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Expression Profiling of Two Rice (*Oryza sativa*) Genotypes Differing in Chilling Tolerance Using cDNA-Amplified Fragment Length Polymorphism

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ABSTRACT

Abiotic stress is a major limiting factor in crop production. Molecular comparisons between contrasting abiotic stress-tolerant genotypes may improve understanding of stress-tolerance mechanisms and can be used in discovery of stress-tolerance genes. We performed a cDNA Amplified Fragment Length Polymorphism (cDNA-AFLP) analysis on chilling tolerant and sensitive rice genotypes in order to identify genes involved in stress tolerance. Seventy-nine percent of the detected transcripts had similar expression patterns in both genotypes (66% constitutively expressed and 13% differentially expressed), whereas 14% and 7% were uniquely up-regulated and down-regulated, respectively, in the chilling-tolerant genotype. Selected up-regulated gene expression patterns represented by transcript-derived fragments (TDFs) differed in response to stress in the two genotypes as shown by Reverse Transcription Polymerase Chain Reaction (RT-PCR). Gene expression was higher for the vacuolar proton ATPase subunit B (*V-ATPase*) and inoistol 1, 3, 4-trisphosphate 5/6-kinase (*IP3K*) genes in response to chilling in the chilling-tolerant genotype compared to the chilling-sensitive genotype. The response of the chilling-tolerant genotype to chilling stress was complex, representing genes involved in signaling, transcription regulation, defense response, and transport-related proteins. Since the total number of TDFs with changed expression pattern was similar in both genotypes while the levels of expression differed, we hypothesize that both genotypes have the same chilling responsive genes but that the genes differ in the manner in which they are regulated. Further experiments are needed to confirm the role of identified genes in rice cold tolerance.

INTRODUCTION

Unlike other cereal crops, such as wheat, barley, and rye, rice (*Oryza sativa* L.) is not well adapted to cold weather and is damaged by temperatures below 15 °C. During rice development, germination and the 3-lf stage are two of the most sensitive stages to chilling stress. In a previous study, CT6748-8-CA-17 (CT) genotype was found to be more tolerant of chilling temperatures than the INIAP12 genotype (CS) (Morsy et al., 2005).

Cold acclimation reduces chilling injury due to chilling stress and is associated with the expression of a large number of genes involved in different cellular processes (Shinozaki and Yamaguchi-Shinozaki, 2000). While discovery of numerous stress-related functional genes and regulatory elements has increased our knowledge of abiotic stress mechanisms, the molecular basis and regulation of chilling tolerance in rice is not completely understood. Analysis of differentially expressed genes provides an opportunity to improve our understanding and utilization of the chilling-tolerant genotypes in rice by integration of these genes through conventional breeding or biotechnological means.

Identification and isolation of low abundant differentially expressed genes is a challenging process; however, cDNA-AFLP is an efficient and reproducible method to identify and isolate such transcripts (Bachem et al., 1998), especially those with low transcript abundance. We used cDNA-AFLP to identify genes with potentially important roles in tolerance to low temperature stress and also used semi-quantitative RT-PCR to identify differences in genotypic gene expression of selected candidate genes. Ultimately, molecular identification of stress-related responses of rice genotypes will provide candidate genes for use in breeding programs or transgenic technology.

PROCEDURES

Biological Material and Treatments

The chilling-tolerant genotype, CT6748-8-CA-17 (CT), and the chilling-sensitive genotype, INIAP12 (CS), of *Oryza sativa* L. (Morsy et al., 2005) were used in this study. Three replicates of each genotype (5 plants each) for each treatment and control were grown in soil pots in a growth chamber under a 12 h photoperiod (30/27 °C; day/night, 600 mE/m^2/sec) regime and 70% relative humidity until the 3-lf stage. Seedlings grown at control conditions were moved to hydroponic media (1X Hoagland's solution), kept at control conditions for 2 days and subsequently all but the control pots were subjected to either low temperature (13/10 °C; day/night), osmotic (Hoagland's solution plus 250 mM mannitol), or salt (Hoagland's solution plus 100 mM NaCl) stress treatments for 4 days. After treatment, shoots were collected and frozen in liquid nitrogen for RNA extraction.

