African Journal of Biotechnology Vol. 10(35), pp. 6725-6732, 13 July, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.1510
ISSN 1684–5315 ©2011 Academic Journals

Full Length Research Paper

Cloning and mRNA expression pattern analysis under low temperature stress of *EAAP* gene in Dongmu-70 rye

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Accepted 19 May, 2011

This research cloned endochitinase-antifreeze protein precursor (*EAPP*) gene of Dong-mu 70 rye (*Secale cereale*) by designing special primers according to Genbank's *EAPP* gene sequence, and analyzing the influence of low temperature stress on the expression of mRNA with RT-PCR. The results indicated that the *EAPP*'s full length coding of Dong-mu-70 rye was 1085 bp, while the homology of its amino acid and that of *Hordeum vulgare* and *Triticum aestivum* was much higher (95 and 91%, respectively). The mRNA of the *EAPP* gene in Dong-mu 70 rye was expressed in roots under normal temperature, and was most active in the leaves. When stressed at a low temperature of 4°C, remarkable improvements were shown in mRNA expression in the root and stems, and the maximum expression appeared 24 h after treatment and decreased afterwards.

Key words: Dongmu-70 rye (*Secale cereale*), endochitinase-antifreeze protein precursor (*EAPP*) gene, low temperature, expression pattern of mRNA.

INTRODUCTION

Plants contain relatively low chitinases which are closely related to the duration of disease normally, but when attacked by virus, bacteria and pathogens, injured or treated with ethylene, its activity strengthens drastically by several times even by hundreds of times (Linthorst, 1991). In addition to its inhibition of plants' infection of pathogens, virus and bacteria (Collnge et al., 1993), it also functions as plant growth adjustment in grapes' ripening (Robinson et al., 1997) and carrot's early embryogenesis (Kragh et al., 1990). Also, it participates in symbiotic nitrogen fixation by degrading nodule formation factors produced by nitrogen fixation (Xie et al., 1996; Minic et al., 1998). Besides, it influences various adversity stresses, among which its anti-freeze quality is gradually discovered. Hon et al. (1994) extracted 6 AFPs with similar amino acid formation from *Secale cereale's* apoplast, found in the homology of 3 N-sequences and 3 anti-bacteria proteins (chitinase, β-1,3-glucanase and sweet protein, having a resemblance with albumin). This was shown in immunoassay as chitinase β-1,3-glucanase and sweet protein, having a resemblance with albumin. Hiilovaara-Teijo et al. (1999) discovered that these proteins displayed dual activity of anti-freeze and anti-disease under low temperature stress with only anti-disease activity expressed under non-low temperature stress.

Yeh et al. (2000) hold that these proteins possibly exist on apoplast other than on vacuole, in that the proteins' expression, induced by low temperature, may be modified and may form a structure domain which inhibits crystallization. Thus, it enables plants to develop anti-disease and anti-freeze quality. They also extracted two chitinase genes coded by I and II from cDNA store of cold-trained winter rye (*CHT9* and *CHT46* expression product), of which delay of crystallization can be inhibited, and the proteins expressed by *CHT9* and *CHT46* genes can be illustrated to have anti-freeze reaction. At present, ethylene is found to function in finding the hydrolyzing

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Abbreviations: ***EAPP,*** Endochitinase-antifreeze protein precursor; ***LRR*****,** leucine-rich repeats; **AFP,** antifreeze protein; **ZmCIPK,** CBL-interacting protein kinase from maize; **OSRLK1,** LRR-type receptor-like protein kinase; **CALRR1,** Capsicum annuum leucine-rich repeat 1.

Table 1. Parameter of PCR primers.

Gene	Primer (5'-3')	Tm (°C)	Product size (bp)
EAPP	F: CTTCATTGCCCAAGATGAGAG R: GGATTGCACCATTATTCGCT	57.7	1085
Tublin	F: GTTGTCCGTCGTGAGGC R: TCAGGCACCGAAATGGC	57.8	509

Tm; melting time.

activity of chitinase in winter rye under low temperature stress (Yu et al., 2001), and it is closely related to the content of Ca^{2+} in mesophyll cells (Stressmann et al., 2004). The cold inducible expression proteins of *CHT9* and *CHT46* genes spread on the parenchyma of sheath, mesophyll, epidermal cells and phloem parenchyma, and are accumulated indirectly as part of growth and development (Yeh et al., 2000). However, reports about anti-freeze protein evolution of *EAPP* chitinase of Dongmu-70 rye and expression pattern analysis under low temperature stress are few. The experiment took *S. cereale* cv Dongmu-70 as the object, designed a special primer based on anti-freeze gene sequence of winter rye in Genbank, clones full length coding sequence of *EAPP* by RT-PCR, and analyzed the system evolution and mRNA expression pattern of the gene under low temperature stress. The research would provide theoretical support for further discovery of the gene's anti-freeze mechanism.

