Plant hybrid proline-rich proteins (HyPRPs) usually consist of an N-terminal signal peptide, a central proline-rich domain, and a conserved eight-cysteine motif C-terminal domain. In this study, one gene (designated as GhHyPRP4) encoding putative HyPRP was isolated from cotton cDNA library. Northern blot and quantitative reverse transcriptase–polymerase chain reaction analyses revealed that GhHyPRP4 was preferentially expressed in leaves: Under cold stress, GhHyPRP4 expression was significantly up-regulated in leaves of cotton seedlings. Using the genome walking approach, a promoter fragment of GhHyPRP4 gene was isolated from cotton genome. 

**Keywords** cotton (Gossypium hirsutum); cold stress; gene expression; hybrid proline-rich protein

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**Introduction**

Plant proline-rich proteins (PRPs) are divided into three classes: hybrid proline-rich proteins (HyPRPs), PRPs containing several copies of the POVEKPOVXK motif, and NHyPRP proteins [1]. NHyPRP protein has a C-terminal region with a high percentage of proline residues, whereas the extended N-terminus of the protein is essentially devoid of proline residues. HyPRPs are characterized by the presence of three different domains: a signal peptide, a central proline-rich domain, and a C-terminal non-repetitive domain that is not specifically rich in proline or glycine but in cysteines. According to the cysteine residue positions in the peptide sequence, the HyPRPs can be subdivided into A and B groups [2]. Proteins belonging to group A present a signal peptide and 4–6 cysteine residues in their hydrophobic domain with the conserved distribution pattern (CXXC–C–C–C–). The second group corresponds to HyPRP group B, which has eight cysteines in a specific pattern in the C-terminal domain (C–C–C–C–C–C–C–) [named as eight-cysteine motif (8CM) domain] associated with different proline-rich repeats and a signal peptide [2].

Till now, >500 proteins containing 8CM domain are classified as 8CM family in different plants. In Arabidopsis, there are 105 8CM proteins, of which 28 proteins are grouped together as HyPRPs [3]. It has been reported that 181 sequences of HyPRP group B were identified from seven plant species in public species-specific sequence databases. Among them, 19 HyPRP cDNA sequences are from tomato, 14 from medic, 52 from maize, 21 from pine, 31 from rice, 28 from Arabidopsis, and 16 from potato. And in accordance with length and structure of the N-terminal domain of these proteins, HyPRPs can be classified into four groups: long proline-rich group, glycine-rich group, short proline-rich group, and proline-, glycine-rich group [4]. Previous studies have revealed that some HyPRP genes are induced or repressed by hormones or stresses. ZmHyPRP is expressed specifically in zygotic embryos of maize, and its expression reaches a peak between 12 and 18 days after pollination [5]. Further study has revealed that abscisic acid (ABA) and salt stress inhibit the gene expression in embryos, whereas the cold treatment enhances its expression [6]. FahyPRP gene is fruit specific and its expression is regulated by auxin in strawberries [7]. Expression of the MsPRP2 in roots is induced by water deficit in salt-tolerant alfalfa plants [8]. Brassica napus BNPRP gene is expressed at a low level in leaves under...
standard growth conditions, but its transcripts are highly accumulated in leaves under low temperature [9]. A previous study has shown that the accumulation of the \( SbPRP \) mRNAs in leaves and epicotyls of soybean seedlings is modulated by ABA, internal circadian rhythm, viral infection, and salicylic acid treatment [10]. Carrot \( DC 2.15 \) gene is down-regulated by auxin in vascular bundles, leaves, and flower discs [11]. In addition, one study demonstrated that \textit{Arabidopsis EARLI1} encoding a HyPRP protein is a cold-responsive gene [12]. More recently, \textit{CcHyPRP} was reported to afford plant tolerance to abiotic stresses, such as PEG, mannitol, NaCl, LiCl, and heat [13].

