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Full Length Research Paper

The clone of wheat dehydrin-like gene *wzy2* and its functional analysis in *Pichia pastoris*

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Dehydrin is one of the responsive proteins under water-deficit stress. The aim of this study was to explore function of dehydrins in cells and the relationship with drought tolerance under water-deficit stress. We used winter wheat (*Triticum aestivum*) Zhengyin No.1 as the material, the complete cDNA sequence of dehydrin *wzy2* was cloned and the code sequence of *wzy2* was transformed into yeast (*Pichia pastoris*) for eukaryotic expression. We also analyzed the relationship between wheat dehydrin *wzy2* gene and drought resistance. Our results indicate that the whole cDNA sequence of dehydrin *wzy2* gene was 819-bp long, containing an open reading frame of 459 bp. The deduced *wzy2* protein had 152 amino acid residues, which formed a 15.5 ku polypeptide with a predicted isoelectric point (p*I*) of 7.17. It was Y_nSK_2 type of dehydrin superfamily. Methanol was used to induce the expression of *wzy2* gene in transformed *P. pastoris GS*115. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting further confirmed that the *wzy2* was successfully transformed into *P. pastoris* and expressed specifically and effectively in yeast. With drought tolerance and cold tolerance treatment to recombinant *P. pastoris*, *wzy2* gene can significantly improve the growth of the transgenic yeast under salt and drought stress compared with the control of pPIC9K yeast cell. However, in low temperature stress conditions, survival rate of the transferred *wzy2* yeast cell had no significant improvement compared with the control of pPIC9K yeast cell. These results further indicate that Y_nSK_2 type dehydrin could be induced by salt and osmotic stress rather than low temperature.

Key words: *Triticum aestivum* L., eukaryotic expression, dehydrin-like, *Pichia pastoris*, drought tolerance.

INTRODUCTION

Water-deficit stresses such as drought and high salinity are some of the most adverse conditions for plants, and they can cause fatal damage to plants and seriously affect crop productivity (Yoshida et al., 2010). To survive under such unfavorable growth conditions, plants have developed a number of unique defense mechanisms and processes for acclimation that enhance their tolerance to detrimental conditions (Rorat, 2006; Xu et al., 2008). The synthesis of hydrophilic proteins such as late embryogenesis abundant (LEA) proteins is a major part of the response to water-deficit conditions (Rorat, 2006). LEA proteins are characterized by a high glycine content, high hydrophilicity and low secondary structure in purified form (Garay-Arroyo et al., 2000, Vaseva et al., 2010), and have been divided into five groups: LEA D19 (group I), LEA D11 (group II), LEA D7 (group III), LEA D113 (group IV) and LEA D95 (group V) (Ingram and Bartels, 1996).

Dehydrins (DHNs), the group II of LEA proteins, are one of the most extensively studied putative dehydration protective molecules and have a wide range of molecular masses from 9 to 200 kD (Allagulova et al., 2003; Close, 1997). They are thermostable, highly hydrophilic proteins that accumulate in vegetative plant tissues under stress conditions such as drought, low temperature, salt stress, or in seeds during maturation drying (Xiao and Nassuth, 2006). This family of proteins is characterized by the EKKGIMDKIKELPG near the carboxyl terminus (Close et al., 1993; Lopez et al., 2003). DHNs have been divided into five subclasses based on their conserved amino acid sequences. These are the Y, S and K segments, which include Y_nSK_2 (n=1 - 3), SK_n (n=1 - 3), K_n (n=2–9), Y_nK_n

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(n=1–2) and KnS sub-types (Mehta et al., 2009; Rodriguez et al., 2005). The precise function of dehydrins has not yet been elucidated, but *in vitro* and expression data suggest that each dehydrin structural type could correspond to a specific function (Allagulova et al., 2003; Brini et al., 2011; Mingeot et al., 2009; Rorat, 2006). The enhancement of drought and salt tolerance by transgenic dehydrin overexpression has been demonstrated for different species (Bajaj et al., 1999; Mingeot et al., 2009; Shen et al., 2003; Yu et al., 2004).

Wheat (*Triticum aestivum L.*) and its related species grow over wide areas with varying climatic conditions (Yu et al., 2005). These plants possess large genetic diversity in terms of their ability to tolerate various abiotic stresses. Some studies reported that a number of DHNs genes such as the Wcsl20 (Houde et al., 1992), Wcs200 (Quellet et al., 1993), Wcor410 (Danyluk et al., 1994), rabl5 (King et al., 1992), pMA80 (Morris et al., 1991) and DHN-5genes (Brini F et al., 2007) have been isolated from wheat. In our previous work, the polymerase chain reaction (PCR) fragments of dehydrin gene-*wzy2* from winter wheat Zhengyin *NO.1* were cloned by the rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) (Huang et al., 2009).

In this study, we isolated and cloned the full-length cDNA sequence of *wzy2* from winter wheat Zhengyin *NO.1*. To further elucidate the role of *wzy2* to abiotic stresses such as osmotic, salt and cold stresses, we conducted the overexpression of *wzy2* in *Pichia pastoris*. The results showed that *T. aestivum L.* LEA2 (*wzy2*) plays a significant role in resuming growth after dehydration stresses, and our data may be applied in improving stress tolerance of wheat crops.

