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Identification of Genes Induced by Low Temperature in Rice

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Abstract : Exposure of seedling of rice (*Oriza sativa* cv.Dongin) to cold stress (6℃, 7day) induced differential gene expression. Differentially expressed polyadenylated RNA induced by low temperature were isolated and identified from the leaves of rice (*Oriza sativa* cv.Dongin) seedling by using the technique, differential display of reverse transcription through polymerase chain reaction (DDRT-PCR). Four bands of cDNAs were differentially displayed on the PAGE gel through DDRT-PCR, and among them three bands were those of overexpressed genes while one band was of an underexpressed gene. One of the overexpressed cDNA was characterized. The size of the DDRT-PCR product was found to be about 200 bp. The sequence of the cloned DNA was compared with those of GenBank through a BLAST E-Mail server, and it was found to have no homologies in the nucleotide sequence with that of any known DNA; therefore, it was designated as RC101. The expression of the cold-stress induced-gene, RC101, was sustained with Northern Blot analysis by using the cloned DDRT-PCR product as a probe.
Key words : cold-induced gene, cold acclimination, rice, differential display of reverse transcription-polymerase chain reation (DDRT-PCR), differential expression.

Rice is one of the major staple articles of food in the world. Nevertheless, it is cultivated in very limited area of the earth under diverse ecological conditions. The common environmental stress affecting growth of rice is the irregular exposure to low temperature. Therefore, the ability of rice to resist low temperature (below 10℃) during growth is an important agricultural property. For example, the cultivars of the Indica subspecies of rice are often damaged by low temperature below 10℃ (Binh and Ooao, 1992). However, the mechanism of chilling tolerance has not been sufficiently understood yet. Thus, a variety of plants have been studied to investigate the mechanism responsible for chilling tolerance. Many plants can acquire increased chilling tolerance during exposure to nonfreezing low temperature (Murata and Nishida, 1994). The process of adaptation to cold, termed as cold-harderning or cold acclimation, has been known to involve changes in gene expression, including the synthesis of new transcripts and polypeptides. So far, several cold-induced genes have been reported to be cloned and characterized (Cattivelli and Bartels, 1990; Binh and Ooao, 1992). Recently, cDNA clones of several cold-induced transcripts have been isolated from alfalfa, maize, arabidopsis, barley etc. (Gilmour *et al.*, 1988; Mohapatra *et al.*, 1988; Cattivelli and Bartels, 1990; Murata and Nishida, 1994). The cold-induced proteins were proposed to play a role in acclimation of the plants to low temperature (Mohapatra *et al.*, 1988; Hong *et al.*, 1994; Murata and Nishda, 1994; Shinozaki and Shinozaki, 1994; Bergantino *et al.*, 1995).

In general, cultivation of rice has been confined to the subtropical area. The aim of the current study was focused on the molecular cloning and characterization of cDNAs of transcripts that accumulated in the seedling of the chilling-tolerant cultivar, Dongjin at low temperature to expand the arable area of rice to a colder area of the earth. An efficient method to screen differentially expressed genes the DDRT-PCR technique (Peng and Pardee, 1992), was applied, and the changes in the expression of genes were investigated.

Materials and Methods

The Seeds of *Oriza sativa cv.* Dongjin were soaked in a 1% sodium hypochlorite for 15 min, washed with sterilized DDI water three times. The sterilized seeds were

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immersed in sterilized water and grown in a growth chamber at 28℃ for three days until germination. The sprouts were planted in several flower pots containing vermilculate and cultivated with sufficient watering in a growth chamber at 28℃. After a week of cultivation, some of the flower pots were transferred to a cold room at 6℃ and treated with a cold stress for a week. The other pots were kept at 28℃.

The leaves of the cold-treated plants along with non-treated plants were frozen with liquid nitrogen separately and ground to a powder. Total RNA was isolated using the method developed by Chomczynski and Sacchi (Chomzynski and Sacchi, 1987) with the RNAzol B RNA isolation Kit (Biotecx Laboratories, Co).

