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Cloning of *TaPRP* from Wheat and Its Cold Tolerance Analysis in Transgenic Tobacco

Zhang Baolei[1], Li Da[1], Zhang Weidong[1,2], Liu Dasheng[3], Gao Qingrong[1,2] & Tian Jichun[1,2]

[1] State Key Laboratory of Crop Sciences, Shandong Agricultural University, Taian City, Shandong Province, China

[2] Genetic and Breeding Department, Agronomy College, Shandong Agricultural University, Taian City, Shandong Province, China

[3] Institute of Environmental Sciences in Shandong Province, Jinan City, Shandong Province, China

Correspondence: Zhang Weidong, Genetic and Breeding Department, Agronomy College, Shandong Agricultural University, Daizong street 61, Taian City, 271018, Shandong Province, China. E-mail: zhangwd@sdau.edu.cn

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Abstract

TaPRP, a proline rich protein (PRP) cDNA, was cloned by RT-PCR from winter wheat. Nucleotide sequence analysis showed *TaPRP* is composed of 1137 bp (378 amino acid residues with a Mr of 42.19 kD). *TaPRP* shows 92.6%, 89.3%, 73.0%, and 73.3% sequence homologies with *PRP* genes from wheat, sorghum, rice, and maize, respectively. The deduced protein includes 170 prolines, presenting a normal *PRP* primary structure. Expression vector pBI-*TaPRP* was constructed, in which *TaPRP* was driven by CaMV35S promoter and stopped by NospolyA. Tobaccos were transformed by *Agrobacterium* containing the constructed vectors. Three transgenic lines were confirmed by PCR detection and Southern blot. Under the same low temperature stress conditions, transgenic plants had lower conductivity rate compared with the non-transgenic plants, suggesting that cold tolerance in transgenic tobacco plants was improved. However, the different transgenic plants showed significant differences in cold resistant, and there also existed significant interactions between plant and treatment temperature. *TaPRP* might have an important role in wheat in cold adaptation process.

Keywords: *TaPRP*, gene clone, genetic transformation, transgenic tobacco, cold tolerance

1. Introduction

Proline-rich proteins (PRPs) exist widely in plant kingdom and represent one of the classes of the so-called cell wall structural proteins (Showalter, 1993). PRPs were distinctive tissue-specific and possess many differentl biological functions in plant, mainly involving in the formation of cell wall. A wealth of information exists regarding the expression patterns of some of the PRP genes. Four proline-rich protein genes were cloned from *Nicotiana alata* and expressed in pollen tube or style (Chen et al., 1993). *MsPRP4*, a proline-rich early nodulin from *Mediccrgo trunccrtulcr*, highly expressed in nodule meristematic cells and might involve in the evolutionary relationships between fabaceous plants and azotobacter (Wilson et al., 1993). Many chemicals in plant affected the PRPs expression level, such as, plant hormones (auxin, cytokinins, gibberellin, ethylene, abscisic acid), jasmonic acid and salicylic acid (Datta et al., 1993; Subramaniam et al., 1994; Ogawa et al., 1999). And many environmental factors, including salt stress (Deutch et al., 1995), water stress (Yu et al., 1996), wounding (Yasuda et al., 1997), and bacterial and fungal infections (He et al., 2002), could also improve the expression level of PRP. In addition, although these proteins may also participate in several developmental processes, such as pollination, wound healing, tissue culture, and secondary metabolites, little information on their expression patterns in these regards (Wilson et al., 1993; Wyatt et al., 1994).

At present, many researches had been documented on the structures, properties and biological functions of the PRPs, and the expression characteristics of these PRPs genes had also been reported in different plant tissues or under environmental stresses. However, limited information is available on these proteins in response to cold stress in wheat. In an effort to understand the role of the protein responding to cold stress, a proline rich protein (PRP) cDNA, *TaPRP*, was cloned and transformed into tobacco by *Agrobacterium*. Electrolyte leakages under

cold treatments were analyzed in non-transgenic and transgenic plants to access these plants' cold tolerance. This research might be helpful to understand the functions of PRP proteins in plants, and lay foundation for the utilization of the proteins in wheat breeding.