MATERIALS AND METHODS

Plant material and growth conditions

The drought-sensitive wheat (*T. aestivum* L.) Zhengyin *NO.1* used was obtained from the College of Life Science, Northwest A and F University, China. Sterilized seeds of Zhengyin *NO.1* germinated at 25°C for 1 day, and then grew in the greenhouse maintained at 22°C under a photoperiod of 16 h/day (Ried and Walker-Simmons, 1993).

Vectors, host strains, and materials

Plasmid vector pPIC9K was preserved by our laboratory. *P. pastoris* strain of GS115 was purchased from Invitrogen, USA. Media components were from Difco (Detroit, MI). MD, MM, MMH, MDG, Yeast extract peptone dextrose (YPD), buffered complex glycerol medium (BMGY), and buffered complex methanol medium (BMMY) were all prepared following the Invitrogen expression manuals (25-0601 2008).DNA restriction enzymes and T4 DNA ligase were purchased from Takara.

Cloning of *wzy2*

Total RNAs were isolated from the leaves of seven-day-old Zhengyin *NO.1* seedlings after being exposed to 20% (w/v) polyethylene glycol (PEG 6000) for 48 h with TRIZOL reagent according to the manufacturer's instructions (Invitrogen, USA). The PCR fragments of *wzy2* were obtained by RACE-PCR in our previous work. In order to obtain the full length of cDNA, the sequence primers (Fw1:5′–GAAAAACCACAAGTTGCAGCCAAGT-3′; Aw2:5′-ACAAAACTCGGAACTCAGACATTAC-3′) were designed based on the sequence of RACE-PCR products. The full-length cDNA sequence of *wzy2* (GenBank accession EU395844) was amplified from cDNA by PCR, which was performed in a total volume of 50 µL in a reaction PCR tube containing 100 ng cDNA template, 20 pmol each primer, and 2× Pfu PCR MasterMix (Tiangen, China). Thirty-five cycles of PCR were performed at 95°C for 45 s, 56°C for 45 s and 72°C for 1 min. The products were separated by a 1% agarose gel and purified with TIAN Gel Midi Purification Kit (Tiangen, China), and then ligated into the pMD19-T vector (TaKaRa, Japan) followed by sequencing. The ligated product was transformed into *Escherichia coli* JM109 competent cells. Positive colonies were screened by blue/white plus ampicillin (Sigma). The colony PCR and the restriction digestion by *Sal* I and *Xba* I were carried out to confirm the recombinant transformants, and the positive clones obtained were verified by sequencing. The plasmid was purified with AxyPrep Plasmid Miniprep Kit (AXYGEN Biosciences, Union City, California, USA) and sequenced at the TaKaRa Biotechnology CO. Ltd (Dalian, China).

Construction of *wzy2*-pPIC9K and sequencing assay

The coding sequence of wzy2

A pair of primers (Fw3:5′-CGGAATTCATGGAGTACCAGGGACAT-CAG-3′; Aw3:5′-AAGGAAAAAAGCGGCCGCTCAGTGCTGTCCG-GGCAGCT-3′) were designed based on the sequencing result of *wzy2* that has been cloned. Fw3 amplified the 5′ end of the *wzy2* coding region followed by an E*co*R I restriction site, while Aw3 amplified the 3′ end of the *wzy2* coding region followed by a N*ot* I restriction site. The amplified product was digested with E*co*R I and *Not* I, and cloned into pPIC9K for the secretory expression. Positive colonies were screened by ampicillin (Sigma). The colony PCR and the restriction digestion by Sal I were carried out to confirm the recombinant transformants, and the positive clones obtained were verified by sequencing. The molecular analysis for *wzy2* was by bioinformatics software Vector NTI Advance 11, ClustalW 1.8, PHD and TopPred2.

Yeast transformations and *wzy2* expression verification

Both the empty vector (EV) and the constructed vector *wzy2*-pPIC9K was linearized with *Sac* I (Takara) and transformed into *P. pastoris* strains of GS115 with MicroPulser electroporator (Bio-Rad, USA) (Shi et al., 2007; Wu and Letchworth, 2004). His^+ transformants were first isolated from MD medium and then replica-plated onto MDG medium containing increasing concentration of G418 (0.2 mg/ml). After the genomic DNA of the His^+G418^- transformants was extracted by the modified "boiling-freezing-boiling method" (Ju et al., 2003), the correct integration of *wzy2*-pPIC9K was confirmed by PCR with the specific primers Fw3/Aw3. Clones with multiple-copy number of *wzy2*-pPIC9K were stocked for further expression studies.