RNA Extraction, cDNA Synthesis, and Amplified Fragment Length Polymorphism Analysis

Total RNA was extracted with an RNeasy kit (Qiagen, Germantown, Md.), followed by isolation of mRNA using PolyATract mRNA isolation system III (Promega,

Madison, Wis.) following the manufacturers' protocols. Then, 500 ng of mRNA of control and chilling-stressed seedlings were each reverse transcribed into cDNA with an oligo-dT primer and 200 units of Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) in the presence of RNase inhibitor (10 units). The 'AFLP Analysis System II' kit (Invitrogen, Carlsbad, Calif.) was used following the instructions of the manufacturer to generate an AFLP fingerprint. The Polymerase Chain Reaction (PCR) products representing both genotypes under control and chilling conditions were separated on a denaturing 5% polyacrylamide gel and stained using Silver Sequence Staining Reagents (Promega) following the manufacturer's protocol.

Characterization of Transcript-Derived Fragments

Clearly visible bands representing differentially regulated gene transcripts were excised from the gel with a clean scalpel and eluted into 100 µl of 50 mM KCl, 10 mM Tris-HCl, 0.1% Triton X-100, and 1.5 mM $MgCl_2$ by heating for 30 min at 90 °C. An aliquot of 1 ml of each eluted DNA was used as template for PCR with 2 pmol each of *Eco*RI-core and *Mse*I-core primers. The PCR products were purified with a QIAquick PCR purification kit (Qiagen), cloned in pCR2.1 vector (Invitrogen), and then sequenced using T3 promoter primer. Nucleotide sequences and derived amino acid sequences were compared with nucleotide and protein sequences of the GenBank databases with the BLAST sequence alignment program.

Semi-Quantitative Reverse Transcription Polymerase Chain Reaction

Semi-quantitative RT-PCR was used to compare the expression levels of differentially expressed genes in response to different abiotic stresses in seedlings of the CT and CS genotypes. Equal amounts of mRNAs were converted into cDNA with a RETROscript kit (Ambion, Austin, Texas) with oligo-dT primer according to the manufacturer's recommendations. Equal amounts (1 ml) of the first strand cDNA were used as templates for PCR amplification using specific primers designed for each clone (Table 1) and repeated 3 times. The RT-PCR products were resolved by electrophoresis through 1.2% agarose-ethidium bromide gels. Expression of the constitutively expressed actin gene was used as the positive control.

RESULTS

Identification of Chilling-Regulated Transcripts

Changes in gene expression within genotypes in response to chilling stress were resolved by the cDNA-AFLP analysis showing 540 bands. Among these bands 66% and 13% were constitutively and differentially expressed, respectively, in both genotypes following chilling stress. Also, 14% and 7% showed increased and decreased abundance,

respectively, of mRNA in the chilling-tolerant genotype only (Fig. 1). Subsequent effort was focused on isolation and cloning of the transcript-derived fragments (TDFs) with increased mRNA abundance from the CT genotype. Sequence homology searches for successfully cloned TDFs (65% of total 75 isolated and cloned TDFs) showed similarities with sequences of genes with both known and unknown functions. Isolated genes with increased mRNA abundance were grouped into categories according to their putative or known functions (Table 2).

Expression Analyses of Selected Transcript-Derived Fragments

Semi-quantitative RT-PCR was performed on the two genotypes to confirm the differential expression of four TDFs with increased mRNA abundance under different stresses (Fig. 2). In agreement with cDNA-AFLP results, expression of the *MADS* box gene in CT was relatively higher than in CS in response to stresses. The *V-ATPase* transcript, which plays an important role in maintaining cell solute homeostasis, increased in response to low temperature, osmotic and salt stresses in the CT genotype. However, its expression in CS was higher than in CT in response to salt stress. On the other hand, clone (AA-CAG) with similarity to 20S proteasome was down-regulated by low temperature and water deficit in the CT genotype, while it was moderately induced by salt in CS and down-regulated in CT. The TDF (AG-CAT11) transcript which encodes to *IP3K* gene was induced by water deficit and salt stress in both CS and CT genotypes, but low temperature stress induced expression of *IP3K* was observed only in the CT genotype.

DISCUSSION

The expression profile obtained by cDNA-AFLP matched the overall changes in gene expression obtained by more global methods applied to *Arabidopsis* and rice (Seki et al., 2001; Rabbani et al., 2003). In this regard, the percentage distribution of genes with altered expression among the general categories was similar with both techniques despite the fact that fewer genes were obtained by cDNA-AFLP (Table 2).

We characterized the expression of four clones, selected for different cellular functions, in response to various abiotic stresses by RT-PCR. For the 20S proteasome gene, the up-regulation of expression by chilling stress revealed by cDNA-AFLP, was not supported by RT-PCR. The difference in expression profile detected by the two techniques might be due to differences among primers and PCR conditions or due to amplification of different isogenes. In the case of the other 3 TDFs (*IP3K*, *MADS* box and *V-ATPase*), their up-regulation in response to different abiotic stresses was confirmed. However, their response differed between the CT and CS genotypes, where the later genotype showed higher expression in response to salt stress than the former genotype. The differential response between the genotypes indicated that the mechanisms regulating gene expression may differ between the two.