2. Materials and Methods

2.1 Plant Materials and Bacterium Strain

The winter wheat cultivar "Shannong 12" was bred in Agronomy College, Shandong Agricultural University and was used as the experimental material. Seeds were surface-sterilized with 1% sodium hypochlorite for 5 min, washed with tap water, and soaked in distilled water for germination in incubator at 25 ^{o}C. After germination, Seedlings were grown in pot filled with sand and cultured with Hoagland nutrient solution under controlled conditions (28 ^{o}C day/23 ^{o}C night, 16 h photoperiod, 500 lmol m^{-2} s^{-1} photons, and 80% relative humidity). After 7 days of growth, the leaves of wheat were frozen in liquid nitrogen and preserved at -80 ^{o}C until use. *Nicotiana tabacun* variety NC89, *Escherichia coli* strains DH5α, and *Agrobacterium tumefaciens* strain LBA4404 were preserved in laboratory.

2.2 Tool Enzymes, Plasmids and Reagents

Trizol regent for total RNA extraction was bought from Invitrongen; Restriction enzymes including *Bam*HI, *Sac* I, and *Eco*R I, Taq DNA polymerase, DNaseI, T4 DNA ligase and cloning vector pGEMT-Easy were all bought from Shanghai Promega Company (Shanghai, China). The expressing vector pBI121 were preserved in laboratory. DNA marker, the plasmid separating kit and DNA reclaiming kit, DIG DNA Labeling and Detection Kit were provided by Boshang Biotech (Beijing, China). All other chemicals and molecular reagents were of analysis grade from TaKaRa (Dalian, China).

2.3 RNA Isolation and Cloning of TaPRP cDNA

Total RNA was extracted from wheat leaves with Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions, treated with RNase-free DNaseI, then the DNaseI was removed according to the instructions of the supplier. The RNA was quantified on a spectrophotometer and visualized on 1.2% agarose/formaldehyde gel to ensure high quality.

The first strand of cDNA was synthesized from 5 μg of total RNA by reverse transcription using Superscript RT II (Invitrogen) according to instructions. Several *PRP* genes in *Poacere* plants were obtained from literature and EST database, and DNA sequences were compared. Degenerate primers flanking the ORF frame were designed with Oligo 6.0 software and two restriction enzyme cutting sites, *Bam*HI and *Sac*I, were added into the primers respectively. The upper stream primer was: P1, 5′-GCG GAT CCA GCA ATG GC(T) GAG (A)GC A(G)C A(C)G C(G)C T-3′ and the down stream primer, P2: 5′-GAC GAG CTC GAG GCT (G)CC (A)GG ACG GGG (C)T-3′. A 25 μl reaction mixture for PCR amplification was prepared containing the following reagents: 10×PCR Buffer 2.5 μL, 5 mmol L^{-1} dNTP 2 μl, the two above primers 10 mmol/L 1 μL, 1.0 unit of Taq polymerase; 5 μL of cDNA sample and 16.5 μL of PCR-Grade Water. PCR conditions were 5 min at 94 ^{o}C followed by 30 cycles of 94 ^{o}C for 45 s, 56 ^{o}C for 1 min, 72 ^{o}C for 1 min and final elongation at 72 ^{o}C for 10 min.

PCR products were visualized by electrophoresis in 1% (w/v) agarose gels. The aimed amplification fragments were recovered and purified with DNA reclaiming kit. The recovered DNA fragments were cloned into pGEMT-Easy vector and transferred into *E. coli* DH5α. After restriction enzyme treatments, DNA sequencing was performed in Shanghai Bioengineering Company. The sequences were analyzed with DNAMAN 5.2.9 Demo version. The obtained cDNA was designated as *TaPRP*.

2.4 Construction of Plant Expression Vector and Gene Transformation

PCR products were digested with *Bam*HI and *Sac*I, and inserted into pBI121 vector with the same digestion. *TaPRP* expression box was constructed with CaMV35S promoter, *TaPRP* sequence and NOS polyA terminator (Figure 1). The expression vector was transformed into *E. coli* DH5α. The correct clone pBI121-PRP was examined by PCR amplification and sequenced by Shanghai Bioengineering Company. Plasmid extraction, digestion, electrophoresis, ligation and *E. coli* transformation were manipulated according to Sambrook et al. (1989).