Test of Wzy2 expression in transformants

The candidate transformants obtained in the pre-expression process were cultured at 28°C in 150 ml baffled shake flasks containing 25 ml BMGY liquid medium until the A_{600} value reached to 2 to 6. The

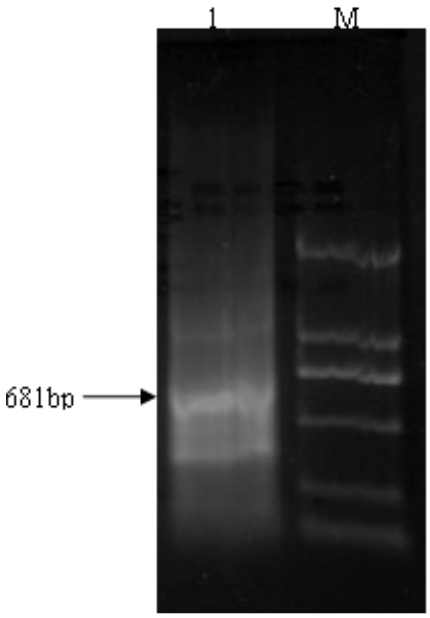

Figure 1. The full length of *Triticum aestivum* dehydrin gene (*wzy2*) obtained by RT-PCR. Lane 1, *wzy2* cDNA; lane M, DNA Marker DL2000.

cultures were centrifuged at 3000 rpm for 5 min and the cell pellets were resuspended in 5:1, the original volume in BMMY. The cultivation lasted for 5 days at 28°C. Methanol was added to the medium to reach a final concentration of 1% every 24 h. Then 4.0 ml of the cell-free culture supernatants was daily collected and concentrated with 10% trichloroacetic acid (TCA) (Sigma). The proteins were separated by 15% acrylamide gels using the Laemmli buffer system (Laemmli 1970). Western blotting detection of *wzy2* specificity in the total proteins of the supernatants was done with rabbit anti-dehydrin antibody (Ab_1) against *wzy2*, goat anti-rabbit antibody (Ab_2) coupled to alkaline phosphates (de Campos et al., 2011) against Ab_1 and Strengthen BCIP/NBT Western Blotting detection kit according to the instructions.

Assay concentration of salts for the drought stress and effect of the temperature stress in transgenic yeast

The abiotic stress tolerance of yeast transformants with *wzy2*-pPIC9K was tested under ionic, osmotic stress and freezing stress, the transformants with pPIC9K was used as a control. The stresses tolerance assay was performed by starting liquid cultures from *P. pastoris* freezer stocks for two days at 28°C with shaking in BMGY. The yeast transformants *wzy2*-pPIC9K and pPIC9K were started from 50 µL of starter cultures in 20 ml BMGY contained in 50 ml conical round bottom tubes with loosely taped caps. The cultures were shaken (250 rpm) at 28°C for 12 h. The cells were then centrifuged at 3000 rpm and the supernatant was completely poured off. Cells were resuspended in 20 ml MMH containing different concentrations of NaCl and sorbitol at 28°C by shaking. The samples were then adjusted to the same A_{600} (usually about 1.0) value. A_{600} value was measured with a spectrophotometer after being cultured at 28°C for 48 h. Three independent experiments were completed.

Aliquots of 100 µL were withdrawn and either immediately frozen in metal blocks pre-cooled to –20°C or serially diluted and plated on yeast extract-peptone-dextrose media (YPD). After 48 h, the frozen samples were thawed individually at 37°C for 2 min and serially diluted and plated for quantification of cells that survived freezing. Growth was measured three times and essentially the same growth was obtained each time (Weyman et al., 2006).

RESULTS

Cloning and structural analysis of *wzy2*

With designed primers, cDNA fragments were amplified from dehydrated Zhengyin *NO.1* seedlings by reverse transcriptase-polymerase chain reaction (RT-PCR) and the clone was identified (Figure 1). The blast and alignment result of the sequence in GenBank confirmed that the gene was a new one. The gene was named *wzy2* (Wheat Zhengyin *No.1* Dehydrin 2) (GenBank accession: EU395844). According to the sequencing results, the first ATG was 79 bp away from the 5′ end. By splicing this gene sequence and 3′ RACE product (Huang et al., 2009), an 819 bp sequence was obtained. Bioinformatics analysis of this sequence shows that this gene has an ORF of 459 bp, which encodes 152 amino acids. The *wzy2* protein sequence has obvious features of dehydrins with two K-segments that consist of 15 amino acids (EKKGVMDKIKEKLPG and RKKGIKDKIKEKLPG respectively) (Figure 2). The *wzy2* protein has a high similarity to the wheat (Zhengyin *No.1 wzy1-1*AF453444; 87%), cone wheat (*Triticum turgidum* L., *DHN15.3* (AM180931); 98%), cone wheat (*T. Turgidum* L. *DHN15.1* (AM180929); (77%), wheat *(T. aestivum L.* AM180929; 77%), wheat (*T. aestivum* L. *RAB* (*X59133*); (79%), durum wheat (*T. durum* dehydrin gene (X78429); 86%) and *Lophopyrum elongatum* dehydrin gene *ESI18-3 (AF031248)* (87 %), respectively.

