU174271	low temperature and salt responsive protein LTI6B	1.1
7113	AU055773	AU055774	metallothionein-like protein	1.3
7645	AU057501	AU057502	metallothionein-like protein	1.3
2142	AU063562	AU172350	*OsSal*T	2.1
8849	AU066156	AU161751	*OsSal*T	2.4
995	AU068611	AU166664	*P. vulgaris* PVPR3 protein	1.3
7493	AU075857	AU075858	pathogenesis-related protein	1.5
8556	D40265	AU163222	peroxidase FLXPER4 (PER4)	1.0
7179	AU162644	AU056017	ascorbate peroxidase	1.4
315	C25998	AU092225	ascorbate peroxidase	−1.0

mentioned RT-PCR method. All of the results were consistent with the microarray data shown in Table 1. Through the application of chilling stress in rice anther, the expression of *OPDAR1*, *Radc1*, and *OsSal*T was repressed and the expression of *SAMDC1* was induced.

Discussion

In this study, we used microarray analysis to determine the gene expression profile in rice anther at the microspore release stage under chilling stress. Normalized log scores were obtained from two inde-

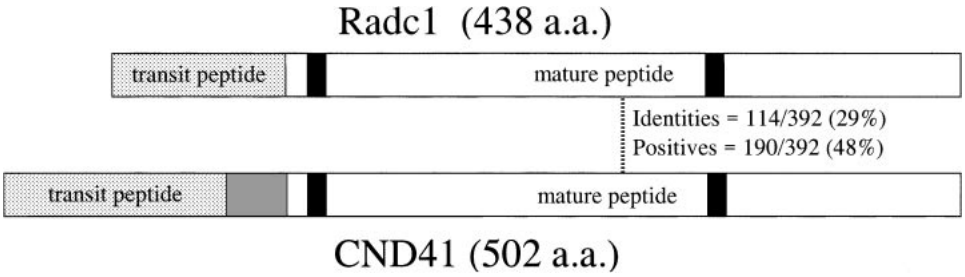

Fig. 1. Domain Organization of Radc1 and CND41.
The dotted box, the shaded box, and the black box represent transit peptide, Lys-rich helix-turn-helix motif, and active site aspartic acid residue respectively.

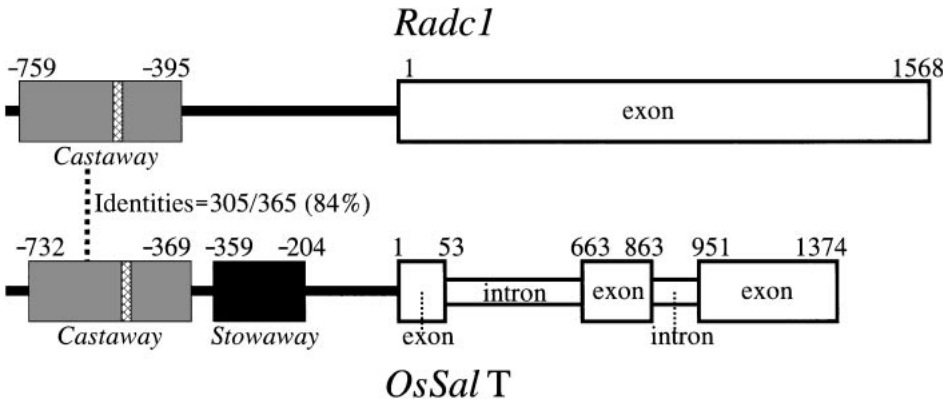

Fig. 2. Comparison of *Radc1* and *OsSal*T Genes.
The shaded box and the black box represent *Castaway* and *Stowaway* respectively. The hatched boxes in *Castaway* represent ABA responsive elements (TACGTGGC).

pendent microarray experiments. As many as 160 ESTs were identified as chilling-inducible or -repressible in rice anther. These genes have a broad spectrum of potential functions based on their sequence similarities (Table 1). The scores of ESTs with similar sequences, such as *OPDAR1*, *SAMDC1* and *Radc1*, showed similar changes in expression level (Table 1). These results indicate that microarray analysis is an efficient and reliable method for screening both up- and down-regulated genes under conditions of interest. But, ESTs homologous to a glucanase gene we isolated from rice anther were not included in the list of 8,987 cDNAs on the microarray that we used. A potential problem of microarray research is that sequence similarity between closely related genes may lead to cross-hybridization. Also, in this experiment, orthologs of *OPDAR1* or *SAMDC1* which have up to 88~89% identity showed similar chilling-responsive expression patterns. RT-PCR experiments with gene-specific primer might be used to confirm the chilling-responsive expression pattern of a particular gene (Fig. 3).

Aspartic proteinases (APs) (EC3.4.23) are one of the major classes of proteolytic enzymes showing acidic pH optima for enzymatic activity.[23] Typical plant APs have a high degree of similarity with those of animals and microbes, but plant AP genes contain an extra plant-

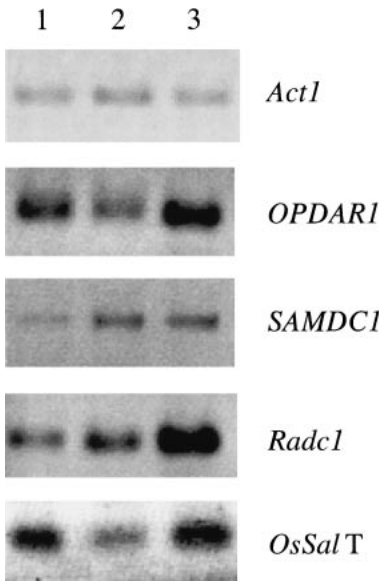

Fig. 3. RT-PCR Analysis of *Act1*, *OPDAR1*, *SAMDC1*, *Radc1*, and *OsSal*T Genes.
The first strands of the cDNA mixture were generated from total RNA from rice anthers. The PCR products were electrophoresed on agarose gel and visualized with ethidium bromide. The template cDNAs are the anthers at meiosis (lane 1), the anthers at microspore middle stage, just after chilling at 12 °C for 5 d (lane 2), and the anthers at the microspore middle stage (lane 3).