The resulted plasmids pBI121-PRP were transferred into *Agrobacterium tumefaciens* LBA4404 by the liquid nitrogen freeze thaw method (Wang et al., 1998). The tobacco plants were transformed by *A. tumefacien*s mediated gene transfer with leaf discs transformation method and the transformed plants were selected by kanamycin. Six tobacco plants were obtained after selection in 100 mg/L kanamycin. The six transformed

tobacco plants and untransformed ones were all cultured on 1/2 MS medium. Shoots were generated from transformed callus after 3-5 weeks on selection medium. The shoots which grew into 2-3 cm were transferred for rooting on MS with 0.1 mg/L IAA, 200 mg/L-1Cefotaxime and 20 mg/L Hygromycin. The rooting plants growing into plantlets were transplanted into soil for continuous growth.

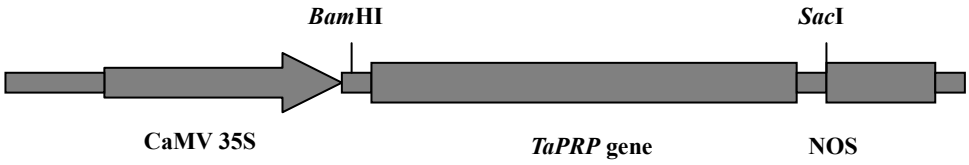

Figure 1. The structure of expression vector pBI121 with *TaPRP*

2.5 PCR Analyses and Blotting Analyses for Transformed Plants

Total plant DNA was isolated from leaves according to CTAB method (Roger et al., 1998). PCR identification of the gene was performed respectively using the DNA from the transformed plants as templates and non-transformed plant and pBI121 as control. Primers, PCR reaction mixture and conditions were the same as those in 1.3.

Southern blotting was carried out according to the Sambrook et al. (1989) method. The DNA samples were restricted with *Eco*R I after agarose gel electrophoresis and were transferred into nylon membrane and randomly labeled with DIG-dUTP according to the instruction of DIG DNA Labeling and Detection Kit.

Total RNA was isolated for RNA gel-blot analysis. Thirty micrograms of total RNA for each sample was separated by electrophoresis in 1% agarose for maldehyde gels. Northern blotting analysis was performed as described by Sambrook et al. (1989). To detect the expression pattern of *TaPRP*, specific probes derived from the coding region were designed. The probes were labeled using the PCR DIG probe synthesis Kit (Boehringer Mannheim, Germany). Before using Northern blotting, the specific respondents to *TaPRP* mRNA was conformed by the hybridization with its cDNA clone, and the probes specifically recognized the corresponding cDNA (data not shown). Non transgenic tobacco plants were as the control. Primers, PCR reaction mixture and conditions were the same as those in 1.3. A photograph was taken to expose the membrane for 5-15 minutes to an X-ray film (Fuji Photo Film Co. Ltd, Tokyo, Japan).

2.6 Cold Resistant Analysis for Transformed Plants

Cold resistance in tobacco plants was evaluated by measuring electrolyte leakage in leaves with EC324 conductivity meter as described by Jaglo-Ottosen et al. (1998). The T_0 generation transformed plants with pBI121-*TaPRP* and the plants transformed with pBI121 were cultivated as experimental materials. The leaves at similar position with similar size were taken out from transformed and non-transformed plants. The leaves were incubated in salt-water bath at 0, -8, -16 ^{o}C for 15 min with constant stirring. Then the leaves were put into the refrigerator at 4 ^{o}C for 30 min. After electrolyte leakage was measured, the leaves were boiled for 10 min, and electrolyte leakage was measured again. Relative conductivity rate were calculated. The conductivity determinations of every sample were repeated three times.