The deduced amino acid sequence of *wzy2* gene was compared with 6 dehydrin sequences. The result is shown in Figure 3, from which the typical conservative domains of dehydrin could be recognized. This sequence contains two K-segments: EKKGVMDKIKEKLPG and RKKGIKDKIKEKLPG, an S-segment: SSSSSSSS and a Y-segment: DEYGNP (Figure 2). According to the classify method previously introduced (Szoke et al., 1992; Tang, 1984; Zhao et al., 2001), *wzy2* is the YSK_2 type. The protein encoded by *wzy2* was analysed by bioinformatics software PHD, and the result showed that its molecular mass is 15.5 kD, isoelectric point is 7.17, and it is a neutral protein. The amino acid composition is shown in Table 1, which demonstrates that this protein is the lacking of Cys and Trp, but rich in Gly (23.7%), Lys (7.9 %), Thr (8.6 %), His (6.6 %) and Gln (8.6 %), known as polar amino acid. Bioinformatics software TopPred2 predicted that this deduced protein has a hydrophilic region which locates about 20 amino acids away from the N-terminus (Figure 4). The transmembrane domain can interact with the hydrophobic group of some membranes. In this way, the denaturation of membrane can be prevented and cell structure can be stabilized.

Figure 2. The full-length cDNA and its amino acids sequence of wheat *wzy2* gene (Huang et al., 2009). The shade is the ORF; regions boxed are the conservative sequence of Y(DEYGNP), S(SSSSSSSS) and K(EKKGVMDKIKEKLPG),(RKKGIKDKIKEKLPG); AATAAA in bold is the poly(A) signal.

Construction of transformant *wzy2*-pPIC9K

After PCR amplification, restriction enzyme digestion and ligation, the fragment was transformed into *E. coli* JM109. The positive clone was screened by ampicillin and colony PCR, as well as sequence. The sequencing result indicated the *wzy2* gene had been successfully transformed into the expression strain pPIC9K.

Transformation and Selection

After being linearized with S*ac* I, the recombinant expression vector was transformed into *P. pastoris* strain GS115 with MicroPulser electroporator. After a stationary cultivation for 5 days under 28°C, about 20 single colonies GS115 His$^+$ were generated on MD plate. The MDG selection identified 2 single colonies GS115 His$^+$. The MD selection results showed that the transformants were Mut$^+$ *P. pastoris.* PCR selection of gDNA with primers Fw3/Aw3 was also conducted (Figure 5), and the DNA of this transformed yeast was isolated. The result reveals that a 495 bp sequence was obtained, consistent with the expected.

Eukaryotic expression of *wzy2* and Western blotting

After a series of selections, the multi-copy transformant *wzy2*-pPIC9K was obtained. Methanol was used to induce the expression of *wzy2* gene, and the supernatant were sampled for SDS-PAGE and Western blotting identification. The electrophoresis image (Figure 6) indicated that specific proteins had been expressed in the

