Cloning and Expression Analysis of OsNADPH1 Gene from Rice in Drought Stress Response

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Abstract: An experiment was conducted to compare the mRNA expression difference in rice leaves and roots under drought stress and normal conditions using Fluorescent Differential Display (FDD) method. One positive fragment was isolated by combination of the H. A. Yellow-PAGE (contained 0.1% H. A. Yellow) separation and macroarray screening methods. Compared to Arabidopsis thaliana NADPH oxidoreductase gene, it has 96% identity. The cDNA was 1423 bp, and contained a complete open reading frame of 1048 bp encoding a protein with 345 amino acid residues. Moreover, the gene expression level was higher under drought stress than that under normal conditions. The possible role of NADPH oxidoreductase gene under drought response was also discussed.

Key words: rice; drought stress; NADPH oxidoreductase-like gene; cloning; gene expression

Drought resistance study in rice has long been one of the important scientific research programs in cereal crops [1-3]. At present, the finding of novel drought genes is common in rice genetic resource development and varietal improvement. According to the action modes, the drought resistance genes can be divided into two classes [4]. The first class constitutes functional genes coding for such enzymes as late embryogenesis abundant protein (LEA) [5], △1-pyrroline-5-carboxylate synthetase (P5CS) [6-7], ornithine-δ-aminotransferase [8], trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase [9] etc. The second one is the collection of regulatory genes such as a transcription factor gene coding for DREB [10-12]. NADPH oxidoreductase gene belongs to the first class, which is a functional gene, and plays an important role in plants suffering from environmental stresses. In Arabidopsis thaliana, this gene has shown a very strong sensitivity to oxidization, high salt, drought, high and low temperature and the non-biological stresses as well [13-14]. Moreover, it plays an important role in oxidant defense response and to control the dynamical balance of NADPH/NADP.

Plant cells produce active oxidative species (AOS) due to the environmental stresses, in particular H2O2. Hung et al [15] alleged that NADPH oxidase and peroxidase was H2O2-generating enzymes in ABA-treated rice leaves. Miao et al [16-17] stated NADPH oxidoreductase as a key enzyme to catalyze and generate superoxide anion. In addition, it might induce stomatal guard cell to generate H2O2 by abscisic acid (ABA). Though a lot of work have been in efforts to elucidate the relationship between NADPH oxidoreductase and osmotic stresses [18-21], but little is known on the participation of NADPH oxidoreductase in signal transmission and gene expression due to the complicated physiological changes of plants subjected to stressed environments and the presence of NADPH oxidoreductase in large number of reactions. In particular in rice, no report so far referring the relationship between gene expression/manipulation and stressed environments, such as drought, high salt and low temperature. In this research, rice seedling was subjected to drought stress. A NADPH oxidoreductase-like gene had been cloned by using Fluorescent Differential Display (FDD) method, while the regulatory expression pattern of this gene under drought stress had also been assayed by RT-PCR. Moreover, the function of the NADPH oxidoreductase-like gene had been also discussed.
MATERIALS AND METHODS

Plant materials and treatments

Before sowing, the seed of Nipponbare (Oryza sativa L. subsp. japonica) was soaked in water and germinated in an incubator at 25°C in a growth chamber until 3-leaf stage. The healthy seedlings were planted on dry quartz sand for drought treatment. The roots and young leaves were collected 0 min (control), 15 min, 1 h, 2 h, 4 h and 6 h later respectively, which had been frozen in liquid nitrogen and stored at -80°C for RNA isolation.

Methods

RNA isolation

Total RNA was extracted using the method described in Molecular Clone Experimental Manual [22]. The RNA was isolated by acid phenol-guanidinium thiocyanate-chloroform and purified with LiCl precipitation. Gel analysis of RNA was performed according to Sambrook et al [23]. DNase was added to eliminate DNA contamination.

mRNA differential display and gene fragment cloning

cDNA was synthesized and the reaction system was adjusted according to the fluorescence differential display kit (TaKaRa). Nine anchor primers and 24 random primers were combined in the first PCR reaction. The PCR was performed as follows. Step 1 (1 cycle): 94°C for 1 min, 94°C for 2 min, 38°C for 5 min, and 72°C for 5 min; Step 2 (35 cycles): 94°C for 30 s, 38°C for 2 min, 72°C for 1 min; Step 3: 72°C for 8 min; 4°C for storing. After the reaction, an equal volume of loading-buffer with 7 mol/L urea PAGE (5.4% PAGE) was added immediately to migrate, and the DNA bands were visualized with a fluorescence scanner (BAS-3000, FujiFilm).