Cold stress is one of the most common abiotic stresses, which adversely affects the growth and development of plants, and significantly constrains the spatial distribution of plants and agricultural productivity [14]. Cotton (\textit{Gossypium hirsutum}), which is the most important textile crop in the world, often encounters cold stress during its early development stages in spring. In most districts of China, temperature in spring is still low and unstable. Cotton seedlings often suffer from low temperature that causes growth retardation, even death, resulting in a decrease in cotton production. Therefore, it is of both biological and agricultural importance to understand the molecular mechanism of cotton cold tolerance. Although some progress has been made in understanding the phenomenon of plant cold acclimation in recent years, the roles of HyPRP genes in cold tolerance of plants (especially cotton) still remain largely unknown so far. In the present study, one cDNA encoding an HyPRP protein (designated as \textit{GhHyPRP4}) was isolated from the cotton cDNA library. Our data indicated that \textit{GhHyPRP4} gene is induced by cold stress in leaves.

### Materials and Methods

#### Plant material, growth conditions, and abiotic stress treatment

Cotton (\textit{G. hirsutum} cv. Coker 312 and Xuzhou 142) seeds were surface sterilized with 75% (v/v) ethanol for 1 min and 10% (v/v) \( \mathrm{H}_2\mathrm{O}_2 \) for 1–2 h, followed by wash with sterile distilled water. The sterilized seeds were germinated on half-strength Murashige and Skoog (1/2 MS medium) under 16 h light/8 h dark cycles at 28°C for 5–6 days. Roots, cotyledons, and hypocotyls were cut from sterile seedlings, and other tissues for RNA extraction were derived from cotton plants grown in the field.

In stress experiments, 6-day-old cotton seedlings were transplanted into soil for 4 weeks on green house. For drought treatment, plants were not watered for 1 week as control, for 8 days as 1-day drought treatment, and for 2 weeks as 1-week drought treatment. For ABA and NaCl treatments, cotton plants were treated with 100 \( \mu \)M ABA and 200 mM NaCl. After 12 h, leaves were collected from the treated seedlings and controls. For cold treatment, cotton plants grew at 4°C for 12 h. Then, leaves of these treated plants and controls were harvested, respectively, immediately frozen in liquid nitrogen, and stored at \(-80°C\) until RNA isolation.

#### Isolation of \textit{GhHyPRP4} cDNA

More than 4000 cDNA clones were randomly selected from the cotton cDNA libraries constructed by Li \textit{et al.} [15] for sequencing. One cotton cDNA encoding PRP (designated \textit{GhHyPRP4}) was identified from these clones, and then completely sequenced.

#### DNA and protein sequence analysis

Unless otherwise stated, nucleotide and amino acid sequences were analyzed using DNAStar software (DNA Star Co., Madison, USA), and protein sequence homology analysis was performed with Clustal W (http://www.ebi.ac.uk/clustalw/). Promoter prediction software (http://www.fruitfly.org/seq_tools/promoter.html) was used to predict the \textit{GhHyPRP4} transcription initiation site. Putative cis-acting elements were identified by submitting the promoter sequence to the PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). Identification of protein domains and significant sites was performed with Motifscan (http://myhits.isb-sib.ch/cgi-bin/motif_scan), and SignalP (www.cbs.dtu.dk/services/SignalP/) was used to determine the N-terminal signal sequence.

#### RNA extraction, purification, and reverse transcription

Total RNA was extracted from roots, hypocotyls, cotyledons, leaves, petals, anthers, ovules, and developing leaves (2–60 day-olds) as described previously [15]. RNAs were purified using Qiagen RNAeasy kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. First-strand synthesis of cDNA was performed using M-MLV reverse transcriptase (Promega, Madison, USA) according to the manufacturer’s instructions.