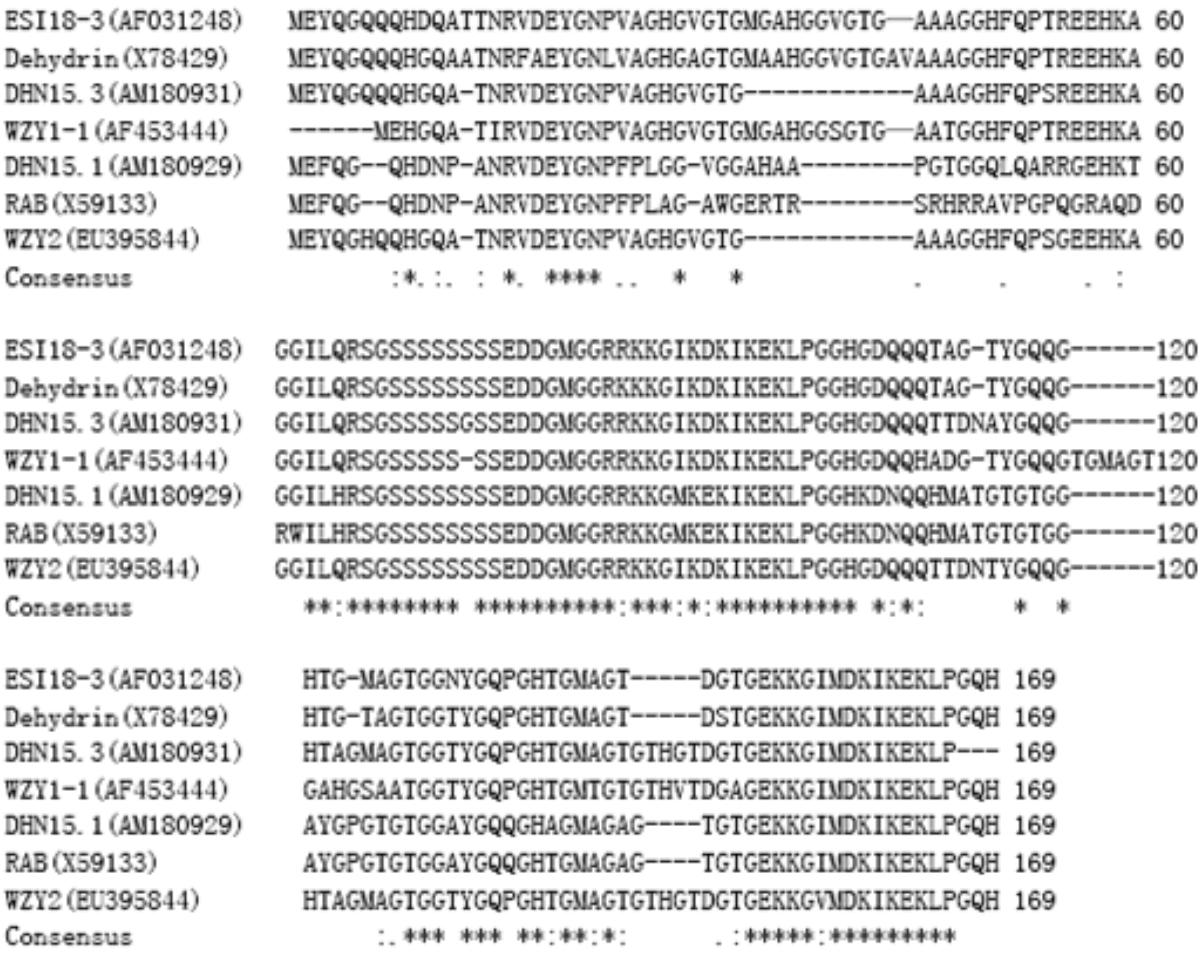

Figure 3. Comparison of amino acid sequence of *wzy2* gene with other 6 dehydrin genes (Huang et al., 2009). Consensus: * represents the same amino acid residues among the seven subunits, ':' represents the same amino acid residues among the six subunits, while '.' represents the same amino acid residues among the five subunits.

Table 1. Residue composition of *wzy2* coding protein (Huang et al., 2009).

Amino acid	Relative composition (%)	Amino acid	Relative composition (%)	Amino acid	Relative composition (%)	Amino acid	Relative composition (%)
A	5.9	H	6.6	K	7.9	L	2.0
G	23.7	N	2.0	Q	8.6	R	2.6
M	3.3	E	5.3	P	3.3	Y	2.6
S	6.6	D	5.3	F	0.7	I	2.6
T	8.6	V	2.6	W	0.0	C	0.0

transformant GS115/*wzy2*-pPIC9K. The Western blotting results (Figure 7) also showed a hybridization band with a molecule mass of about 15.5KD, which was identical with the anticipated, The result also revealed that under the induction of menthol, the expression became strengthened when the induction time prolonged gradually, and at the third day the expression was maximum. The gene *wzy2* in transgenic yeast had the highest expression on the third day after being induced.

The stress resistance assay of transgenic yeast GS115 with *wzy2* gene

NaCl and sorbitol gradient concentrations were used to treat the transgenic yeast transferred with *wzy2* gene to test their growth activity. Yeast with an empty vector pPIC9K was used as the controls. The results show that when being treated with NaCl at concentrations higher than 0.8 mol/L or sorbitol at concentrations higher than

