

Tibetan hulless barley dehydrin, *dhn4*, cloning and transforming into tobacco

J.-H. Wang, K.-L. Chen, H.-W. Li, J. He, B. Guan

Horticultural Institute of Sichuan Academy Agricultural Sciences, 610066, Chengdu, China, e-mail: kevin_wangjb@126.com

J.-B. Du

Department of botany and microbiology of University of Oklahoma, 7301, Norman, USA

J.-J. Liu

Sichuan Academy Agricultural Sciences, 610066, Chengdu, China, e-mail: sc.liujianjun@163.com

Abstract: A dehydrin, *dhn4*, cDNA fragment has been obtained via RT-PCR from Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f.). It indicated that *dhn4* encoded a YSK2 type dehydrin (DHN4). One Y segment (VDEYGNP), one S segment (SGSSSSSS) and two K segments (RKKGIKEKIKEKLP and EKKGIMDKIKEKLP) were identified in the deduced amino acid sequence of *dhn4*. The secondary structure of DHN4 protein predicated with software Anthepro 5.0 is prone to α -helix, and the tertiary structure predicated by SWISS-PORT indicated intrinsically unstructured. The coding region of the *dhn4* cloned into pBI121 binary vector with the 35S promoter was transformed into the *Agrobacterium tumefaciens* strain DHA105. The *Agrobacterium* mediation was transformed *dhn4* into the leaf disc of tobacco and then the tobacco plantlets with kanamycin resistant were regenerated using callus induction mediums supplemented with kanamycin and carbencillin. The regenerated plants were transferred into plots with peat moss and grown in the greenhouse. The inserting *dhn4* of regenerated plants were identified separately by PCR, PCR southern blot and DNA sequencing using the gnomonic DNA.

Keywords: Tibetan hulless barley, dehydrin, cloning, tobacco, transforming

Introduction

Dehydrins, also referred to group II of the late embryogenesis abundant

(LEA) proteins, are typically accumulate in the embryo or in vegetative tissues in response to an environmentally imposed dehydrative effects, such as drought, salinity or freezing. The different dehydrins with motifs combination were induced by a number of physiological responses against environmental signals. Dehydrins are believed to play a protective role during cellular dehydration to induce to prevent loss function of enzyme. They are structurally characterized by several conserved domains known as the K-segment (EKKGIMDKIKEKLP), S-segment (a Ser stretch), and Y-segment (V/TDEYGNP). The K-segment can be found in all dehydrins and is believed to fold into an amphipathic α -helix that facilitates interactions with various cell components (Close *et al.*, 1997).

Tibetan hulless barley (*Hordeum vulgare* L. var. *nudum* Hook. f.) is the main cereal crops in plateau areas with high altitudes from 2500 to 3000 meters in southwestern of China, in particular wide distribution among Tibet, Qinghai, and parts of Gansu, Yunnan and Sichuan provinces. The annual yields for Tibetan hulless barley in China takes account of 70% of the world total production. Lacking water and low temperature in the mentioned regions, it is unable to widely cultivate alternative cereal crop to replace it. Tibetan hulless barley, a variety of *Hordeum vulgare*, is adaptable for growth in the highland to feed large local population.

To better understand the mechanism of resistant cold and drought stress for Tibet hulless barley, the Tibet hulless barley dehydrin, *dhn4*, has been successfully cloned. Sequentially, the PCR products of the complete dehydrin ORF, open reading fragment, was recombinant into the expression vector pBI121 controlled by the constitutive promoter CaMV35S with *nptIII* as the selectable marker. *Agrobacterium tumefaciens* DHA105 mediation was carried out to transform into tobacco, *Nicotiana tabacum*. The regenerated tobacco seedlings were further identified using PCR and PCR southern blot. Meanwhile the PCR products for regenerated tobacco seedlings were subjected to DNA sequencing.