#### Quantitative reverse transcriptase–polymerase chain reaction

Expression of the \textit{GhHyPRP4} gene in cotton leaves was analyzed by real-time quantitative reverse transcriptase–polymerase chain reaction (RT–PCR) using the fluorescent intercalating dye SYBR-Green in the Option 2 detection system (MJ Research, Watertown, USA). A cotton polyubiquitin gene (\textit{GhUBI1}, GenBank accession no. EU604080) was used as a standard control. A two-step RT–PCR procedure was performed in all experiments using a method described earlier [16]. In brief, total RNA was reverse transcribed into cDNA and used as templates in real-time PCR with gene-specific primers as follows: \textit{GhHyPRP4}, forward
primer: 5’-CCTTCTTGGCCTAAAGACACAT-3’ and reverse primer: 5’-CTAGAAATGAGTAAGTGAAC TTAG C-3’. GhUBII, forward primer: 5’-CTGTAATCTTGGT TACAGTTATC-3’ and reverse primer: 5’-GGGATGCAA ATCTTGGAAAAAC-3’. The PCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) according to the manufacturer’s instructions. The cycle threshold (Ct), defined as the PCR cycle at which a statistically significant increase of reporter fluorescence was first detected, was used as a measure for the starting copy number of the target gene. Relative quantity of the target GhHyPRP4 expression level was determined using the comparative Ct method to achieve optimal amplification. PCR conditions for each primer combination were optimized for annealing temperature and Mg²⁺ concentration. PCR products were confirmed on agarose gel. The efficiency of each primer pair was detected using GhHyPRP4 cDNAs as the standard templates, and the RT–PCR data were normalized with the relative efficiency of each primer pair.

RNA gel blot analysis
The 3’-untranslated region (3’-UTR) fragment and partial open reading frame of GhHyPRP4 cDNA was prepared as a probe by PCR amplification. The gene-specific primers are same as quantitative RT–PCR primers. RNA samples (20 μg per lane) from cotton tissues were separated on 1.2% (w/v) agarose-formaldehyde gels for 4–5 h and then transferred onto Hybond-N nylon membranes (Amersham, Little Chalfont, UK) by capillary blotting. Gene-specific probe was labeled with [32P] dCTP using the random primer method (Random primer DNA labeling kit; TaKaRa, Dalian, China). Northern hybridization was carried out as described previously [15]. The membrane was exposed to X-film (Eastman Kodak, Rochester, USA) with two intensifying screens at −80°C for 1–3 days.

Isolation of GhHyPRP4 promoter by genome walking
GhHyPRP4 5’-flanking sequence was isolated according to the Genome walker kit (Clontech, Mountain View, USA) as described previously [15]. Primers for PCR-based DNA walking in Genome Walker Library were gene-specific GhHyPRP4-P1 (5’-GGATCCCGACTGTTAGTGAATGTTGA GTG-3’) and GhHyPRP4-P2 (5’-GGATCCCGGAGGAC AGCAGAAAGAGGGC-3’), adapter sequences AP1 (5’-GTAATACGACTCACTATAGGGC-3’) and AP2 (5’-A CTATAGGGCAAGCCTG-3’). Two Genome Walker PCR reactions were carried out successively using the Advantage 2 PCR Kit (Clontech), which is a KlenTaq-based system with PfU-like high fidelity and efficiency in the amplification of the DNA template. A 1009 bp 5’-upstream region of GhHyPRP4 was amplified by PCR.

Construction of the GUS reporter cassette and Arabidopsis thaliana transformation
SalI and BamHI sites were introduced at the 5’-end and 3’-end of the GhHyPRP4 5’-upstream region (including the putative promoter fragment and 5’-UTR before translational initiation codon ATG) by PCR using primers 5’-CTTGTGCGACCGTTTTAATTTATATG-3’ and 5’-CTTGGATCCCTGCGAGAGGCGAAGAC-3’, respectively. The SalI/BamHI fragment (1009 bp) of GhHyPRP4 5’-upstream region was subcloned into pGEM-T vector (Promega). Plasmid DNA containing the GhHyPRP4 5’-upstream fragment was digested with SalI and BamHI, and subcloned into the SalI/BamHI sites of the pBI101 vector, to generate the chimeric GhHyPRP4:GUS construct.

For plant transformation, the chimeric GhHyPRP4:GUS gene was introduced into Arabidopsis by the floral dip method [17]. Transformed seeds were selected on 1/2 MS medium containing 50 mg/L kanamycin. To test the effect of cold, 2-week transgenic T2 seedlings were transferred to the incubator set at 4°C for 1, 3, 6, 12, and 24 h.