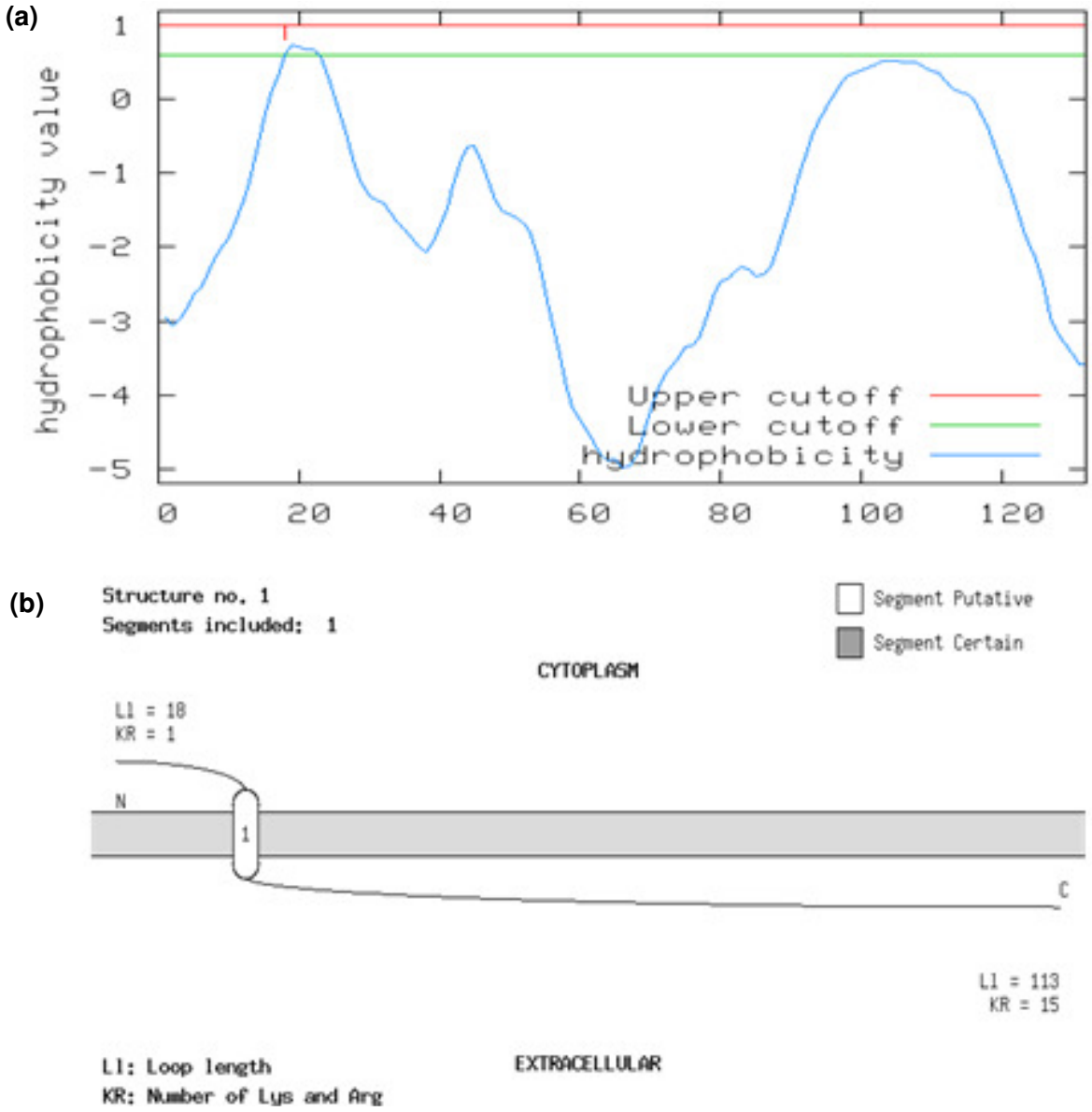

Figure 4. Prediction of membrane-spanning regions and their orientation (Huang et al., 2009). (a) Prediction of transmembrane and hydrophilicity. (b) Transmembrane orientation; Ll, loop length; KR, number of Lys and Arg.

1.6 mol/L, the growth of wzy2 transgenic yeast group improved compared with the controls. It indicates that the *wzy2* gene could improve the tolerance capacity of yeast to stressful conditions (Figure 8A and B).

The stress resistance assay of transgenic yeast GS115 carrying *wzy2* gene

The expression of a gene in response to one stress may derive protection against other types of stress (Imai et al., 1996). Therefore, it is meaningful to determine if the expression of *wzy2* altered the cold tolerance of transgenic yeasts. The experimental group (yeast carrying *wzy2* gene) and the controls (yeast with an empty vector pPIC9K), which were in the same growth status were treated at -20℃ for 48 h. Then the number of colonies was counted after plate culturing. The survival rate of the experimental group was 37.83 ± 9.26%, while that of the control was 32.85 ± 6.33%. This result indicates that under the low temperature, the survival rates of yeast carrying *wzy2* gene are not significantly different from the control (Figure 9).

DISCUSSION

Water-deficit stresses are some of the most adverse

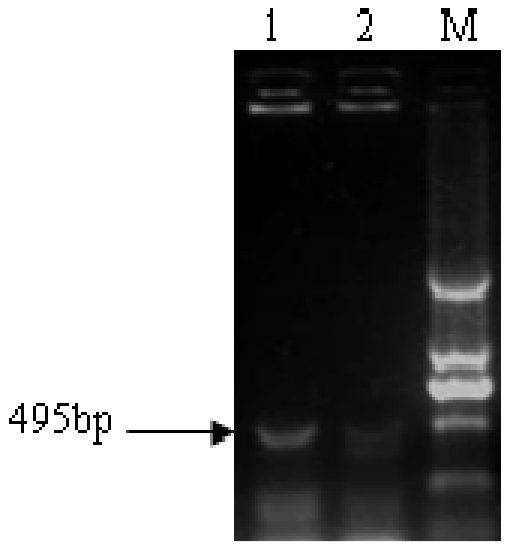

Figure 5. Recombinant *wzy2*-pPIC9K gPCR identification. Lanes 1 and 2, gDNA PCR results; lane M: DNA Marker DL2000.

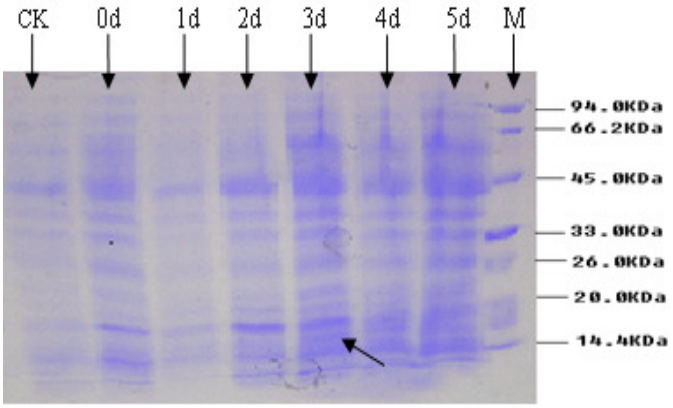

Figure 6. SDS-PAGE analysis of the expression level of *wzy2* at different times. Supernatants from GS115/ *wzy2*-pPIC9k were collected at different time points after methanol induction were processed and separated on SDS–PAGE on 15% polyacrylamide gels with the negative control of the 3 day supernatant of GS115/pPIC9K(CK). Lane M is protein standards. The positions of the special proteins induced by methanol are indicated by the arrow.

