EXPRESSION PROFILES OF STRESS RESPONSIVE GENES IN RICE (ORYZA SATIVA L.) UNDER ABIOTIC STRESSES

Aloka Lanka Ranawake1, Naoki Mori2 and Chiharu Nakamura2
1University of Ruhuna, Faculty of Agriculture, Mapalana, Sri Lanka
2Kobe University, Graduate School of Agricultural Science, Laboratory of Plant Genetics, Kobe, Japan

Correspondence to: Chiharu Nakamura
E-mail: nakamura@kobe-u.ac.jp

ABSTRACT
Abiotic stress is one of the major limiting factors reducing crop yield worldwide. We studied the expression profiles of 12 known stress responsive genes including transcription factor genes in japonica standard rice cultivar Nipponbare under low temperature, drought, NaCl and ABA stresses by RT-PCR in a time series up to 24 h. Expression of all the selected candidate genes was induced at least under one abiotic stress. The observed levels of expression of the same genes differed under different stress conditions; some were positively regulated under some stresses but negatively regulated under other stresses. Our results showed the presence of both specific and common gene regulatory pathways for these stresses and provide information on selective markers useful in evaluating cereal germplasms.


Keywords: abiotic stress, gene expression profiling, rice (Oryza sativa L.), transcription factors

Introduction
Abiotic stresses such as low temperature, drought or dehydration and high salinity are serious constraint on crop production. Plants use a variety of signal transduction pathways to regulate abiotic stress responses. Understanding the stress response mechanisms in plants therefore is a prerequisite for the development of new varieties of crops with durable levels of tolerance to these abiotic stresses. In both monocots and dicots including rice and Arabidopsis, the modes of expression of a large number of abiotic stress responsive genes, cDNAs and ESTs (expressed sequence tags) have extensively been studied under various environmental conditions (3, 9, 13, 14, 19, 20, 24, 25, 28, 31, 35). Among them, DREB (dehydration responsive element binding) and CBF (C-repeat/DREB binding factor) genes, AOX (alternative oxidase) genes, genes involved in alcohol fermentation, dehydration and sugar metabolism are important players.

Rice DREB genes (OsDREB1A, OsDREB1B, OsDREB1C, OsDREB1D and OsDREB2A) have been identified as low temperature, drought and high-salt responsive transcription factor genes in rice, all of which are orthologous to Arabidopsis thaliana DREB and CBF genes (6, 13, 15, 27, 33). WDREB1 and WDREB2 of wheat are also their homologs (7). Another transcription factor gene NAC6 (nitrogen assimilation control-type DNA biding protein) of rice is known to be up-regulated by low temperature, drought, NaCl and ABA stresses (16). OsAOX1a and OsAOX1b encode rice alternative oxidase proteins acting in mitochondrial alternative pathway and are up regulated by a variety of abiotic stresses including low temperature (11, 19, 22). Waox1a, a wheat ortholog of rice OsAOX1a, is induced not only by low temperature but also by inhibitors of the cytochrome and alternative pathways (29). Genes involved in alcohol fermentation are known to respond to abiotic stresses. Transcripts of ADH (alcohol dehydrogenase) and ALDH (aldehyde dehydrogenase) genes, which are involved in alcohol fermentation, are accumulated in response to abiotic stresses in rice and Arabidopsis thaliana (1, 5, 8, 12, 18, 28). Abscisic acid (ABA) is a phytohormone regulating various abiotic stress responsive pathways (36). Proteins inducible by dehydration and ABA are termed DHN (dehydrins) or RAB (responsive to ABA) (2). DHN is a group of late embryogenesis-abundant (LEA) proteins known to protect protein or membranes in plant tissues under abiotic stresses such as desiccation or low temperature (4, 17, 32). The protein phosphatase 2C gene (PP2C) encodes a class of evolutionarily conserved serine/threonine protein phosphatase involved in stress responses in yeasts, mammals and plants (26). Sugar transporter (SUT) genes have been found in plants including rice, and their structure, function and regulation have been studied (10).