Histochemical assay of GUS activity
Histochemical assay for GUS activity in transgenic plants were conducted according to the protocol described previously [18] with some modifications. In brief, fresh tissues from the plants were incubated in 5-bromo-4-chloro-3-indolylglucuroniode (X-gluc) solution consisting of 0.1 M sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, and 0.1% (w/v) X-gluc (Clontech) for 8 h. The stained plant materials were then cleared and fixed by rinsing with 70% (v/v) ethanol, and the samples were examined and photographed directly or under a microscope.

Overexpression of GhHyPRP4 in fission yeast
The cDNA sequence of GhHyPRP4 gene was amplified by PCR, using primers P1: 5’-CTTGTGCGACATGCC TTCTGCTACTCCTGTGTAATGATTGAATGTTGA GTG-3’) and P2: 5’-CTTGGATCCCGGAGGAC AGCAGAAAGAGGGC-3’, cloned into yeast vector pREP5N with SalI and BamHI sites introduced at the 5’-end and 3’-end of the GhHyPRP4 5’-upstream region (including the putative promoter fragment and 5’-UTR before translational initiation codon ATG) by PCR using primers 5’-CTTGTGCGACCGTTTTAATTTATATG-3’ and 5’-CTTGGATCCCTGCGAGAGGCGAAGAC-3’, respectively. The SalI/BamHI fragment (1009 bp) of GhHyPRP4 5’-upstream region was subcloned into pGEM-T vector (Promega). Plasmid DNA containing the GhHyPRP4 5’-upstream fragment was digested with SalI and BamHI, and subcloned into the SalI/BamHI sites of the pBI101 vector, to generate the chimeric GhHyPRP4:GUS construct.

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control samples (non-frozen cells) were also stained with methylene blue. Cell survival rate was determined as the percentage of freezing survival rate of frozen samples relative to the control samples.

Overexpression of GhHyPRP4 in A. thaliana
The coding sequence of GhHyPRP4 gene was amplified by PCR, using primers up: 5'-CTTGGATCCATGGCTT CCTACAAAACCTCTG-3' and down: 5'-CTTGAAGCTCT ATTACATGGAGCCTG-3', and cloned into pBI121 vector with BamHI/SacI sites. The pBI121:GhHyPRP4 construct was introduced into A. thaliana by the floral dip method. Transformed seeds were selected on 1/2 MS medium containing 50 mg/L kanamycin (T1 generation). Bioassay of cold tolerance of wild-type and transgenic Arabidopsis plants was performed using the method described previously [21], using constitutively expressed ACTIN2 gene (AT3G18780) as an internal control.

Results

Isolation and characterization of GhHyPRP4 gene
Over 4000 cDNA clones from the cotton cDNA library were randomly sequenced. Among them, one clone encoding a putative HyPRP was selected and designated as GhHyPRP4 (GenBank accession number: HM989876). GhHyPRP4 cDNA is 634 bp in length, including 369 bp of coding region that encodes a protein of 122 amino acids (13 kDa, pl 8.25). Amino acid composition analysis showed that GhHyPRP4 protein is rich in proline (10.6%), leucine (17.1%), cysteine (10.6%), alanine (8.9%), serine (7.3%), and threonine (5.7%) residues. Most importantly, GhHyPRP4 protein has three-domain architecture as occurred in all other HyPRPs. As shown in Fig. 1, the first 25 residues represent a putative signal peptide, and the predicted cleavage site is between residue 25 (Ala) and 26 (Cys). A central domain is proline rich, and its length varies among different HyPRP proteins. A long cysteine-rich hydrophobic domain is named as 8CM at C-terminus. The conserved spacing of residue Leu is also observed in GhHyPRP4, like other plant HyPRPs. In addition, a potential N-glycosylation site (NVI) is located in 8CM.

GhHyPRP4 shares high similarities with a salicylic acid-induced HyPRP protein (SbPRP, 71% identity) from soybean and a cold-induced HyPRP (DEA1, 60.7%) from tomato. Furthermore, sequence comparison revealed that GhHyPRP4 displays 63.1% and 54% identities with GhHyPRP2 and GhHyPRP3, respectively.