The differentially displayed bands were excised from the gel and the DNA fragments were eluted from the gel slices by soaking the gel with 50 µL of ddH2O in micro-centrifuge tubes for 10 min. The tubes containing the recovered DNA were then placed at 100°C water-bath for 10 min and subjected to centrifugation. The supernatant was decanted and used as template for the second round of PCR reaction. Fragments were amplified in reference to anchor primers and random primer, which were used in the first reaction. Step 1: 94°C for 1 min (pre-treatment); Step 2 (15 cycles): 94°C for 30 s, 40°C for 2 min, 72°C for 1 min, Step 3: 72°C for 3 min, 10°C for storing. Then the samples were separated by using urea PAGE containing 0.1 % H. A.-Yellow (TaKaRa) and the main band was excised from the gel and used as the substrate for the third round of PCR reaction and the same procedures were followed as in step 2 except the number of cycles which was 42. After finishing the PCR reaction, 1 µL of each amplified product was pipetted onto nylon membrane and exposed to UV for 2 min after dried in normal conditions. The samples were then used for macroarray screening. The positive colonies were screened with dUTP-dig probe synthesized by M-MLVRT enzyme (Promega) using 3 µg of mRNA. The residual PCR products were recovered by electrophoresis on 1.5 % (W/V) agarose gel. The interest DNA fragment was purified with Glassmilk (BIO 101), ligated to T-easy vector and finally transferred into Escherichia coli JM109.

Sequencing and sequence analysis

Plasmid DNA was isolated with QIAprep Miniprep Kit (QIAGEN), and samples were adjusted using CEQ™ DTCS Quick Start Kit (Beckman) and sequenced by Beckman Coulter CEQ 8000 (BECKMAN 8000, Beckman). The homology analysis was blasted on DNAMAN and NCBI Websites.

Semi-quantitative RT-PCR

The first-strand cDNAs were synthesized by Revertra Ace (MMLV Reverse Transcriptase RNaseH-(TOYOBO, Tokyo, Japan) according to the manufacturer’s protocol. Each reaction encompassed 20 µL of reaction mixture containing 1 µg of total RNA. For each RT-PCR reaction, a pair of rice β-actin primers was treated as an internal PCR control: 5′-GAACTGGTATGGTGCAAGGCTG-3′ and 5′-ACA CGGAGCTCGTTGJAGGAG-3′. Two specific primers, namely 5′-AGGGCAAACAATCACCCCAAGGAA CG-3′ and 5′-AACAGGAAGAGGACTACAGAAGG CGG-3′, were designed using the software primer primer 5.0. All genes were subjected to PCR.
reactions with GeneAmp PCR System 9700 in 26 cycles: 95°C for 5 min (pre-denaturation), 94°C for 30 s, 60°C for 40 s, and 72°C for 60 s, and finally at 72°C for 10 min for extension. Then 8 µL of each PCR product was subjected to electrophoresis on 1.5% (W/V) agarose gel with 0.01 % (W/V) ethidium bromide. The gels were digitally photographed with the BIO-RAD system (Bio-Rad, Hercules, CA, USA).

**RESULTS**

**Sequence analysis**

An interest DNA fragment of about 700 bp was obtained by FDD. After sequencing, the fragment was ligated to the RACE fragment sequence, generating a sequence of 1423 bp in length. The results of homology search in GenBank indicated that it was located on chromosome 4, which harbors the BAC clone named OSJNa0067K08 (the accession number is AL606627), and its function has not yet been reported. The full-length cDNA obtained by cloning might be a putative NADPH oxidoreductase gene [24-25]. We named this gene \textit{OsNADPH1} temporarily. Through Motif search (http://motif.genome.jp/), a potential NADP binding-site and a zinc-binding-dehydrogenase motif.