In the present study we focused on 12 genes belonging to the above described groups including both trans-acting transcription factor genes and cis-acting down stream genes. We herein report expression profiles of these representative genes under low temperature, drought, NaCl and ABA stresses using seedlings of a standard japonica rice cultivar Nipponbare. Gene expression profiles were monitored by reverse transcriptase polymerase chain reaction (RT-PCR) at different time intervals up to 24 h under each stress.
TABLE 1

Primers used for gene expression analysis

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>OsDREB1A</td>
<td>5' UCG AGC AGA GCA AAA UCA ACB GU 3'</td>
<td>5' AUC GGA AGC CAG AAA AGA GA 3'</td>
</tr>
<tr>
<td>OsDREB1B</td>
<td>5' AUG GAG GUG GAG GAG GCG GC 3'</td>
<td>5' GUC CUC CCA CCA CGC UCC GG 3'</td>
</tr>
<tr>
<td>OsNAC6</td>
<td>5' GGA TTT GAT TAG ACA GAG GA 3'</td>
<td>5' TTT TGT AIT AAT TCC ACG CT 3'</td>
</tr>
<tr>
<td>JRC0549</td>
<td>5' TAG CTG TAG TAA TCG ATC 3'</td>
<td>5' TAC AAT AAT GAT TGC CCG 3'</td>
</tr>
<tr>
<td>OsAldh2a</td>
<td>5' ATT GTG TGT GTG GTG AAT ATT ATT ATT 3'</td>
<td>5' AAT AAC ACG TCG TAT AAT AAT AGT 3'</td>
</tr>
<tr>
<td>OsAOX1a</td>
<td>5' TAG GAG GUG GUG GAG GAG GCG GC 3'</td>
<td>5' GUC CUC CCA CCA CGC UCC GG 3'</td>
</tr>
<tr>
<td>OsAOX1b</td>
<td>5' TCA TCA TAC ATC AAC GGG CGA TGC 3'</td>
<td>5' TGG GCA CGG GTC AGC CCA CCG CCA 3'</td>
</tr>
<tr>
<td>OsADH</td>
<td>5' ATT ATG GTG TTG GTG AAT AAG ATT 3'</td>
<td>5' AAT AAC ACG TCG TAT AAT AAT AGT 3'</td>
</tr>
</tbody>
</table>

PCR program for OsDREB1A, OsDREB1B, OsNAC6, OsDHN, OsPP2C, JRC0549 and OsSUT: 94 °c for 5 min, 94 °c for 30 sec, 55 °c for 30 sec, and 72 °c for 30 sec; for OsAOX1a, OsAOX1b, OsAldh2a and OsAldh2b: 94 °c for 5 min, 94 °c for 1 min, 55 °c for 1 min, and 72 °c for 30 sec; for OsADH: 94 °c for 5 min, 94 °c for 1 min, 50 °c for 1 min, and 72 °c for 30 sec.

TABLE 2

Classification of 12 abiotic stress responsive genes based on the mode of induction and expression profiles according to Rabbani et al. (20)

<table>
<thead>
<tr>
<th>Classification</th>
<th>Low temperature</th>
<th>Drought</th>
<th>NaCl</th>
<th>ABA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td></td>
<td></td>
<td>OsDREB1A</td>
<td>OsHDN</td>
</tr>
<tr>
<td></td>
<td>OsDREB1A</td>
<td></td>
<td>OsDREB1B</td>
<td>OsSUT</td>
</tr>
<tr>
<td></td>
<td>OsDREB1B</td>
<td>OsAOX1a</td>
<td>JRC0549</td>
<td>OsSUT</td>
</tr>
<tr>
<td></td>
<td>OsAOX1a</td>
<td>OsAOX1b</td>
<td></td>
<td>OsSUT</td>
</tr>
<tr>
<td>Group 2</td>
<td>OsAOX1b</td>
<td>OsAOX1a</td>
<td>OsDHN</td>
<td>OsDHN</td>
</tr>
<tr>
<td></td>
<td>OsAldh2a</td>
<td>OsAldh2a</td>
<td>JRC0549</td>
<td>OsDHN</td>
</tr>
<tr>
<td></td>
<td>OsAldh2b</td>
<td>OsAldh2b</td>
<td></td>
<td>OsDHN</td>
</tr>
<tr>
<td>Group 3</td>
<td>OsADH</td>
<td>OsAldh2a</td>
<td>OsAOX1a</td>
<td>OsNAC6</td>
</tr>
<tr>
<td></td>
<td>OsDHN</td>
<td>OsAldh2a</td>
<td>OsAldh2b</td>
<td>OsPP2C</td>
</tr>
<tr>
<td></td>
<td>OsNAC6</td>
<td>OsAldh2a</td>
<td>OsNAC6</td>
<td>OsADH</td>
</tr>
</tbody>
</table>