Materials and methods

Materials

The seeds of Tibetan hulless barley were kindly provided by Professor Lin Honghui. The cloning vector pMD18-T, rTaq DNA polymerisase, DNase, endonuclease were purchased in company (TAKARA, Japan). M-MLV was provided by supplier (Invitrogen, USA). The competent cell of *Escherich coli* strain DH5 α , *Agrobacterium tumefaciens* strain DHA105 and binary vector expression

pBI121 (Clontech, USA) have been long term conserved in our laboratory.

Methods

Tibetan hulless barley dehydrin, dhn4, clone and analysis

Seven days after germination, the seedlings of Tibetan hulless barley were transferred onto Hoagland solution for plant propagation under the at 25°C under a 16-h photoperiod. The seedlings have been treated with 15% (W/V) PEG6000 for three days. The total RNA was extracted according to the previous protocol (Zhang *et al.*, 2004). The primers for following procedures have been designed by the primer premier 5.0 (Premier, Canada). The degenerate dehydrin primer Yinwu-1, Yinwu-2 and Yinwu-3 have been designed for the 3'-RACE (Table 1). To remove contaminating DNA, RNAs were treated with RNase-free DNase. The resulting RNA samples then were reverse-transcribed by M-MLV reverse transcriptase to produce the first strand of the cDNA using Yinwu-3 containing Oligo (dT). The PCR fragments obtained using Yinwu-2 and Yinwu-1 was cloned into the pMD18-T vector and sequentially transformed into *Escherich coli* strain DH5 α referring to protocol (Wang *et al.*, 2006). The PCR protocol was carried out under following condition: 94°C for 2 min, the 30 recycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and then final extension at 72°C for 8 min.

The inserting fragment of clones obtained by 3'RACE was subject to DNA sequencing (Invitrogen, USA) and to analyze by BLAST in the Genebank. The resulting PCR sequence was identified to be a part of *dhn4* due to the considerable high homology with the known dehydrin *dhn4* originated from *Hordeum vulgare* at the level of nucleotide. In order to obtain the full length of cDNA, the specific Yinwu-4 and Yinwu-5 (table 1) were designed based on the known barley dehydrin cDNA sequence. The RT-PCR products were cloned into the pMD18-T vector to generate pMD18-DHN using the specific Yinwu-4 and Yinwu-5 followed by DNA sequencing. The resulting nucleotide sequence was subjected to analyze by Editseq of software package (NASTar, USA) and to predict for protein structure of protein by Anthepro5.0 and SWISS-PORT (Wang *et al.*, 2008).

The recombinant expression vector pBI121-DHN construction

The recombinant binary vector, pBI121-DHN, has been constructed by following procedures (Fig.1). The full length of *dhn4* fragment have been amplified by PCR with the recombinant vector pMD18-DHN as template using two specific primers DHN5-2-1 and the DHN5-1-2 (table 1) adding with *Xba* I and *Sac* I endonuclease recognition sites for each. Sequentially, PCR products were treated