The total RNA was subjected to a reverse transcription reaction with an oligo- dT ($T_{12}MG$) primer by using the methods of Liang and Pardee (1992). Two hundred ng (0.1 μg/μl of freshly diluted solution) of total RNA was mixed in reverse transcription buffer with 20 μM dNTP and 1 μM $T_{12}MG$ to make a total volume of 19 μl. The mixture was incubated at 65℃ for 5 min and at 37℃ for 10 min. After adding 1 U of MMLV reverse transcriptase to the mixture, it was mixed well and incubated at 37℃ for 60 min. The reaction was stopped by heating at 95℃ for 5 min.

The single strand of cDNA was used as a template to amplify the DNA in a thermal cycler by polymerase chain reaction (PCR). The reaction was carried out with incubation in a thermal cycler at 94℃ for 30 s, at 40℃ for 20 min, at 72℃ for 30 s. The reaction was repeated for 40 cycles and followed by an additional extention with an incubation at 72℃ for 5 min.

The PCR products were incubated at 80℃ for 2 min and chilled on ice. The DNA fragments of the PCR products were analyzed by electrophoresis on a standard Urea-PAGE gel at 60 W. The gel was dried in a vacuum gel dryer and autoradiographed with exposure on an X-ray film in a dark room for 72 h.

One of the sustained bands of the fragment cDNA was excised out of the gel with a sterilized razor blade and was subjected to a reamplification reaction. A reaction mixture of 20 μM dNTP, 0.2 μM arbitrary primer, 1 μM $T_{12}MG$, 1 U of Ampli Taq (Perkin-Elmer, Norwalk, USA), PCR buffer, and the extracted cDNA was mixed to make a final volume of 40 μl for each primer set combination and was incubated in a thermal cycler for a reamplification reaction. The PCR reaction was carried out at 94℃ for 30 s, at 40℃ for 2 min, and at 72℃ for 30 s, and the whole procedure was repeated for 40 cycles. The reaction was followed by an additional elongation at 72℃ for 5 min. The resulting PCR products were validated by an electroporesis on a 1.5% agarose gel.

The reamplified cDNAs were extracted from agarose gel by using a Jetsorb Kit (Genmed. Co) and were inserted into the pGEM-T vector (Promega). The recombinant DNA was used to transform the JM 109 cells. The transformed cell bearing the insert (white colony) was isolated in a plate (LB/Amp/IPTG/X-Gal), and the recombinant DNA was validated. Manual nucleotide sequenceing of the insert DNA was carried out by the standard dideoxy chain termination method using a sequenase version the 2.0 DNA sequencing kit (USB Biochemical) with SP6, T7 promoter primers.

For Northern blot analysis, 20 μg of total RNA was loaded on a formaldehyde / 1% agarose gel, and an electrophoresis was carried out at 150V. The electrophoresed RNAs were transferred to a nylon membrane and crosslinked in a UV-crosslinker (UV Stratalinker 1800). The [α-^{32}P] dNTP labeled probe of cDNA was hybridized to the membrane for 17 h and autoradiographed (Maniatis *et al.*, 1989).

Results

Among the strains tested, Dongjin appeared to be the more cold-tolerant cultivar than others such as Chilsung and Hyangdo. Therefore, Dongjin was chosen to study the effect of cold on the expression of cold-induced genes. Cold-treatment on rice caused a growth retardation when compared with that of the normal condition (28℃) resulting in very short height and narrow leaves.

The total RNA was extracted and used in a reverse transcription reaction using $T_{12}MG$ as primers. Two different combinations of primer sets made of one anchored oligo dT primer ($T_{12}MG$: 5'-TTTTTTTTTTTTMG) and two kinds of short arbitrary primers (two 10-mers, AP6; 5'-GCAATCGATG-3', AP7; 5'-CCGAAGGAAT-3') were used for the PCR essentially as described by Liang and Pardee except for modifying the annealing temperature from 42℃ to 40℃. With the combination of $T_{12}MG$ and AP7 primers, four bands of differentially expressed genes by the cold-treatment were displayed on the DDRT-PCR gel (Fig. 1). Three of them were those of overexpressed genes (cold-induced genes), and one was that of an underexpressed gene (cold-depressed gene).