According to the classification by Rabbani et al. (20) group 1 contains genes whose induction is rapid and transient, i.e. they reach maximum levels at 1 to 2 h after stress and then decrease. In group 2, gene expression increases within 1 to 2 h after stress and thereafter the level remains fairly constant. Group 3 shows maximum levels of expression at 5 to 10 h after stress and then decreases, while group 4 shows gradual increase in their gene expression to reach maximum levels at 24 h.

Materials and Methods

Plant material

Dehulled seeds of a japonica standard Nipponbare cultivar were subjected to overnight imbibition to stimulate synchronous germination. Imbibed seeds were surface-sterilized with 1% (w/v) solution of sodium hypochlorite (NaClO) for 10 min and rinsed in distilled water. Sterilized seeds were allowed to germinate for 3 days on wet blotting papers in glass Petri dishes (70-mm-diameter) in a dark incubator adjusted at 35 °c. Germinated seeds were planted in soil-filled trays and kept at 28 °c with 16 h/8 h light/dark cycles for one week.

Abiotic stress and ABA application

For low temperature stress, one-week-old seedlings were subjected to cold treatment at 4 °c in the dark. For drought stress, seedlings were dried at 25 °c after they were carefully uprooted and excess soils around the root system were washed...
away. For salinity stress, seedlings were placed in 400 mM NaCl solution in Petri dishes at 25 ºC. For ABA treatment, seedlings were sprayed with 200 µM exogenous ABA and kept at 25 ºC. After all these stress treatments, leaf samples were collected serially at 0 h, 0.25 h, 0.5 h, 1 h, 2 h, 3 h, 4 h, 6 h, 8 h, 12 h and 24 h.

**RT-PCR analysis**

Primers for amplifying fragments of *OsDREB1A* and *OsDREB1B* were designed by DNASIS. Primer information for *OsAOX1a* and *OsAOX1b* was kindly provided by Dr. Mikio Nakazono, Tokyo University (22). Primers for *OsDHN* and *OsPP2C* were synthesized based on EST clones JRC0022 and JRC0121, respectively (20). Stress responsive EST clone JRC0549 was also used for expression analysis. Primers for *OsNAC6* and *OsSUT* were designed based on EST clones JRC0528 and JRC2455 (20). Seedling leaves were collected, frozen with liquid nitrogen and were then subjected to RNA extraction using guanidine thiocyanate. The amount of transcripts was determined by RT-PCR analysis using a first strand cDNA synthesis kit (TOYOBO, Osaka, Japan). The total template RNA samples for the cDNA synthesis were treated with DNaseI to remove contaminated DNA. RT-PCR was performed with specific primers, which were designed and synthesized by Invitro Lifetech Oriental (Nacalai). Primer information is shown in Table 1. For each sample 4 µl cDNA template was added to 16 µl reaction mixture containing 1 µl of each forward and reverse primer, 2 µl 10x buffer, 0.8 µl MgCl₂, 1 µl dNTPs, 10 µl Q water and 0.2 µl rTaq Polymerase to make a 20 µl PCR mixture. RT-PCR was carried out by amplification with 22 cycles under conditions described in Table 1 using a thermal cycler, Gene Amp PCR System 9700 (Applied Biosystem). For each sample, 4 µl of cDNA template was added to 16-µl reaction mixture. Rice actin gene was used as a control. RT-PCR products were resolved on 1.2% or 2.0% agarose gel, stained with ethidium bromide, and pictures of images were taken under UV light.