SIGNIFICANCE OF FINDINGS

Expression profiles of chilling-responsive genes in rice genotypes contrasting in chilling tolerance showed notable differences in response to chilling. The general trend in gene expression was similar in both genotypes but they differed in the level of expression of specific critical genes. These results suggest that both genotypes probably have the same stress-responsive genes, but that the regulation of these genes differs between genotypes. The difference in regulation may be related to differences in the levels of signaling molecules, such as inositol, or to the activity of transcription factors and to the promoter motifs present in the genotypes. This latter suggestion could be verified only by promoter sequence analyses of the genes present in each genotype. Understanding the critical differences in molecular responses of contrasting genotypes will be helpful in gene selection to improve stress tolerance of crops by gene manipulation or by marker-assisted breeding methods.

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Table 1. Polymerase Chain Reaction primers used to validate mRNA expression patterns of selected chilling-regulated transcript-derived fragments isolated by cDNA-AFLP techniques from rice.

Gene	Forward primer	Reverse primer
MADS box-like protein	aacgctgcaaaagacgcat	atcccagatatcaccagctga
Inositol 1, 3, 4-trisphosphate 5/6-kinase (IP3K)	tgctacacaaaataactgatg	taaaaagcatggtgctcg
V-ATPase	ttgacggtgaaaaggctgtt	agcagcagaaaagaggggaat
a2-20S proteasome	gaggtgccttctatttta	tgtctccaatgcaaagac
Actin	caaggccaatcgtgagaag	agcaatgccagggaacatagt

Table 2. Relevant homologies of up-regulated cDNA-AFLP sequence fragments of rice to sequences in the database.

No. of TDFs[a]	Putative function/homology	GenBank accession no.	e-value
Stress-related (35.4%)			
6	Paraneoplastic encephalomyelitis antigen	CX056242	1.00E-18
3	Non-cyanogenic beta-glucosidase precursor	CX056238	2.00E-29
2	Similar to Metallothionein-like protein type 3	CX056273	2.00E-23
1	20S proteasome alpha subunit B	CX056269	1.00E-65
1	RING-H2 finger protein ATL3F	CX056281	1.00E-27
1	Similar to Catalase isozyme 2	CX056280	6.00E-22
1	NADH-ubiquinone oxidoreductase B18	CX056279	2.00E-47
1	Floral organ regulator 1	CX056266	2.00E-53
1	snRNP protein	CX056258	4.00E-50
Transport (18.7%)			
3	Similar to Vacuolar ATP synthase subunit E	CX056274	8.00E-14
2	Similar to Peroxisomal ABC transporter	CX056246	7.00E-23
2	Antiporter/ drug transporter	CX056259	1.00E-60
1	Vacuolar ATPase B subunit	CX056276	1.00E-58
1	Similar to Peptide transporter 1	CX056278	2.00E-67
Biosynthetic pocesses (16.7%)			
3	Similar to Eukaryotic initiation factor 4A-3	CX056253	3.00E-43
2	Heat shock protein DnaJ family protein	CX056247	4.00E-38
1	Similar to Mannose-1-phosphate guanyltransferase	CX056264	3.00E-76
1	Peptidyl-prolyl isomerase FKBP12	CX056268	5.00E-56
1	Eukaryotic translation initiation factor 2 gamma subunit	CX056243	5.00E-32
Signaling and transcription (14.6%)			
2	Inositol 1, 3, 4-trisphosphate 56-kinase family protein	CX056249	2.00E-27
2	Pyruvate kinase	CX056261	1.00E-19
1	Similar to Transcription factor MADS55	CX056267	2.00E-16
1	BHLH transcription factor	CX056270	2.00E-21
1	Transcription factor Dp-1	CX056257	9.00E-14
Unknown function (14.6%)			
1	ATP-dependent RNA helicase-like protein DB10	CX056245	5.00E-42
1	Conserved hypothetical protein	CX056277	5.00E-20
1	Hypothetical protein	CX056272	2.00E-22
1	Similar to RNA-binding protein BRUNOL5	CX056234	2.00E-09
1	Conserved hypothetical protein	CX056250	1.00E-05
1	Retrotransposon protein, Ty3-gypsy subclass	CX056236	5.00E-20
1	Retrotransposon protein, unclassified	CX056251	5.00E-05

[a] TDFs = transcript-derived fragments.