One of the overexpressed gene (O3: Fig. 1) was excised from the polyacrylamide-urea gel and subjected to a reamplification reaction with the same primer set of $T_{12}MG$ and AP7. For some unknown reason, the DNA appeared to be two separated bands on a 1.5% agarose gel electrophoresis (Fig. 2). One was about 220 bp estimated by the marker on the gel, and the other was 500 bp. One of the DNA (220 bp) was used to make a recombinant DNA with pGEM-T. After validation of the

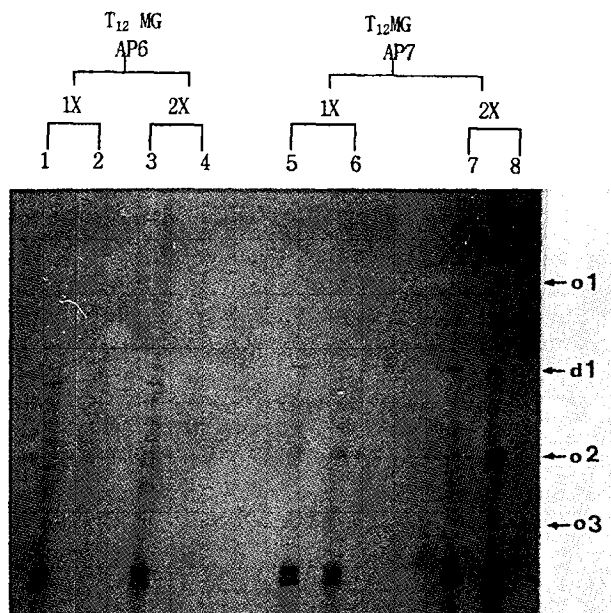

Fig. 1. Differential display of mRNA's from a control *Oryza sativa cv.* Dongjin vesus the cold-treated *Oryza sativa cv.* Dongjin. The purified total RNA was reverse transcrived with T12MG. Two different combinations of primer sets made of one anchored oligo dT primer(T12MG5'-TTTTTTTT TTTTMG) and two short arbitary primers(10-mers. AP6: 5-GCAATCGATG-3; AP7: 5-CC GAAGGAAT-3)were used for the PCR reactions. The odd-numbered lanes represented the total RNA from control cultivar while the even-numbered lanes represented the mRNA from the cold-treated cultivar. The cDNA tags that appear to be differentially expressed were marked by arrowheads. (d1: the DNA band of depressed gene; o1, o2, o3: the bands of over-expressed gene)

recombinant DNA based on size, the recombinant DNA was used to analyze the base sequence of the insert DNA, and the resulting nucleotide sequence appeared

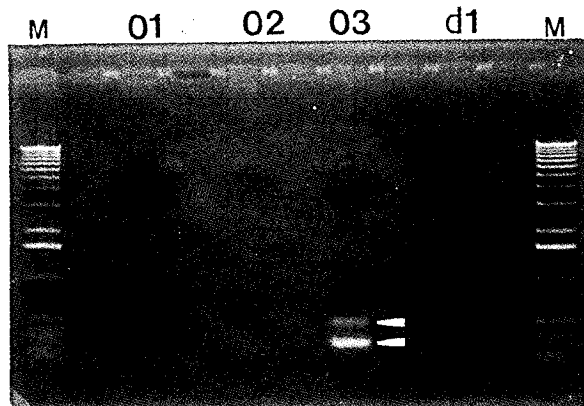

Fig. 2. Electrophoresis of the specific reamplification products from differentially expressed cDNA template with primer set of T_{12}MG AP7 (5-CCGAAGGAAT-3) on 1.5% agarose gel electrophoresis (M, 1 kb ladder; O1, O2, and O3, reamplified cDNAs).