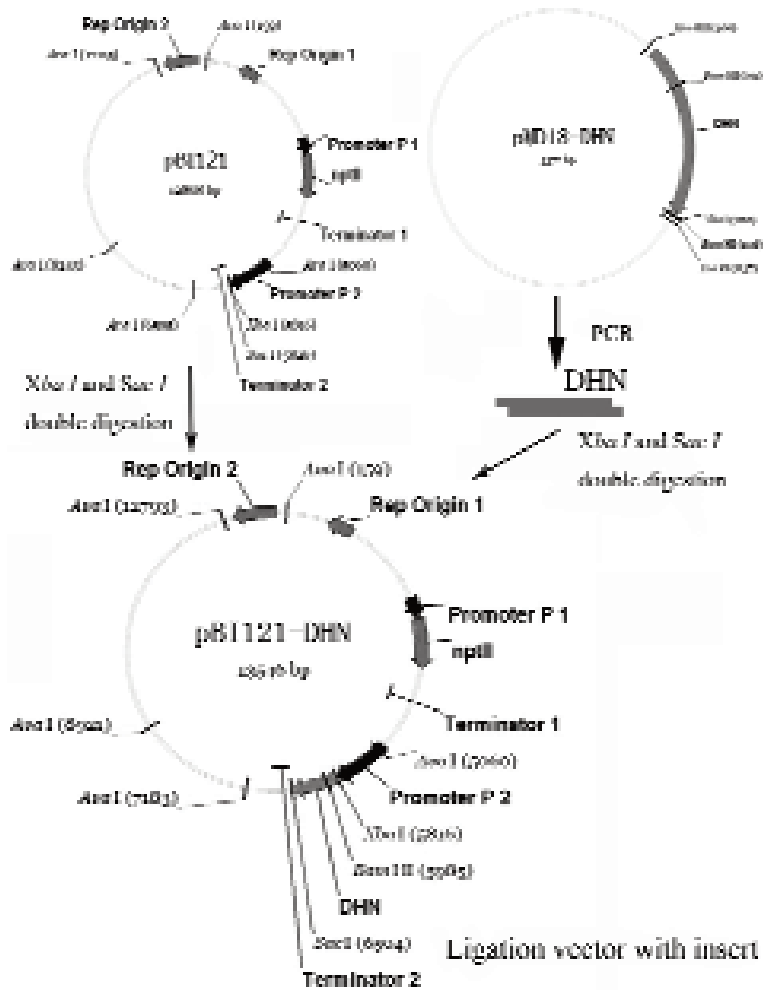


Fig. 1 - Scheme of recombinant construct of pBI121-DHN

by *Xba I* and *Sac I* then was purified again with the extraction kit (OMEGA, USA). The endonuclease restricted products were recombinant into expression vector pBI121 that was treated with same two enzymes digestion prior to purify again with kit. The recombinant vector pBI121-DHN has been transformed into the competent cell of *Agrobacterium tumefaciens* strain DHA105 and then cultured on the culture LB medium with kanamycin.

Table 1 - The nucleotide sequence of primers used in this study. W=A, T R= A,G S= G,C V=A, G, C

PRIMER CODE	NUCLEOTIDE SEQUENCE (5'-3')
Yinwu-1	AG ATG GAG TWC CAR GGS CAG CAS
Yinwu-2	GAC CAC GCG TAT CGA TGT CGA C
Yinwu-3	GAC CAC GCG TAT CGA TGT CGA C(T) ₁₆ V
Yinwu-4	CAA GTT CAG CGG CAG CGC AAG A
Yinwu-5	GCG AGG ACC ATA CCG TAA GCA TAC ATA GA
DHN5-2-1	TCTAGAATGGAGTACCAGGGACA
DHN5-1-2	CGGAGCTCTGCCGCTAGTGGCTC

Regenerated kanamycin resistant tobacco seedlings

Sterilized seeds of tobacco were sown on MS medium (Murashige and Skoog, 1962) and maintained at 25°C under a 16-h photoperiod. The mature leaves on the low positions of tobacco were harvested to cut into pieces in 5 cm wide and then were dipped with infiltrating LB medium cultures containing DHA105 transformed with pBI121-DHN for approximate 5 min. After co-culture in the callus induction medium (MS basic medium, 2mg/L 6-BA, 1mg/L IAA) for 5 d in dark, the infiltrated leaves disc were then subjected to the selective medium (callus induction medium supplemented with 100 mg/L Kanamycin and 500 mg/L carbencillin) and to maintain at 25°C under a 16-h photoperiod for 49 d. The resulting shoots about 10 cm in length were subjected to rooting medium for 24 d (1/2 MS medium supplemented with 250 mg/L cefotaxime sodium and 50 mg/L Kanamycin). The regenerated kanamycin resistant tobacco seedlings were transferred to plots with sterilized peat moss mixing with vermiculite.