3. Results

3.1 cDNA Cloning and Characterization of TaPRP

The complete cDNA of wheat *TaPRP* was obtained using a combination of bioinformatics tools and PCR cloning. In order to obtain the full length of *TaPRP* gene, RT-PCR was carried out with the template of wheat total RNA and 5′ and 3′ degenerate primers of *PRP* cDNA. A specific product of 1.1 kb was amplified (Figure 2). The amplified PCR product was gel-purified and inserted into the pGEMT-Easy vector. Sequence of positive clone was determined by dideoxynucleotide sequencing. Sequence analysis showed that the cDNA was 1 197 bp in length. The start codon ATG and stop codon TGA were at 6 bp and 1142 bp, respectively. The full *TaPRP* cDNA sequence contained 1137 bp with a single ORF and it encoded a peptide of 378 amino acids (Figure 3). The deduced molecular mass and isoelectric point of *TaPRP* were 42.19 Kd and 9.69, respectively. The *TaPRP* cDNA sequence in this study was released in GenBank with accession number GQ331032.

The similarity of amino acid sequence with the reported sequences in GenBank, including wheat (DQ286560.1), sorghum (AJ234401.1), rice (EF408055.1) and maize (AJ130830.1) were 92.6%, 89.3%, 73.0%, and 73.3%, respectively (Figure 3). The deduced secondary structure showed that 3-31aa is a membrane-spanning domain. The proline number in the whole protein accounts for 44.97% of the whole numbers amino acid, and the mass of all proline accounts for 39.95% of the mass of whole protein. These characteristics showed that *TaPRP* is a member of *PRP* family in *Poaceae*.

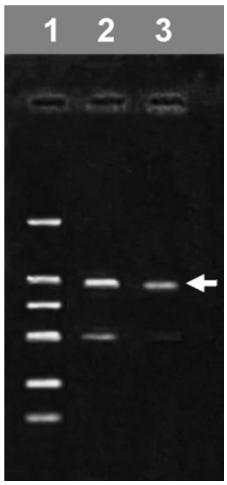

Figure 2. Agarose gel analysis of RT-PCR product of *TaPRP*

Note. 1: DNA molecular-weight; 2:3: PCR products, Arrow for 1.1kb RT-PCR stcrip.