GhHyPRP4 gene is preferentially expressed in leaves
To investigate the expression pattern of GhHyPRP4 gene, RNA gel-blot analysis was carried out using total RNAs isolated from different cotton tissues. The results showed that GhHyPRP4 mRNA was accumulated in an organ-specific manner. It was expressed strongly in leaves and moderately in petals, but was not detected in other tissues [Fig. 2(A)].

To investigate whether its expression was developmentally regulated in leaves, GhHyPRP4 transcript levels in developing leaves were determined by quantitative RT–PCR. The experimental results revealed that GhHyPRP4 expression reached a peak in 1-week-old leaves, and gradually declined with further leaf development [Fig. 2(B)].

GhHyPRP4 gene is induced by cold stress in leaves of cotton
Quantitative RT–PCR analysis was performed to elucidate the effect of abiotic stress on GhHyPRP4 expression. As shown in Fig. 3, under high salinity (200 mM NaCl) and drought stresses, GhHyPRP4 expression was dramatically down-regulated in 2-week-old leaves, whereas its mRNA level was significantly up-regulated after 4°C treatment for 12 h. In addition, the results also indicated that GhHyPRP4 expression in leaves was remarkably down-regulated by ABA. The independent t-tests revealed that there were significant differences in the expression of GhHyPRP4 in leaves between the different treatments and control.

Isolation and activity analysis of GhHyPRP4 promoter
On the basis of the cDNA sequence of GhHyPRP4, a 1009 bp 5′-flanking sequence was isolated by genome walking PCR. Analysis of the sequence revealed a putative transcription initiation site in −57 bp upstream of the ATG translation start codon (http://www.fruitfly.org/seq_tools/promoter.html), and typical TATA and CAAT boxes were present in this promoter. The predicted transcription initiation site matches with available EST sequences in NCBI. In addition, several putative cis-acting regulatory elements corresponding to known cis-elements of plant genes were present, such as heat stress responsiveness element (AAAAAATTTC), MeJA-responsive element (CGTCA and TGAGC), MYB binding site involved in drought-inducibility element (CAACTG), elicitor-responsive element (TTGGACC), ethylene-responsive element (ATTTCAAA), gibberellin-responsive element (TCTGTGTG), and wound-responsive element (AAATTT CCT) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Fig. 4).

Because generating transgenic cotton is time consuming and labor intensive, it is very difficult to use transgenic cotton plants for assaying promoter activity. Then we employed the model Arabidopsis system for analyzing the cotton promoter activity. Expression of the GUS gene driven by the GhHyPRP4 promoter was examined in transgenic Arabidopsis plants. As shown in Fig. 5, at a very
early stage of seedling development, \textit{GUS} staining was only detected in cotyledons of 3- and 5-day-old seedlings [Fig. 5(A,B)]. In 2-week-old seedlings, \textit{GUS} activity was detected in petioles and leaves, and very low levels of \textit{GUS} expression were observed in cotyledons [Fig. 5(C)]. Thereafter, \textit{GUS} signals were restricted in leaves from the vegetative stage to the reproductive growth phase [Fig. 5(D,E)], and no \textit{GUS} expression was detected in the
With longer cold treatments [Fig. 5(I,J)]. In addition, quantitative PCR analysis also indicated that GUS transcripts were significantly higher in the 2-week-old transgenic seedlings treated with cold for 2 h than those of control seedlings [Fig. 5(K)]. These results indicated that the isolated GhHyPRP4 promoter is active in leaves, and is cold-inducible in transgenic Arabidopsis.