specific sequence (PSS) of approximately 100 residues, which shows high sequence and topological similarity to saposins, sphingolipid-activating proteins in mammalian cells. PSS has a putative membrane-binding region and may play a role in vacuolar transport of plant APs.[24–26] The tobacco chloroplast nucleoid DNA-binding protein CND41 and Radc1 protein lack the PSS sequence. As shown in Fig. 1, an aspartic protease motif of Radc1 shows significant similarity to CND41, but lacks the N-terminal Lys-rich helix-turn-helix motif that is essential for DNA binding in CND41.[27] The results shown in Fig. 3 indicate that *Radc1* is notably repressed by chilling in rice anther. Hence, *Radc1* may play some regulatory role that is different not only from typical plant APs associated with cell death or plant defense, but from chloroplast DNA-binding protein. It is possible that *Radc1* may degrade some proteins related to anther development and its response to chilling temperatures in rice plants.

Over 40% of the rice genomic sequence is repetitive DNA and most of this is related to transposable elements.[28,29] The class 1 long-terminal repeat retrotransposons form the largest component of transposable elements, comprising 14% of the rice genome, but numerically, MITEs constitute the largest group, covering about 6% of the genome with over 100,000 elements classified into hundreds of families.[30,31] MITEs are non-autonomous class 2 elements, but *Stowaway*-like and *Tourist*-like MITEs can now be connected with two superfamilies of transposases: *Tc1/mariner* and *PIF/harbinger* respectively.[32] Recently, an active MITE family, *miniature Ping* (*mPing*), was found in rice, and the correlation between *mPing* insertion in the *slender glume* allele and the slender mutation of glume was confirmed.[33–35] These *mPing* elements have undergone amplification more extensively in the temperate *japonica* than in the tropical *japonica* cultivar.[33] In wild rice, *Oryza eichingeri*, two MITEs, *Castaway* and *Stowaway*, in the 5′ upstream region of *OsSal*T, were shown to be not inserted.[22] ABA-responsive elements, which are known to regulate cold-inducible gene expression,[14] were also conserved in the same position of *Castaway*, inserted in the 5′-flanking region of *Radc1* and *OsSal*T. *OsSal*T mRNA accumulates very rapidly in sheaths and roots from mature plants and seedlings upon treatment with ABA.[21] Therefore, the similar down-regulated expression pattern of *Radc1* and *OsSal*T under chilling in rice anther and the nearly identical position of the same *Castaway* insertion in the 5′-flanking region of the two genes is reminiscent of the possibility that the rice plant adapted to environmental extremes through transposon activation by stresses such as chilling and drought during the domestication of temperate *japonicas*. Since the *Castaway*-like sequence was not found in the 2 kbp 5′-flanking regions of 156 other chilling-responsive ESTs (data not shown), a further search for chilling-responsive *Castaway* elements in the rice genome and promoter-GUS assay under chilling conditions will be necessary in order to define the functions of *Castaway* in the chilling response in rice anther.

Jasmonate (JA) is involved in plant responses to several biotic and abiotic stresses and is a signal compound that regulates plant growth and development.[36] In *Arabidopsis*, the function of JA in anther development and pollen fertility is known to be essential. The JA defective mutants in *Arabidopsis* are male-sterile because anther filaments do not elongate enough, and anther locules do not dehisce, and moreover, pollen grains on the mutant plants are inviable even though they develop to the trinucleate stage.[37,38] JA has been shown to increase the chilling tolerance of tomato fruit.[39] OPDAR is the key enzyme in JA biosynthesis. To our knowledge, there is no information regarding the relationship between JA and anther development in rice and other cereal crops. The results shown in Fig. 3 indicate that *OPDAR1* expression is remarkably repressed by chilling in rice anther, implying that *OPDAR1* and JA play some role during anther development and its response to chilling temperatures in the rice plant.

Polyamines (PAs) are involved in many biological processes in plants.[40] The stress tolerance of plants is correlated with their capacity to enhance the biosynthesis of polyamines under stressful conditions.[41] A close correlation between the chilling tolerance of rice cultivars and putrescine accumulation in leaves under chilling stress has been found.[42] In a chilling-tolerant cultivar of cucumber, synthesis of spermidine was increased in leaves during chilling treatment, while it was not in a chilling-sensitive cultivar.[43] SAMDC is the key enzyme in PA biosynthesis that decarboxylates S-adenosylmethionine and supplies an aminopropyl moiety to putrescine and spermidine.[44] Low activity of SAMDC is a major cause of poor performance of tomato pollen germination at high temperatures. To our knowledge, no prior study has elucidated the relationship between PA and microspore development in the rice plant. The results shown in Fig. 3 indicate that expression of *SAMDC1* is remarkably increased by chilling in rice anther, implying that *SAMDC1* plays some role in microspore development and that it is tolerant to chilling temperatures in the rice plant.

In conclusion, we identified many rice anther genes regulated by chilling using high-throughput microarray analysis. The results suggest a network of gene functions and plant hormones involved in the chilling response at the early microspore stage of rice anther. Further experimentation including promoter-GUS assay or histochemical analysis of chilling-responsive genes will be necessary in order to define their functions in the chilling response in rice anther.

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