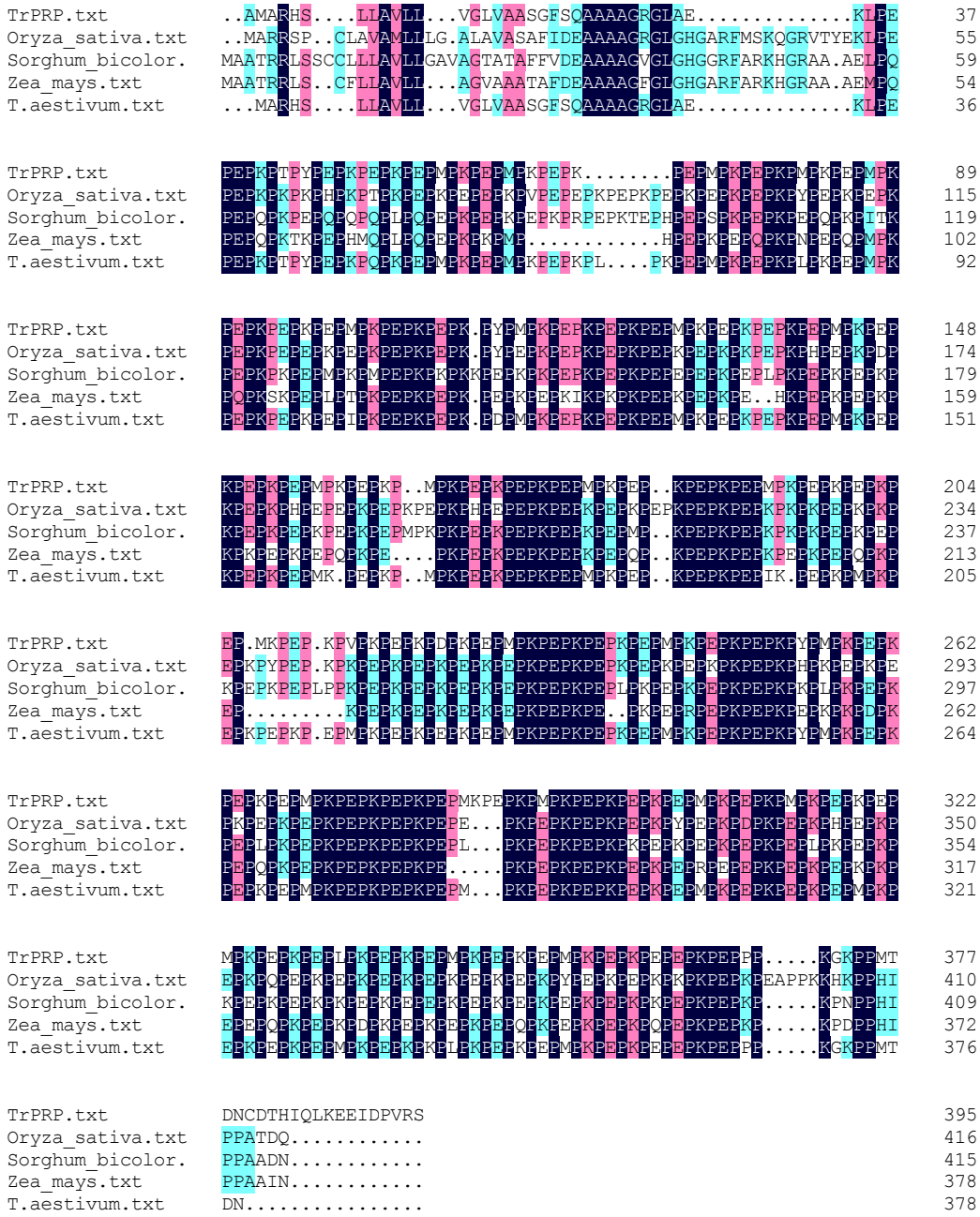

Figure 3. Alignment of full amino acid sequences from *TaPRP* and 4 other PRP proteins in *Poaceae*

Note. 1. *TaPRP*; 2. *Oryza sativa*; 3. *Sorghum bicolor*; 4. *Zea mays*; 5. Wheat.

3.2 Construction and Identification of Plant Expression Vector

The recombinant plasmid pBI121-*TaPRP* was digested for identification with *Bam*HI and *Sac*I. The digestion products displayed a band with about 1.1 kb (*TaPRP* gene) on the gel. The size of the bands was similar with the anticipated sequences and sequencing also indicated that *TaPRP* inserted correctly into pBI121. The expression vector of pBI121-*TaPRP* was constructed successfully.

3.3 Identification of Transformed Tobacco Plant

Nicotiana tobacum was transformed by *Agrobacterium tumefaciens* using the heredity conversion system. After screening with kanamycin, six survived plants were obtained. Primers P1 and P2 were used to detect the transformed plants using the total DNA of the six plants and the control (tobacco plants transformed with

plasmids pBI121) as templates. DNA fragments of 1100 bp were obtained from three kanamycin-survived plants, but not from the control plant and other three kanamycin-survived plants (Figure 4). The total DNA of transformants and the control were digested with *EcoR* I. The plants with 1100 bp nof fragment obtained were considered as transgenic plants (transformed plants) and designated transformants I, II, and III.

Southern blotting analysis, using the *TaPRP* as the probe, showed that all the three transformants produced differential hybridization signals, but no hybridization signal was observed in the control. This indicated that the *TaPRP* ORF was inserted into the genome of tobacco, but the sites of insertion were different from each other (Figure 5). The total RNA of transformants and the control were extracted respectively. Northern blotting analysis using *TaPRP* DNA fragment as the probe showed that all the three transformants had a differential hybridization signal, but the control did not produce any signal (Figure 6). This indicated that the *TaPRP* fragment was transcripted in the tobacco, but the levels of expression of *TaPRP* in the three transformants were not identical. The different expressions might result from the difference of inserting site or different physiological activity.