conditions for plants, and they can cause fatal damage to plants and seriously affect crop productivity (Yoshida et al., 2010). When plants are under adverse conditions such as drought, salt and low temperature, DHNs are induced to generate and accumulate (Bohnert et al., 1995). In this study, dehydrin gene *wzy2* was cloned from the leaves of seven-day-old wheat Zhengyin *NO.1* seedlings treated with PEG 6000. The cDNA of wzy2 is absent or unclear in seedlings without treatment of PEG 6000. The gene characteristics were similar with the dehydrin gene *wzy1-1* reported by Zhang et al. (2007a),

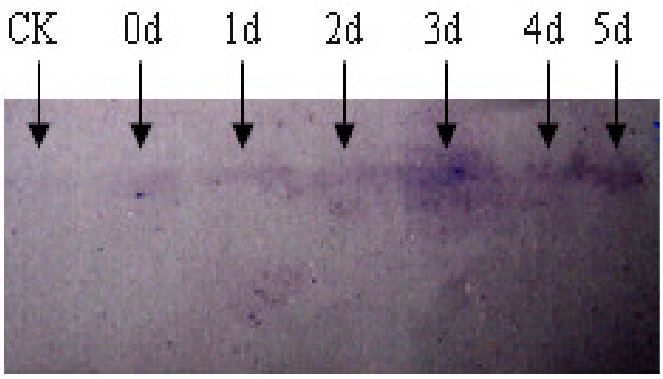

Figure 7. Western blotting detection of the *wzy2*-pPIC9K recombinants. The un-purified supernatants from GS115/*wzy2*-pPIC9K were concentrated by 10% TCA, separated on SDS–PAGE on 15% polyacrylamide gels, transferred to PVDF membrane and detected with Ab1, Ab2 and Strengthen BCIP/NBT Western Blotting Detection kit as described under the "material and methods".

and thus we speculated that the expression of this type of DHNs is related to drought and osmotic stress. Lack of water results in the increment of free radicals and peroxidation of plasma membrane, and consequently changes the cell structure. And small proteins such as DHNs play some roles in protecting the plasma membrane under drought conditions (Koag et al., 2003; Zhang et al., 2007b). Rorat (2006) speculated that the Y_nSK_2 type DHNs may stabilize the membranes by an inhibitory effect on the transition of the PA-derived vesicle to the hexagonal phase, or by altering membrane interfacial charge density to decrease the facilitated fusion of negatively charged molecules. The analysis of the deduced protein shows that the Wzy2 protein is highly hydrophilic and may be in a highly extensional status because of the abundance of the polar amino acids such as Gly (23.7%), Lys (7.9%) and His (6.6%). Thus, these findings suggest that Wzy2 protein may have a specific role in antioxidation of cell membrane in response to water stress.

There are many examples of the induction of α-helical structures that accompany the binding of proteins to membranes, notably the adoption of α-helical structure by the binding of α-synuclein to liposomes containing anionic phospholipids (Davidson et al., 1998; Koag et al., 2003; Ladokhin and White, 1999). The random coil structure of DHNs may maintain protein structure and binds water to protect plant cells against dehydration (Rorat, 2006). In our study, the prediction of the secondary structure of the protein encoded by *wzy2* gene shows that 87.5 % of the amino acid residues is in random coil, 9.21% in α-helix and 3.29% in fold domain. Further analysis of the secondary structure of *wzy2* protein shows that the transmembrane domain may also exist, which is presumed to be an amphipathic α-helix formed by K-segments, and is involved in binding part of the proteins and membranes, so that free water in the cell can be bounded and the