PCR and PCR southern blot to identify transgenic tobacco

About total 1 g of tobacco mature leaves were harvested and then were grinded into fine powders adding with liquid nitrogen. The resulting powders were mixed with 3 ml pre-incubated CTAB and incubated the mixtures at 65°C for 45 min. When the mixtures decreased to room temperature, 1 ml of 5 M KAc was added and kept still at 4°C for 20 min. The mixtures then was added with chloroform and isoamyl alcohol (1:1) and subjected to centrifuge at 10000 rpm to collect the supernatant. 1.2 ml isopropyl alcohol was added into the mixture and collected the DNA precipitates. The DNA was transferred to clean tube with 1.5ml 70% ethanol for one night incubation at 4°C. The collecting precipitates were subjected to dry under the laminar flow cabinets and dissolved in the 2ml TE buffer. The RNase has been added and incubated for 1 h at 37°C. The treated

mixtures were transferred to 10 ml tube adding with 2ml chloroform and isoamyl alcohol (1:1) and then centrifuged at 8000rpm for 10 min. Supernatant were collected and added with 2ml 100% ethanol and 200 μ L NaAc (pH6.8) to stand for 2 h at -20°C. The DNA were obtained by centrifugation for 10 min at 8000rpm and then dissolved in the TE buffer.

The extracted DNA were subjected to identify by PCR under the following conditions: pre-denatured for 5 min at 94°C and then run for 35 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C with a final extension of 5 min at 72°C. The PCR products were separated by 1.5 % agarose electrophoresis. The resulting PCR products then were submitted to southern blot according to the DIG High Prime Labeling and Detection starter Kit's protocol (Roche, USA) and to DNA sequencing (Invitrogen, USA).

Results and analysis

Full length of *dhn4* ORF sequence cloning

The amplified 3' terminal partial sequence with 300 bp in size containing a conserved K motif was obtained using the degenerate primers by RT-PCR (fig. 2, 633bp-961bp). It was indicated it has the higher homology with the known barley *dhn4* from the Genebank.

Based on the known dehydrin sequence to design specific primers, the full length 678bp of sequence was produced by RT-PCR (fig.2). The complete sequence consisted of two specific conserved K motif, RKKGIKEKIKEKLPG and EKKGIMDKIKEKLPG, S motif rich in lysine, SGSSSSSS, and Y motif, VDEGNP, which was clearly indicated it belonged to a type of YSK2. The Tibetan hulless barley dehydrin has 98% identity with known *dhn4* dehydrins originated from barley at level of nucleotide. Therefore the cloned sequence was named by *dhn4* of Tibetan hulless barley.

The secondary structure of DHN4 predicated with software AnthePro 5.0 is prone to α -helix, which amphiphilic α -helices found in dehydrins are considered to bind to intracellular molecules, especially membranes and proteins to prevent cell membrane disassociation. The tertiary structure predicated by SWISS-PORT was indicated intrinsically unstructured.