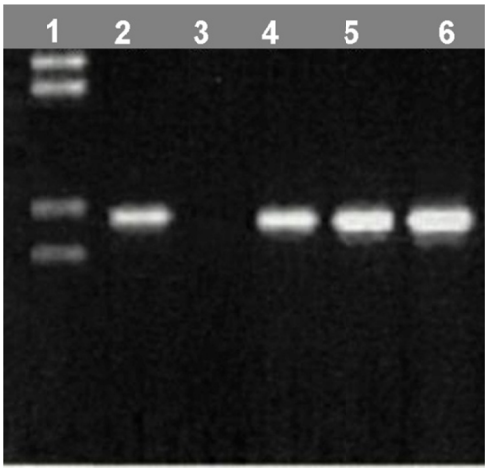

Figure 4. PCR amplification of some Kan resistant plants of transgenic tobacco

Note. 1: DNA ladder; 2: Positive control, pBI121-*TaPRP* Plasmid; 3: Negative control, non-transgenic plant; 4-6: 3 transgenic tobacco plants.

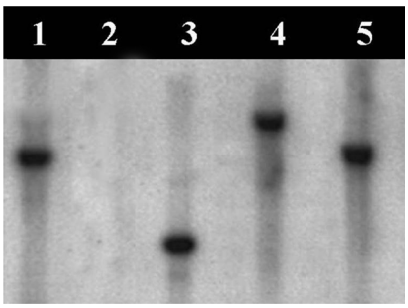

Figure 5. Southern blot analysis of transgenic plants

Note. 1: pBI121-*TaPRP*, positive control; 2: Untransgenic tobacco plant; 3-5: Transgenic tobacco plants.

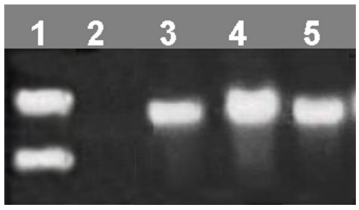

Figure 6. Expression of *TaPRP* in the transgenic plants detected by Northern blot

Note. 1: DNA marker; 2: Wild-type plants; 3-5: No. 1-3 transgenic tobacco seedlings *TaPRP*.

3.4 Cold Resistant Experiment on the Transgenic Tobacco Plants

Leaves from three transformants plants, and plants transformed with empty vector (no *TaPRP*) were frozen at the three indicated temperatures (0, -8, -16 °C) and the extent of cellular damage was estimated by measuring electrolyte leakage (Table 1). With duration of cold stress, the electrolyte leakage from detached leaves gradually increased in different degree. This is due to the increasing cell leakage for frozen injury in leaves. At the three cold stress treatments (0, -8, -16 °C), the relative conductivity rate in transformed plants were significantly higher than that from control plants. The results indicated that PRP protein might improve the ability of tobacco resist to cold. More stability of membrane in transformed plants could be kept under cold stress. In addition, electrolyte leakage to cold stress showed significant differences among the three cold stresses ($P < 0.05$), but no differences among the three transformed plants (Figure 7). The interactions were significant between plant and treatment ($P < 0.05$).

Table 1. Conductance rate in leaves of three transgenic tobacco plants under low temperature

source of variation	sum of squares	freedom of motion	uniform	*F*-value	*p*-value
Inter-plant	9.21	2	4.60	0.236	NS
Frozen	4888.48	2	2444.24	125.135	***
Plant × Frozen	78.13	4	19.53	3.115	*
Error	112.87	18	6.27		
Total variance	5088.69	26			

Note. *, *** indicated significance $P < 0.05$ and $P < 0.001$ probability levels, respectively.

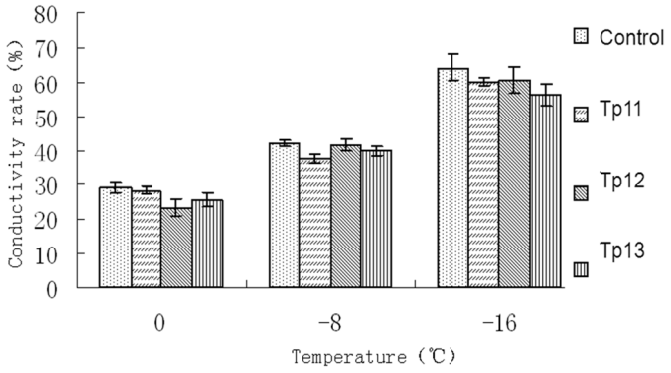

Figure 7. Conductivity rate of transgenic and non-transgenic tobacco at low temperature

Note. Conductivity rates with the same letter at the same temperature have no difference ($P < 0.05$).