Overexpression of GhHyPRP4 gene in fission yeast and in A. thaliana

Since expression of GhHyPRP4 was up-regulated by cold stress, we supposed that GhHyPRP4 might be involved in cell cold defense. To determine the potential contribution of GhHyPRP4 in protecting cells against freezing damage, GhHyPRP4 was expressed in fission yeast, and the survival rate of cells was examined after a 60-h freezing period. GhHyPRP4 was constructed into pREP5N vector, which was then placed under the control of nmt-1 promoter activated in the absence of exogenous thiamine. Ten transformed yeast cell lines with GhHyPRP4 and the control lines containing empty vector (pREP5N) were randomly selected for figuring out cell survival probability under cold stress (at −20 °C for 60 h), calculated as percentage of the same cell lines incubated at 4 °C. After being frozen for 60 h, there were obviously more cells remaining alive in the transformed cell lines expressing GhHyPRP4 in induction medium than those of the same lines without GhHyPRP4 expression in non-induction medium [Fig. 6(A,B)]. In contrast, there were much less cells alive in the control cells harboring the empty pREP5N vector either grown in induction medium or non-induction medium [Fig. 6(C,D)]. Statistical analysis revealed that the freezing survival rate of transformed cells expressing GhHyPRP4 was 19.08% ± 3.62%, whereas that of control cells was 11.32% ± 2.39% [Fig. 6(E)].

To further investigate its possible role in plant response to cold stress, the transgenic Arabidopsis plants expressing GhHyPRP4 were selected for further analysis. Over 30 homozygous lines of T2 and T3 generations were obtained, and the expression levels of the GhHyPRP4 gene in these transgenic progeny plants were examined by RT–PCR using gene-specific primers. As shown in Fig. 7(A), the transgenic lines (L3 and L8) displayed high levels of GhHyPRP4 overexpression, whereas GhHyPRP4 transcripts were not detected in the wild-type plants. However, survival rate of L3 and L8 transgenic plants was identical to wild-type plants after 4 °C treatment for 2 days and subsequent recovery at 22 °C for 5 days (data not shown). Measurement and statistical analysis indicated that there was no significant difference in root length between the transgenic lines and wild type after cold treatment for 2 days and subsequent recovery at 22 °C for 5 days [Fig. 7(B)]. The above data suggested that GhHyPRP4

Figure 2 Analysis of expression of GhHyPRP4 in cotton tissues  (A) RNA gel blot analysis of GhHyPRP4 transcripts in cotton tissues. Total RNA (20 μg/lane) from root (1), cotyledon (2), leaves (3), hypocotyl (4), petal (5), anther (6), fiber (7–9) at 5, 10, and 15 days post-anthesis (DPA) and ovule (10 and 11) at 10 and 15 DPA was fractionated on a 1% denaturing agarose gel and transferred onto a nylon membrane, the equal loading of RNA on the gel was confirmed by ethidium bromide staining of the gel before transfer to the nylon membrane. Top panel, autoradiograph of RNA hybridization; bottom panel, RNA gel before transfer to membrane showing equal loading of RNAs. (B) Quantitative RT–PCR analysis of expression of GhHyPRP4 during leaf development. Relative value of the GhHyPRP4 expression in the development of cotton leaves is shown as a percentage of GhUBI1 expression activity. Error bars represent standard deviation. wk, week.

Figure 3 Quantitative RT–PCR analysis of expression of GhHyPRP4 in leaves of cotton under NaCl, ABA, drought, and cold treatments Relative value of GhHyPRP4 expression in cotton leaves is shown as a percentage of GhUBI1 expression activity. 1, 4-week seedlings cotton plants (control plants); 2, 12-h ABA treatment; 3, 12-h NaCl treatment; 4, 12-h cold treatment; 5, 1-day drought treatment; 6, 1 week drought treatment; 7, 1 week drought treatment and rewatering for 1 day. **P < 0.05, ***P < 0.01 compared with the control (t-test). The experiments were repeated three times, means represent average values, and bars show standard errors.
gene may be not directly related to plant cold tolerance, although this gene is induced by cold in cotton leaves.

Discussion

In the present study, one cotton cDNA, GhHyPRP4, encoding HyPRP was isolated and characterized. GhHyPRP4 shared high similarity with many plant HyPRP B group proteins (42.6%–71.0% identities). According to the classification principles of HyPRPs proposed by Dvorákova et al. [4], GhHyPRP4 can be placed into HyPRP B and short proline-rich domain subgroup. Previous study has revealed that the expression of most HyPRP B group genes is specific to the defined plant development stage and/or organ specific [2]. Cold-induced SbPRP gene is preferentially expressed in leaves and epicotyls [10]. Another PRP gene (PVR5) from bean is specifically expressed in the roots of bean seedlings [22]. The expression of the strawberry Fahyprp gene is fruit specific and varies through the different fruit development and ripening stages [7]. MtPRPD1 is specifically expressed in the embryo axis during radicle emergence and early postgermination [23]. ZmHyPRP gene is found to be transcribed in immature maize embryos and ovary prior to pollination [5]. Likewise, our data indicated that the expression of GhHyPRP4 is leaf specific and developmentally regulated, suggesting that the gene may be involved in leaf development of cotton.