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Fig. 1. Nucleotide sequence of \textit{OsNADPH1} and the putative amino acid sequence.

Fullface indicates potential NADP banding site; Underline indicates zinc-binding-dehydrogenase motif.
zinc binding dehydrogenase motif were discovered in 149-166 (boldface in Fig.1) and 155-302 (underlined in Fig.1) regions, respectively. Therefore, the translational product of this gene might be responsible for functioning of NADPH oxidoreductase.

Homology analysis

As shown in Fig. 2, OsNADPH1 shared a 65% homology in amino acid sequence with Nipponbare oxidoreductase zinc-binding dehydrogenase family (The accession number is ABA96821). The latter located on chromosomes 11 and 12 in rice was involved in disease resistance. The OsNADPH1 and probable NADP-dependent oxidoreductase P1 protein in Arabidopsis thaliana (The accession number is CAC01710) shared a 65% amino acid homology. The latter had possibly the anti-oxidant defense function or probably participated in manipulating of NADP/NADPH dynamical balance in plants. Moreover, OsNADPH1 and zeta-crystallin homologue in Arabidopsis thaliana (The accession number is CAA89262) shared a 65% homology in amino acid sequence. These results indicated that OsNADPH1 is a novel gene, which has been submitted to DDBJ, EMBL and GenBank, with an accession number of AB246888.

Expression analysis

RT-PCR analysis indicated that the expression level of OsNADPH1 gene increased gradually with the increase in treatment time (Fig. 3). The expression level reached its peak 30 min after treatment in leaves, with 4 times stronger signal than the control according to the results of semi-quantitative analysis (Bio-Rad GEL-Quantity One 1-D analysis software). The expression level changed slightly between 30 min and 2 h after treatment, and began to drop gradually after 2
and reached the value equivalent to that of control 6 h after treatment. The expression level in roots increased firstly and then decreased, being more sensitive than that in leaves. The level began to express rapidly after 15 min, which was 3-fold than that of control, and peaked at 1 h after treatment, as about 5-fold as that of control. Once reached the top level, then it dropped gradually to only 1/3 of that in control 6 h after treatment.

**DISCUSSION**

Water stress influences the vital activities of plants in several ways, such as disrupting cell walls, inhibiting normal growth, attenuating photosynthesis, and misbalancing endogenous hormone metabolism etc. In response to drought stress, plants have a set of defensive mechanisms in order to adapt the environmental variations, including recognition of stress signals, gene expression and plant adaptability. These defensive activities are undoubtedly aiming to ease the degree of damage caused by drought stress. During this study we found that the expression level of OsNADPH1 gene sharply rose within a short period of time, indicating its participation in induction of AOS (H2O2). In general, the activity of ribonuclease elevates as the length of drought-stressed increases. Meanwhile, DNA and RNA are degraded gradually, with the decrease in synthase activity. Thereby, the protein synthesis is blocked, resulting in a decrease of NADPH oxidoreductase content. The leaves and roots will dry gradually and even die if the drought period is further prolonged.

The water stress not only affects the efficiency of plasma membrane in redox system, but also reduces the oxidation rate of protoplasmic membrane NADP and NADPH in large scale. Moreover, AOS including H2O2, caused by water stress, may possibly have an effect on activities of various oxidation inhibitors and activating expression of genes related to defense and detoxication. It has been reported that H2O2 is mainly produced by PMRS, and the NADPH oxidoreductase is an important ingredient of PMRS. In the process of drought stress, NADPH oxidoreductase might be the direct electron donor in protoplasmic membrane. Usually, it produces H2O2, suggesting NADPH oxidase as one of the essential enzymes involved in H2O2 generation. Therefore, during the early phase of drought, the H2O2 content in plant may increase due to the elevating activity of NADPH oxidoreductase. However, the active mechanism(s) of OsNADPH1 in drought stress along with its function(s) will need to be further studied.

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**REFERENCE**