Recombinant expression binary vector construction

The recombinant vector, pBI121-DHN, was extracted from LB medium

```

1      GTGCCAAGCTTGCATGCOCTGCAGGTGACGATTCAAGTTCAGCGGGCAGCGCAAGCTGGAG
1      M E
61      TACCAGGGACAGCAGCAGCGGCCGCTCGACGAGTACGGCAACCOCTGTGGCCGGACATGGC
21      Y Q G Q Q H G R V _ D _ _ E _ _ X _ _ G _ _ N _ _ P V A G H G
121     GTCGSCACCGGCATGGGAACGCACGGCGGGCTGGAACTGGCGCGGGCCCGGTGGGCAT
41      V S T G M G T H G G V G T G A A A G G H
181     TACCAGCCCATGAGGGACASGCACCAGACTGGCCGTGGGATCCTGCACCGCTCCGSCAGC
61      Y Q P M R D E H Q T G R G I L H R S G S
241     TCCAGCTCCAGCTCGTCCGAGGACGATGGCATGGGTGGCAGGAGGAAGAAGGGCATCAAG
81      S S S S S S S E D D G M G G R R R K K G I K
301     GAGAAGATCAAGGAGAAGCTCCCTGGTGGCCACGGTGAACCAGCAGCACAACCGCTGGCACC
101     E K I K K K L P G G H G D Q Q H N A G T
361     TACGGCTACGGACAGCAGGGTACTGGCATGGCCCGGCACCGGGCAACCTACGGSCAGCAG
121     Y G Y G Q Q G T G M A G T G G T Y G Q Q
421     GGTCACTGGGATGACGGGATGGGAGCCACTGACGGCACCTACGGACAGCAAGGCCAC
141     G H T G M T G M G A T D G T Y G Q Q G H
481     ACTGGGATGGCCGGCACCGGGGCACATGGCACCGCGGCCACCGGGCGSCACCTACGGSCAG
161     T G M A G T G A H G T A A T G G T Y G Q
541     CAAGSCCACCGGGATGACAGGCACGGGCATGCACGGCACCGGGCGSCACCTACGGSCAG
181     Q G H T G M T G T G M H G T G G T Y G Q
601     CAGGSCCACCGGGATGACAGGCACGGGCATGCACGGCACCGGGTGGCACCTACGGSCAG
201     Q G H T G M T G T G M H G T G G T Y G Q
661     CACGGCACCGGACCGGGGAGAGGAAGGGCATCATGGACAAGATCAAGGAGAAGCTCCCT
221     H G T D T G E K K G I M D K I K E K L P
721     GGCCAGCAGTGAAGCGCTCCGAGCCCGCCCGCGCCCGCCACTTCTGAGTGTGGAGGTG
241     G Q H *
781     CCGTCCGACCCCGTTCAGAAATCAATAATAAGATGGCGATAACAATAAAATCCACCATA
841     CAACSTGAGCATAGTTCACCTAGCTCACTTGGCTGTGGAGGGGGGAATGTATCTAGTTC
901     AAGTTTACGTGAACAACACTGTGTTAAGAGTGTTCCTGCTGTTTACACTATATAATCTT
961     GTAATTTCTTTTGGTTCAACCCATAAAAAAAAAAAAAAAAAA

```

Fig. 2 - *cDNA* sequence and predicted amino acid sequence of *dhn4* from Tibetan hulless barley

supplemented with kanamycin. The inserting fragment, *dhn4*, has been determined by PCR with 678bp in size (fig. 3A). Furthermore, double endonuclease digestion tests were subjected to confirm the inserting fragment *dhn4*. The digested products with 680 bp and 1540 bp in size have been identified using different pairs of endonuclease (fig. 3B).

Tobacco seedlings with kanamycin resistant regenerated

The leaf discs of tobacco were submitted to medium culture with kanamycin

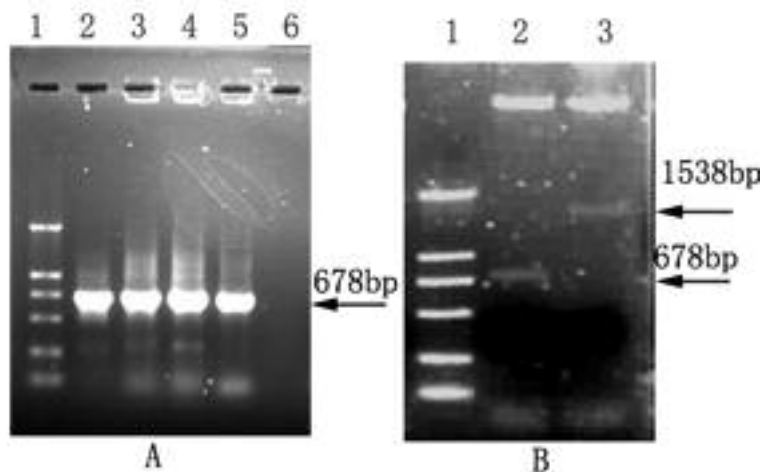


Fig. 3 - A The clones for *Agrobacterium tumefaciens* transformed with pBI121-DHN were identified by PCR. The 2,3,4,5,6 electrophoresis lanes were indicated using the PCR products by the DNA templates originated from recombinant vector, random picked *Agrobacterium* clone 1, random picked *Agrobacterium* clone 2, random picked *Agrobacterium* clone 3 and empty as control respectively.