4. Discussion

Genomic and cDNA sequences of *PRPs* indicate that the PRP proteins can be placed into two classes based on their primary structure. The first of these classes is characterized by *PRP* genes isolated from carrot and soybean,

which encode tandem copies of the pentapeptide PPVX(K/T), where X is often Y, H, or E (Hong et al., 1990). In contrast, a second group of *PRP* cDNAs predicts two-domain proteins containing a Pro-rich N-terminal domain and a C-terminal domain that lacks Pro-rich or repetitive sequences. This group of PRP genes includes TPRP-F1 in tomato (Salts et al., 1991). In the present study, a full length of a wheat proline-rich protein gene, named *TaPRP* was cloned and described in its molecular characterization. *TaPRP* encoded a putative protein which contains 170 proline, and 44.97% of all the total number of amino acids is the protein. *TaPRP* represents the first subset of *PRPs* in higher plants. Homology analysis revealed that TaPRP shared similarity with many PRPs from *Poaceae*. Its genebank accession number is GQ331032.

PRPs play an important role in plant development, pathogen responses and stress-resistant responses. Generally, PRPs are thought to contribute to the enhancement of cell wall structure in specific cell types during plant development, and they could fall into cross-linked or non cross-linked PRPs based on their ability to cross-link with components within the cell wall (Cassab, 1998), Both kinds of PRPs could participate into e responses to one stimulus, but might involve in different response mechanisms (Wang, 1996; Marshall et al., 1999). It has been reported that these proteins may involve interactions with other components within the extracellular matrix and possibly with proteins within the cell membrane, and thus induce cascade reactions in stress or pathogen-resistant responses (García-Gomez et al., 2000; Murphy et al., 2002). *TaPRP* cloned in this study has no linked tyrosine and Val-Tyr-Lys domains, belonged to non cross-linked PRP (Kieliszewski et al., 1994).

Temperature is one of the most important environmental factors in plant growth and development, crop yields and quality and species distributions in the world. Improving the cold tolerance in crops has significances for agricultural production. In recent years, cold mechanism has been deeply researched. Many cold resistant-relevant genes have been cloned from different plants and transgenic plants with cold tolerance have been obtained. These genes involve in stability of cell membrane (Los et al., 1997; Warren et al., 2000; Steponkus et al., 1998; Cyril et al., 2002; Vega et al., 2004), antioxidant and radical scavenging activity (Kang et al., 2002; Sato et al., 2001), antifreeze protein (Wallis et al., 1997; Huang et al., 2002), transcriptional activator in low temperature signal transduction (Xiang et al., 2007; Hsieh et al., 2002; Gilmour et al., 2000) and osmotic regulation (Jin et al., 2005; Su et al., 2006; Kumar et al., 2004).

The transgenic tobacco plants with *TaPRP* significantly deduced conductivity rate under low temperature treatment. This indicated the plants improved the freezing tolerances. But the cold tolerances mechanism of PRP might be different from those of other proteins. It has been reported that PRP involved in maintaining the stability of cell membrane under many abiotic stress. As one main components in cell cytoplast and cell wall, PRP could be an antifreeze protein in cell, or have significance in osmotic regulations. So, it is necessary to study the cold tolerance mechanism of *TaPRP* further.

In this study, electrolyte leakage was measured by conductivity rate under cold treatments to judge the plant cold tolerances. Conductivity rate is one index to evaluate if the cell membrane system has been injured under low temperature. Besides electrolyte leakage, contents of sugar, free praline, soluble protein, organic acids and the ingredients of membrane lipids could all be indices in evaluating plant cold tolerances. In order to unambiguously clarify the functions of *TaPRP*, several different detecting measures should be utilized in future experiments.

Our research proved that cold tolerance plant materials could be obtained by transgenic *TaPRP*, however, it should be pointed out that past researches indicated that transgenic single genes into a plant has less effective than transgenic several genes in cold tolerance. Improving the crop stress resistance by gene engineering is one important field in plant genetic transformation. Categories, transformation modes and expression characteristics of foreign genes in transformed plants should coordinate together for cold resistance. We believe that this research might be helpful for breeding new wheat cultivars with stress resistance.

Acknowledgements

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