**Results and Discussion**

We studied the expression profiles of 12 representative abiotic stress responsive genes using seedlings of a standard *japonica* rice cultivar Nipponbare subjected to four different abiotic stress conditions. The results of the time course study of gene expression in response to low temperature, drought,
OsDREB1A and OsDREB1B occurred under the low temperature stress. Two paralogs each of OsDREB1A and OsDREB1B showed a similar response to low temperature. In a previous study, we showed that both OsDREB1A and OsDREB1B responded more strongly to the low temperature stress than to the other three stresses and that a low temperature tolerant japonica cultivar accumulated more abundant OsDREB1 transcripts than a sensitive indica cultivar (21). These results agree with the findings that CBF/DREB1 transcription factors are key regulators of the cold signal transduction in various plant species (6, 7, 13, 15, 27, 30, 31, 33, 35). Alternative oxidase proteins are up-regulated by a variety of abiotic stresses including low temperature in rice (11, 22), wheat (29), Arabidopsis (23) and others (19). OsAOX1a did not respond to any of the four stresses but OsAOX1b showed marked response to the low temperature stress. Similar patterns of AOX1a and AOX1b expression were reported under low temperature in etiolated rice seedlings (22).

Under the drought stress, transcripts of two paralogs of OsAOX1 genes, OsDHN and JRC0549, showed maximum levels at 5 to 10 h and then decreased, while those of OsAldh2a, OsNAC6 and OsPP2C genes showed steady accumulation. By contrast, dehydration stress markedly inhibited OsDREB1A transcript accumulation. The salinity stress given by NaCl treatment led to rapid and temporal accumulation of OsDREB1A transcript until 3 h, followed by a rapid decline thereafter. A similar rapid but temporal accumulation was observed in transcripts of OsAOXb1, two paralogs of OsAldh2b, and OsDHN. Transcription of Arabidopsis thaliana ALDH3I1 gene was induced by osmotic and oxidative stresses and over-expression of ALDH3I1 conferred stress tolerance in transgenic plants (12). Up-regulation of some ALDH genes by drought and high salinity stresses and ABA application was reported in rice, indicating that ALDH genes are involved in osmotic stress tolerance (8, 9). It was also reported that expression of OsAldh2a was induced under anaerobic conditions in rice (1). Transcripts of both OsNAC6 and OsPP2C accumulated steadily under the drought stress.

The signaling pathways regulating low temperature, drought and salt stresses are largely overlapped and mediated by ABA (3, 14). ABA application caused some increases in the transcripts of OsADH, OsDHN, OsSUT and OsNAC6. Sugars are distributed into different tissues via transporters and play...
important roles in biological reactions in plants. Under abiotic stress conditions, plants accumulate sugars as a means to increase stress tolerance (34). In fact, OsNAC6 was reported to be induced by all four abiotic stresses (16), agreeing with our present result. ABA treatment however caused marked inhibition of OsDREB1A and OsDREB1B.

Overall expression patterns of these 12 abiotic stress responsive genes are grouped into five classes using a Venn diagram based on induction and/or up-regulation by low temperature, drought, NaCl, and ABA stresses (Fig. 3). OsDHN and OsNAC6 were induced by all four stresses and two paralogs each of OsAOX1 and OsAldh2 were induced by all but ABA stresses. No gene was grouped into a class uniquely responsive to drought. Gene expression patterns of these genes were also categorized into four groups according Rabbani et al. (20), although some did not match the classification very well (Table 2). Taken together, our results showed differential expression profiles of different genes to different abiotic stresses, agreeing with the previous observation of the abiotic stress induced gene expression in plants including rice and Arabidopsis (13, 14, 19, 20, 24, 25, 35, 36). These differential gene responses to various abiotic stresses likely confer high adaptability upon plants grown under different environments.

Conclusions
Gene expression analysis provides valuable information on how plants respond to abiotic stresses through particular or common signal transduction pathways. It also helps evaluate the levels of contribution of particular genes to relevant stress tolerance at different growth stages. We studied the expression profiles of 12 representative genes in rice under low temperature, drought, NaCl and ABA stresses. RT-PCR analysis in a time series up to 24 h revealed differential expression of genes under different abiotic stresses, showing the presence of both specific and common regulatory pathways for these abiotic stresses. Our results provide information on selective markers that might be useful in evaluating cereal germplasms.

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REFERENCES