B The 2,3 electrophoresis lanes shown the recombinant vectors restricted digestion by Xba I combined Sac I and Sac I combined Hind III separately. Molecular Weight Maker DL2,000 (TAKARA, Japan)

selection pressure after infiltrating with *Agrobacterium* (fig. 4A). The callus with kanamycin resistant was induced in callus inducing medium adding with kanamycin (fig. 4B).

The roots of seedlings with ability of anti-kanamycin have been produced in root inducing medium supplemented with lower amount of kanamycin (fig. 4C). The regenerated plantlets were transferred to small pots with peat moss mixing with vermiculites (fig. 4D).

The transgenic tobacco identification

The regenerated tobacco seedlings with kanamycin resistant were further investigated to confirm the *dhn4* was already inserted into the plant genome. Comparing PCR electrophoresis patterns between wide type plants, plants transformed with empty binary vector pBI121 and with pBI121-DHN, it clearly indicated kanamycin resistant seedlings produced more intensities bands with

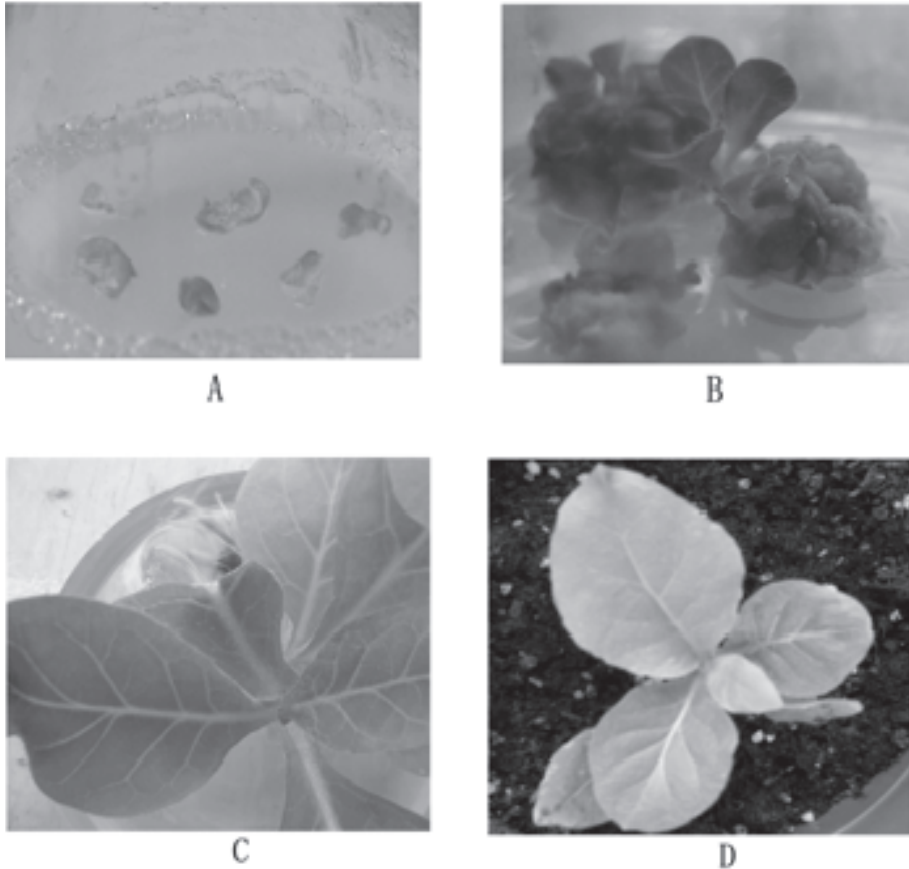


Fig. 4 - Photographs were shown the regenerated tobacco seedlings during the different culture steps

678bp in size but previous two seedlings only unspecific bands (fig. 5B).