MATERIALS AND METHODS

Seeds of *S. cereale* cv Dongmu-70 were bought in Jindao Seedling Company, while the RNA extraction kit was gotten from Invitrogen Company, RevertAidTM First Strand cDNA Synthesis Kit from Fermentas Company, Taq enzyme from Invitrogen Company, dNTP from Invitrogen Company, DNA Maker from Tiangen Biochemistry Company, DNA Recovery Kit from Tiangen Biochemistry Company, pMD19-T vector from TakaRa Company, and *E. coli* DH10B was gotten from a lab store. However, plasmids mini-extraction kit was gotten from the Invitrogen Company.

Treatment of plant materials

Seeds of Dongmu-70 were flushed with aseptic water three times after 30 s of soaking in ethanol, sterilized with 4% sodium chlorate for 60 min, and washed three to five times. The seeds were planted in plates with three-layer-filter paper, placed in MS nutrient solution for hydroponic cultivation and covered by Handi wrap with air vent. They were allowed to sprout up in a culture box at 25 ± 1°C. The Handi wrap was opened and the seeds or seedlings were washed with MS nutrient solution everyday, to ensure that the bottom of the plate was dump. The Handi wrap was covered and abundant MS nutrient solution was drained off to prevent too which makes seeds develop slowly for humus respiration. When sprouts were about 5 cm long and roots were about 7 cm long, they were moved into flasks and put in a culture box at 25 ± 1°C with 4000 lx (12 h day^{-1}) illumination. There was a continuation of the hydroponic cultivation with MS nutrient solution, while RNA was extracted using trizol.

RNA expression pattern analysis of EAPP under low temperature

According to *EAPP* sequence (AF28043) of GenBank (Hon et al., 1994), using Primer Premier5.0 software, the designed PCR amplimer of the gene chose house keeping gene of *Tubulin* as an inner reference, as shown in Table 1. The PCR used a thermocycle of 95°C for 5 min, 32 cycles of 94°C for 30 s, 60.5°C for 30 s, 72°C for 1 min and with a final extension at 72°C for 10 min. Analysis of the tested PCR of *EAPP,* by 1% agarose gel electrophoresis, and the scanned line density results of the amplimer, was done by Gel-Pro analyzer. When the OD of Tublin was compared with its converted OD, a relative abundance ratio of *EAPP* was gotten. At the same time, when it was compared with Dongmu-70 nurtured under 25 ± 1°C, the tissues of leaves and stems were taken at different times and the different RNA expressions were tested.

Biology information analysis

BLAST was used to compare the homology and search sequence in GenBank database, and was used to visit the ORF finder of NCBI. The ORF analysis was done on an open reading frame of nucleic acid of the sequence obtained by electronic expansion, using standard genetic code. The sequence was converted into amino acid sequence with Primer Premier5.0. However, the cluster analysis was done with DNADIST (PROTDIST), NEIGHBOR, TREEVIEW, FITCH and COBSENSE of PHYLIP (http://evolution.genetics.washington.edu/phylip.html).

RESULTS

EAPP gene cloning

As shown in Figure 1, as tested by 1% agarose gel electrophoresis, the cDNA of Dongmu-70 *EAPP* was about 1000 bp as predicted, and the length was 1085 bp.

System evolution of *EAPP* gene

The *EAPP*'s complete cDNA sequence was searched for in an online free database, to obtain 25 cDNA sequences of different plants. When compared by the homology, the *EAPP* of Dongmu-70 shared relatively high homology with *Hordeum vulgare* and *Triticum aestivum*. The resemblance ratio of the nucleotide's sequences was seen as 97 and 96%, respectively, while for amino acids, it was seen as 95 and 91%, respectively. The system evolution showed (Figure 2) that Dongmu-70 had the closest kinship

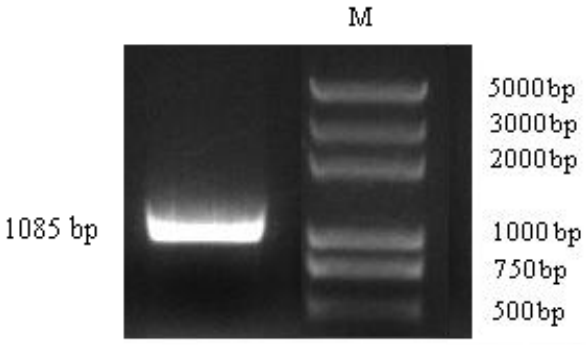

Figure 1. Transcription of *EAPP* gene.

with panacea plants including *Triticum aestivum* AY437443, *Zea mays* EU72, NM001156110, *Oryza sativa* RICCHT1, Z29961, *Festuca arundinacea* EU837265 and *Poa pratensis* AF000964. The same kinship was found with *Medicago sativa* U83592.1 and *Medicago truncatula* Y10373.1, but the farthest kinship was found with panace plant *Bromus inermis* (AB428423).