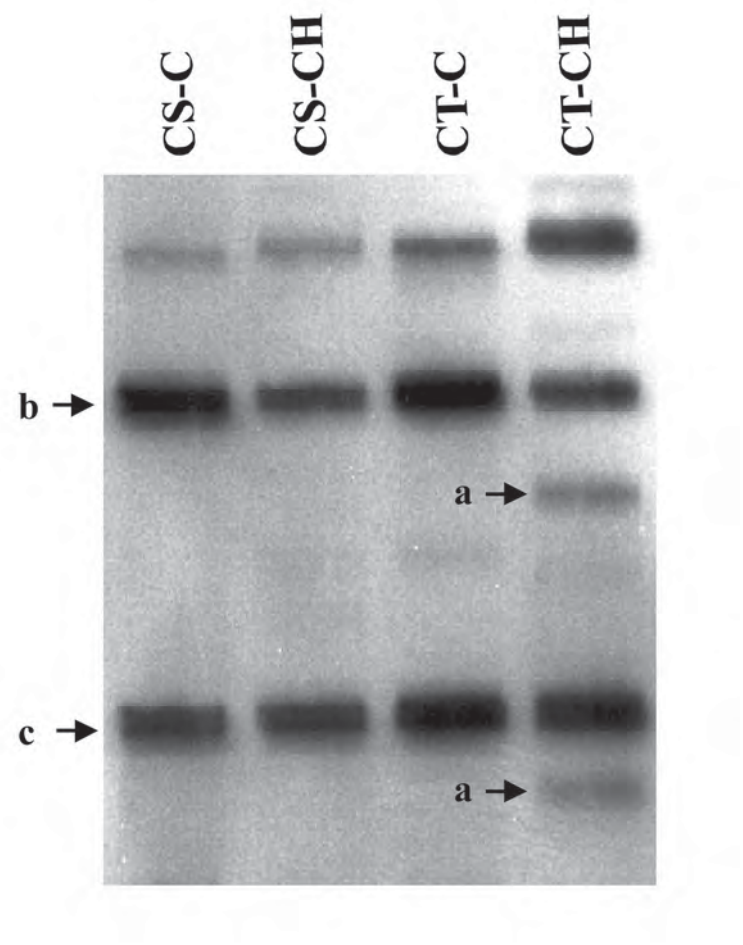

Fig. 1. Representative pattern of the cDNA-AFLP displays changes in gene expression of chilling-tolerant (CT) and chilling-sensitive (CS) genotypes in response to chilling stress (CH) compared to control plants (C). (a) Transcripts induced only in CT genotype, (b) Transcripts repressed in both genotypes, (c) Transcripts constitutively expressed in both genotypes.

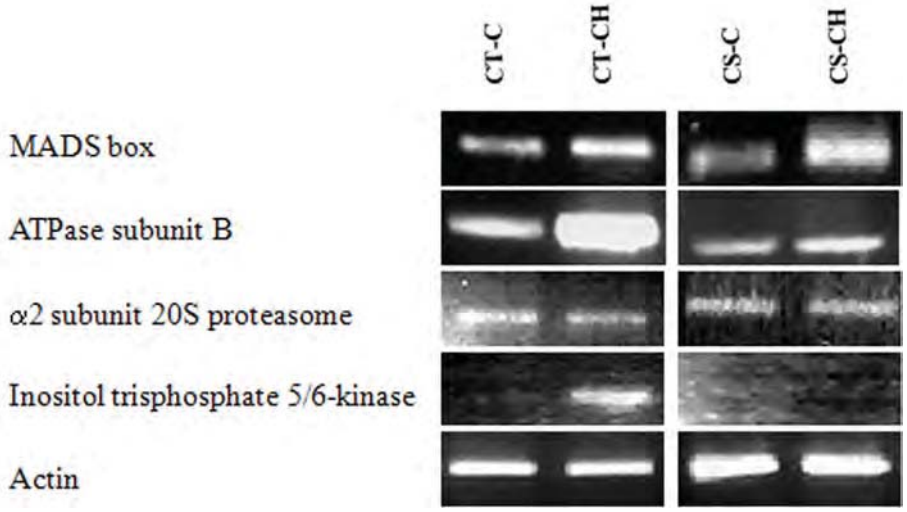

Fig. 2. RNA blot analysis for differentially expressed Transcript-Derived Fragments identified by comparing the chilling tolerant (CT) and chilling-sensitive (CS) genotypes under chilling stress (CH) against control (C) conditions. The actin gene was used as positive control for RNA loading.