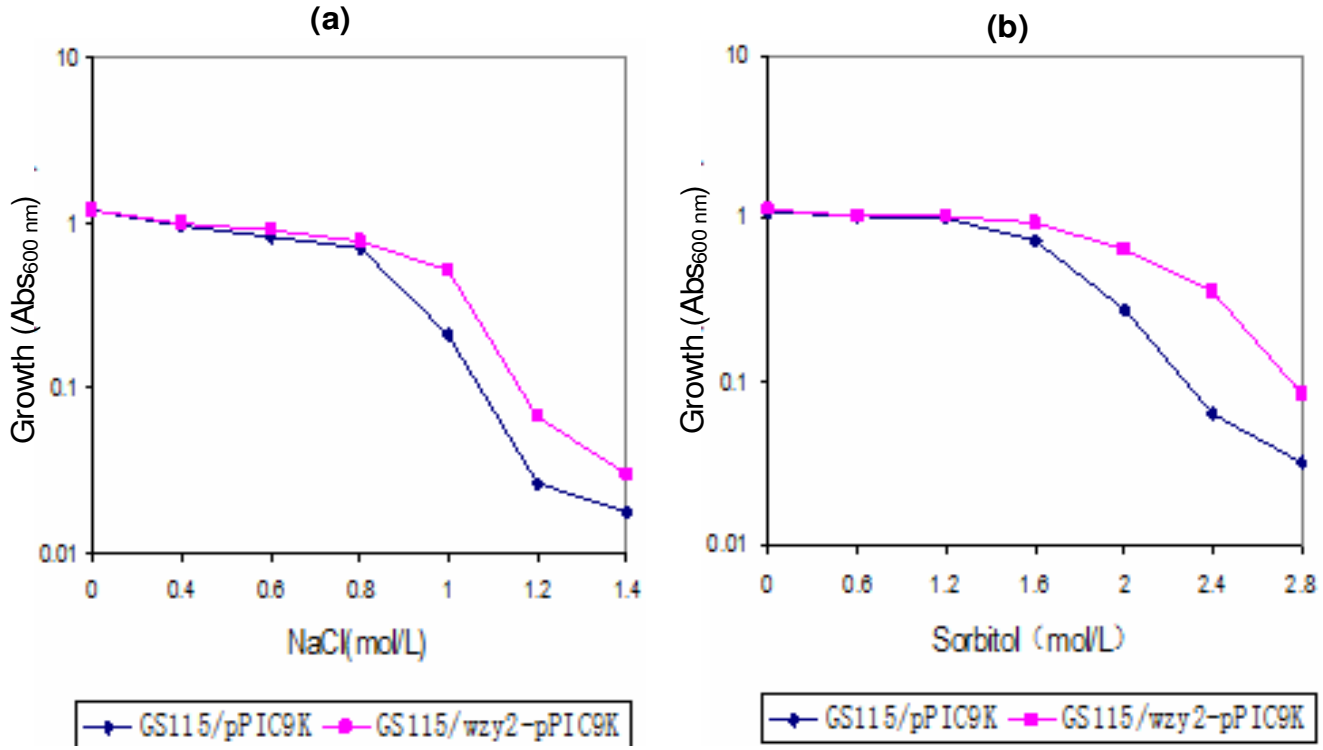

Figure 8. Yeast growth analysis with overexpression of *wzy2* under ion and osmotic stress. (a) NaCl concentration from 0 to 1.4 mol/L. (b) Sorbitol concentration from 0 to 2.8 mol/L.

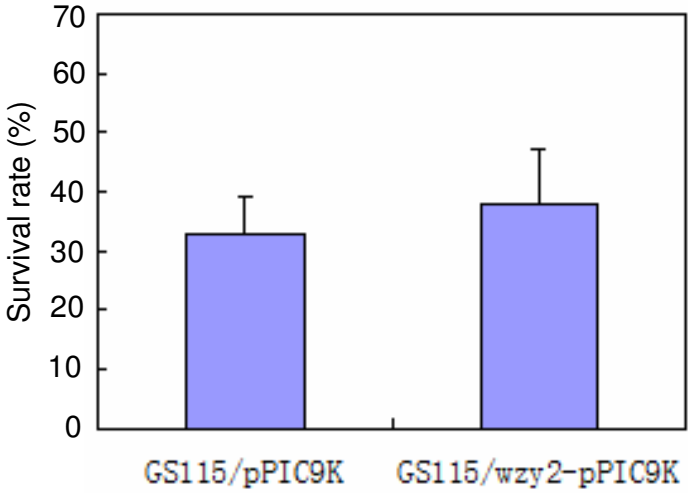

Figure 9. Cold resistance analysis for *wzy2* overexpression in yeast.

structure of the membrane can be stabilized (Campbell and Close, 1997; Svensson et al., 2002). The high-content of the random coil may play a role in maintaining water content in plant cells under adverse conditions such as drought, salt and low temperature stress.

To initiate the functional analysis of DHNs, *P. pastoris* cells have been used as a model organism. Yeast cells have been considered a good model system for eukaryotic cells and used to address the function of some heterologous genes (Gellissen et al., 1992; Zhang et al., 2000). In the present study, we cloned plant dehydrin gene *wzy2* into *P. pastoris* GS115 and examined their effects on growth performance under ionic, osmotic and temperature stress. Generally, the basic or neutral Y_nSK_2-types are induced by drought or abscisic acid (ABA), but not by low temperature (Allagulova et al., 2003; Xiao and

Nassuth, 2006). Our data demonstrates that *P. pastoris* cells expressing *wzy2* showed improved tolerance to salt and osmotic stress, but not to low-temperature stress, and further proved the assumption that the expression of Y_nSK_2 dehydrin can be induced by drought, but not by low temperature. Similar results were also reported in the dehydrin gene DHN1, DHN2, DHN3, DHN4, DHN6 and DHN9 in *Hordeum vulgare*. Furthermore, we also identified that the *wzy2* gene can also be induced by ABA, according to the report of Zhao (2010). But not all Y_nSK_2 DHNs can be induced by drought and ABA. For example, the DHN12 (Y_nSK_2) in *H. vulgare* is only express during the period of the embryo development, and any treatment cannot induce its expression in other tissues (Choi and Close, 2000). Recently, great progress had been going on in the constitution and structure, location and transportation, gene expression and regulation, function and mechanism of dehydrin. But further studies in the area of its expression regulation and the accurate mechanism are still interesting and necessary.

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