It has been demonstrated that both biotic and abiotic stresses could induce or repress the expression of HyPRP B genes [2]. ShPRP expression is down-regulated by 0.6%–2% NaCl and 200 µM ABA treatment [10], while BNPRP gene is highly expressed in B. napus under low-temperature conditions [9]. Similarly, Arabidopsis EARLI1

Figure 4 Nucleotide sequence of the promoter region of GhHyPRP4 gene. Numbering is relative to the first base of the ATG codon, which is shown in italics. The transcription start site predicted by http://promotor.biosino.org/cgi-bin/promoter.cgi is indicated with a bent arrow. Motifs with significant similarity to previously identified cis-acting elements are underlined, which include CAAT and TATA boxes, an ethylene-responsive element, an MYB binding site involved in drought inducibility, an elicitor-responsive element, a MeJA-responsive element, a wound-responsive element, the heat shock element, and a gibberellin-responsive element.
and its three orthologs are induced by cold [12], and tomato DEA1 transcripts are accumulated in response to cold and the rapidity of the cold-induced transcript accumulation is regulated by circadian rhythm [24]. In this study, the expression of GhHyPRP4 was dramatically down-regulated in response to 2 weeks of water stoppage, 200 mM NaCl and 100 μM ABA treatments and up-regulated by cold (4°C for 12 h). Significant accumulation of GhHyPRP4 transcripts in leaves under cold treatment denotes that GhHyPRP4 is cold-inducible in cotton. The presence of hormone and stress response elements in the GhHyPRP4 promoter suggested that hormones and abiotic stresses might be involved in the regulation of GhHyPRP4 expression. In addition, our results showed that GUS gene driven by GhHyPRP4 promoter was expressed in leaves, and GUS activity was induced in leaves and petioles of the transgenic Arabidopsis plants treated at 4°C, consistent with GhHyPRP4 expression in cotton.

As plants (including cotton) appear to express a large number of HyPRP B group paralogues, it is difficult to investigate the role of a single HyPRP protein in plants [4]. Thus, we chose a relatively simple system, yeast cell (S. pombe) to investigate whether GhHyPRP4 protein is involved in cell cold tolerance. We chose yeast because its genome does not have any HyPRP genes, and it was previously shown that putative stress tolerance gene from plants has the potential to increase the freezing survival of S. pombe cells [20]. The results showed that overexpression of GhHyPRP4 in fission yeast significantly increased the freezing survival rate of the host cells under freezing stress. Likewise, previous studies indicated that DEA1, a tomato HyPRP protein, significantly increased the freezing survival of Pichia pastoris cells [25], and Saccharomyces cerevisiae cells expressing EARLI1 gene and its orthologs efficiently protected them from freezing stress [12]. A previous study indicated that overexpression of DEA1 in tomato did not improve the freezing tolerance of transgenic tomato plants [25]. Likewise, our results revealed that transgenic Arabidopsis plants with overexpressing GhHyPRP4 did not display higher cold tolerance than wild type. However, overexpression of EARLI1 partially improved freezing tolerance of Arabidopsis plants, whereas a trend to increase freezing damage was observed in EARLI1-knockdown plants [12,26]. GhHyPRP4 shares 60.7% identity with DEA1, and 42.6% identity with EARLI1. Sequence comparison revealed that EAELI1 protein contains a longer proline-rich domain than GhHyPRP4 and DEA1 proteins. So the length of...
proline-rich domain in HyPRPs may be important for HyPRPs’ function. On the other hand, yeast cells overexpressing GhHyPRP4 significantly increased the cell survival rate under freezing stress, unlike the transgenic GhHyPRP4 Arabidopsis plants. This fact implicated that the molecular mechanism of plant response to cold stress may be different from that of yeast cell.

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