To deeply investigate the unspecific bands of PCR products for wide phenotype, the PCR southern blot were carried out. But the PCR Southern blot produced similar results for the wide type, which have detected a band with less molecular weight than those of transgenics (fig. 5A). Therefore, all of the PCR products for wide phenotype, seedling transformed with pBI121-DHN and seedling transformed with empty vector were subjected to DNA sequencing. It was found the PCR sequence of transgenic transformed with pBI121-DHN only were definite *dhn4* but wide phenotype and seedling with empty vector failed to be determined.

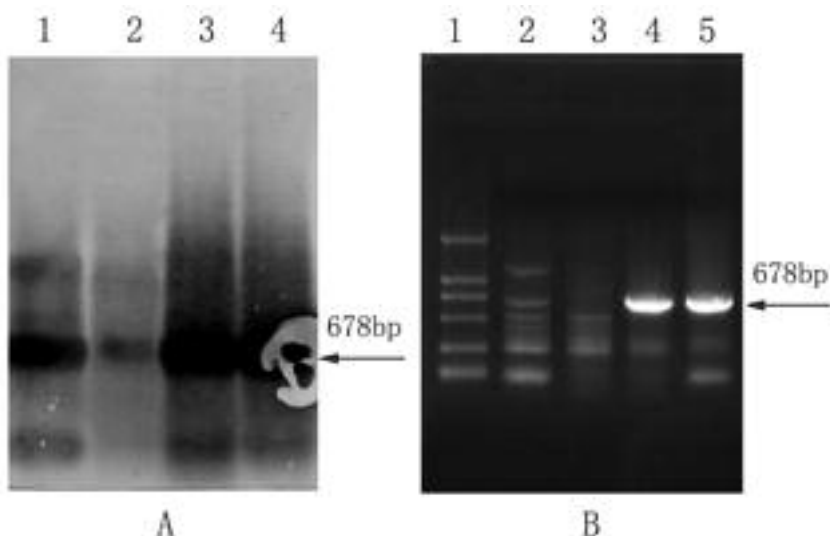


Fig. 5- A The electrophoresis lane 1,2,3 and 4 indicated the PCR southern blot using extracted total DNA originated from wide phenotype, transgenic seedling transformed with empty vector, transgenic seedling clone 1 transformed with pBI121-DHN and transgenic seedling clone 2 transformed with pBI121-DHN.

B The electrophoresis lane 2,3,4 and 5 indicated the PCR products using extracted total DNA originated from wide phenotype, transgenic seedling transformed with empty vector, transgenic seedling clone 1 transformed with pBI121-DHN and transgenic seedling clone 2 transformed with pBI121-DHN. Molecular Weight Maker DL2,000 (TAKARA, Japan)

Discussion

Citrus (*Citrus unshiu* Marcov.) dehydrin, CuCOR19, in response to chilling stress was overexpressed in tobacco (*Nicotiana tabacum* L.) and the cold stress tolerance of dehydrin transgenic at low temperature facilitates plant cold acclimation by acting as a radical-scavenging protein to protect membrane systems under cold stress and has inhibitory activity against lipid peroxidation (Hara *et al.*, 2003). In addition, the transgenic *Arabidopsis thaliana* with wheat *dhn-5* dehydrin have higher proline contents, lower water loss rate under water stress and Na^+ and K^+ accumulate to higher contents in the leaves of the transgenic plants (Brini *et al.*, 2007). However, other study indicated no difference in freezing tolerance improvement was found between the different plants, *wor40* wheat dehydrin over-

expression transgenic strawberry or wide phenotype, under non-acclimated conditions, suggesting that the WCOR410 acidic dehydrin protein needs to be activated by another factor induced during cold acclimation (Hound *et al.*, 2004). Furthermore, expression levels of the DHN24 protein, *Solanum soganandinum* SK3 dehydrin, varied among transgenic lines and overall, no correlation between the DHN24 protein level and the degree of chilling tolerance was found (Yin *et al.*, 2006). So far, it still needs deep investigations to point out the physiological and molecular roles induced by the environmental signals, especially under the stress of drought and freezing.