NGGGGAGGTT TTTCCAGTGA GCCATGTCAG ATTTGGTACA
TAGAGGATCT CATGTTTTTG GAACTTTAGA GTCGCCATTC
TTTTCTTTGT AGTGTCGATG TTAAATTGAT TATAGGTGAT
AGTGATATTC TGGGCCATAT GTTGTTGACT CGGTATGGAT
TATTGGTTAT TGAATGTCTA TGCTTGTGCC AAAAAAAAAA
AAAA

Fig. 3. Nucleotide sequence of the DDRT-PCR product.

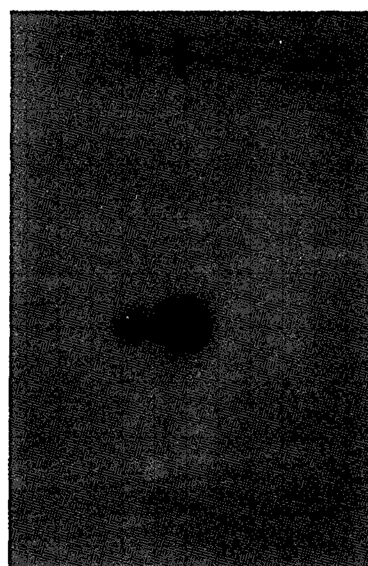

Fig. 4. Northern blot analysis showing the difference in the expression of the cold-induced gene, RC101 (lane 1: The control Dongjin cultivar, lane 2: The cold-induced Dongjin cultivar).

to be around 200 bp, as shown in Fig. 3.

When the sequence of the cloned DDRT-PCR product (Fig. 3) was compared with that of GenBank through a BLAST E-mail server, it appeared to have no homology in nucleotide sequence to that of any known DNA. The expression of a differentially expressed gene by cold was sustained by Northern blotting, as shown in Fig. 4.

Discussion

Many plants can acquire cold-tolerance named cold-acclimation when they are exposed to a nonfreezing low temperature (Murata and Nishida, 1994). The mechanism of cold-acclimation has not been fully understood; however, it has been predicted that plants can adopt to cold-stress through synthesis of diverse proteins (Mohapatra *et al.*, 1987; Gilmour *et al.*, 1988; Hong *et al.*, 1994; Bergantino *et al.*, 1995).

As a matter of fact, changes in the protein synthesis pattern and mRNA levels in the leaves of cold-sensitive and cold-tolerant rice cultivars have been reported by Hahn and Walbot (1989). In addition, recently genes

which code the proteins regulating cold-acclimation have been cloned and characterized (Cattivelli and Bartels, 1990).

In our study, four genes (Fig. 1) were appeared to be regulated by the cold treatment. Among the genes, three of them were induced to be overexpressed (O1, O2, O3; Fig. 1) and one of them was underexpressed (d1; Fig. 1). Therefore, it may be said that the cold-treatment in Dongjin cultivar successfully induced expression of several genes to adopt to the cold-stress. The DNA of one of the overexpressed genes on the DDRT gel (O3; Fig. 1) was chosen for characterization. The sequencing data are shown in Fig. 3, and the size of the DNA was around 200 bp, which does not exactly coincide with the value estimated from the gel electrophoresis. There was a substantial difference in the size of the DNA between the estimated value from gel electrophoresis and the size determined from the sequencing gel for some unknown reason; however, the small difference between estimation and sequencing could be acceptable. When the sequence of the cloned DDRT-PCR product was compared with those of GenBank, there found to be no homology in the nucleotide sequence to that of any known genes, so that it was temporarily designated as RC101.

The expression of cold-induced gene(RC101) was successfully confirmed by Northern analysis using mRNA from a cold-treated Dongjin cultivar (Fig. 4).

In conclusion, the response of Dongjin cultivar to a temperature of 6℃ alters the expression of genes. One of the cold-induced DNA was successfully isolated and characterized, and the expression of the cold-induced gene, RC101, was confirmed by Northern analysis. Work is now in progress to analyze the altered relative pattern of protein synthesis, to screen more relevant cold-induced genes, to clone full-length cDNA, to construct cDNA library, and to find another mechanism of tolerance against cold stress e.g., desaturase(Navendra et al.,1993).

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