In this study, a dehydrin *dhb4*, full length cDNA fragment has been obtained *via* RT-PCR from Tibetan hulless barley. It indicated that *dhb4* encoded YSK2 type dehydrin (DHN4). One Y segment, one S segment and two K segments were identified in the deduced amino acid sequence of DHN4. Tobacco was transformed with the recombinant constructs using the *Agrobacterium tumefaciens* mediating transformation method to generate transgenic plants. In this paper, the PCR and PCR- Southern blot didn't conclude a clear or definite answer to identify inserting fragment of transgenics due to many unspecific amplified bands with wide phenotype. It could be speculated tobacco could have similar own dehydrin, as house keeper gene, which may be a little higher homology with *dhb4* of Tibetan hulless barley at the level of nucleotide. Therefore the PCR products for kanamycin resistant seedlings and wide phenotypes were further subjected to DNA sequencing and found it was definite *dhb4* for PCR product of transgenic but no clear results for wide type. In future, the transgenic tobacco, over-expressed alkaline dehydrin protein YSK₃, should be investigated against proper biochemical indicators under the water stressed condition. Drought and salt stresses are two major factors that lower plant productivity. Transgenic approaches offer powerful means to better understand and then minimize loss of yield due to these abiotic stresses. Thus, the development of genetically engineered plants with enhanced tolerance, dehydrin, presents a new opportunity for horticulture crops breeding in future.

Acknowledgement

The authors want to thank Dr. Luciano Conticini, Dr. Stefano Del Debbio at IAO in Florence of Italy for their kind of helps. This work was partially supported by the project, Center for the production of fruit plants in Sichuan province, found by Directorate General for development co-operation (DGCS) of Italian

Ministry of Foreign Affairs and by Young Researchers funding of Sichuan Academy of Agriculture Sciences and by Fruit Breeding Project In Sichuan Province.

Bibliography

- Brini F., Hanin F., Lumbreras V., Amara I., Khoudi H., Hassairi A., Pages M., Masmoudi K., 2007. *Overexpression of wheat dehydrin DHN-5 enhances tolerance to salt and osmotic stress in Arabidopsis thaliana*. Plant Cell Rep., 26:2017-2026.
- Close T.J., 1997. *Dehydrins: a commonality in the response of plants to dehydration and low temperature*. Physiol. Plant, 100:29-296.
- Hara M., Terashima S., Fukaya T., Kuboi T., 2003. *Enhancement of cold tolerance and inhibition of lipid peroxidation by citrus dehydrin in transgenic tobacco*. Planta, 217: 290-298.
- Houde M., Dallaire S., N' Dong D. and Sarhan F., 2004. *Overexpression of the acidic dehydrin WCOR410 improves freezing tolerance in transgenic strawberry leaves*. Plant Biotechnology Journal, 2 :381–387.
- Murashige T., Skoog F., 1962. *A revised medium for rapid growth and bioassays with tobacco tissue cultures*. Physiol. Plant, 15:473-497.
- Wang J.-H., Liu X., Chen J., Deng J.-L., Chen K.-L., Li H.-W., He J., Jiang G.-L., Liu J.-J., 2006. *Grapevine Leafroll associated Virus-3 in Sichuan, China*. Journal of Agriculture and Environment for International Development, 101 (3/4): 131-141.
- Wang J.-H., Liu X., Xi D.-H., Yuan S., Jiang Y., Yang H., Du J.-B., Zhang Z.-W., Chen K.-L. and Lin H.-H., 2008. *Cloning and Prokaryotic Expression of CP Gene of Grapevine virus A Sichuan Isolate*. Acta Horticulturae Sinica, 35(7) : 967-972.
- Yin Z., Rorat T., Szabala B.M., ĩkowska A.Z., Malepszy S., 2006. *Expression of a Solanum soganandinum SK3-type dehydrin enhances cold tolerance in transgenic cucumber seedlings*. Plant Science, 170 :1164-1172.
- Zhang N.-H., Wei Z.-Q., He J.-X., Du L.-F., Liang H.-G., 2004. *An efficient and economic method for preparation of high quality plant RNA*. Prog. Biochem. Biophys., 31 (10): 947-950.