EAPP gene structure and features analysis

The Dongmu-70's *EAPP* gene sequence test was converted into amino acid sequence as the following:

MRGVVVVAMLAAAFAVSAHAEQCGSQAGGATCPNCLC
CSKFGFCGSTSEYCGDGCQSQCNRCGGTPVPVPTPT
GGGVSSIISQSLFDQMLLHRNDAACLAKGFYNYGAFIA
AANSFSGFATTGGTDVRKREVAAFLAQTSHETTGGWP
TAPDGPYSWGYCFNQERGAPSDYCSPSSQWPCAPGK
KYFGRGPIQISYNYNYGPAGRAIGTDLLNNPDLVATDAT
VSFKTALWFWMTPQSPKPSSHDVITGRWSPSGADQAA
GRVPGYGVITNIINGGLECGRGQDARVADRIGFYKRYC
DLLGVSYGDNLDCYNQRPFA

As shown in Figure 3, the proteins of Dongmu-70 rye *EAPP* had continual sequences. They were composed of 11 repeated fractions of 12 to 13 amino acids, while wheat consisted of 10 repeated fractions, seven for maize subspecies, seven for corn, five for Japanese subrice and one for oryza. It indicated that the sequence of amino acid of *EAPP* proteins for Dongmu-70 complies with the structure of anti-freeze protein, thus rye has an anti-freeze quality. The protein consisted of four repeated sequences, and had three anti-freeze sequences of LXX: LAA, LCC and LNN, where only LCC existed in the six compared species, while the other two, LAA and LNN, can be found in rye and wheat. The other four plants had gene mutation, which might be a vital sequence related to the anti-freeze quality of rye. In particular, LAA and LNN were more closely related to this quality. However, the other four plants showed gene mutation in LAA and LNN, which might influence their anti-freeze quality.

Different expressions of mRNA in different organs

As shown in Figure 4, there was *EAPP*'s mRNA expression in the roots, stems and leaves of Dongmu-70 rye, but were mainly expressed in leaves under room temperature (25℃). The second most obvious expression existed in the roots, while the least existed in the stems. As shown in Figures 5 and 6, after 48 h of 4℃ low temperature induction, mRNA of *EAPP* had roughly the same tendency of expression under room temperature, but the relative amount of expression was distinctively higher than that in the normal temperature, which proved that *EAPP* of different tissues of Dongmu-70 exhibited low temperature induction expression. As such, it was relatively lower under normal temperature, and obviously higher under low temperature stress.

DISCUSSION

Since Devies (1969) first discovered anti-freeze protein in 1969, *AFP* has been found in microorganisms (Gilbert et al., 2005), insects (Graham et al., 1997; Graether et al., 2004) and other advance plants (Huang et al., 2002; Griffith et al., 1997; Worrall et al., 1998) in rye. However, the *AFP*'s molecular weight showed six proteins with clear anti-freeze activity in 11-36 KD. Yin et al. (2001) compared the *AFP* sequences of carrots of Wuzhong County in Ningxia Province, Hua County and Hanzhong in Shaanxi Province with those of British carrots, and found that the homology was 98.5%. Lu et al. (2007) extracted the anti-freeze proteins of 14-90KD molecular weight and heat activity inhibition from leaves of *Ammopiptanthus nanus* in Xinjiang. The experiment showed that *A. nanus* obviously has inter-specific genetic consistence, and strongly supported the conclusion that anti-freeze proteins existed in plants of the same genus. The research for the first time extracted the full length of *EAPP* of coded plant from Dongmu-79 rye, and found out that high homology existed between Dongmu-70 rye barley and wheat, with 95 and 91%, respectively.

The experiment showed that *EAPP* was expressed in the stems, leaves and roots. Some studies indicated that the seedlings of carrots were treated for 30 min in low temperature of 4℃, and its anti-freeze gene (*DcAFP*) starts to express and becomes constant within 24 h (Meyer et al., 1999). Bian et al. (2008) cloned *ZmCIPK3* of corn protein kinase, in which the transcription of *ZmCIPK3* cannot be tested in embryo, coleorhiza, roots and leaves of seedlings, but increased the obvious linesin tassel, ovary and ripe leaves. Nonetheless, it was sensitive to 4℃ low temperature induction, while the transcription level increased clearly within 2 h, and was maintained at high level of transcription after 24 h. In the study, the transcription of *EAPP* was tested in normal temperature in roots, stems and leaves. The grayness showed that its expression was at a low level and was

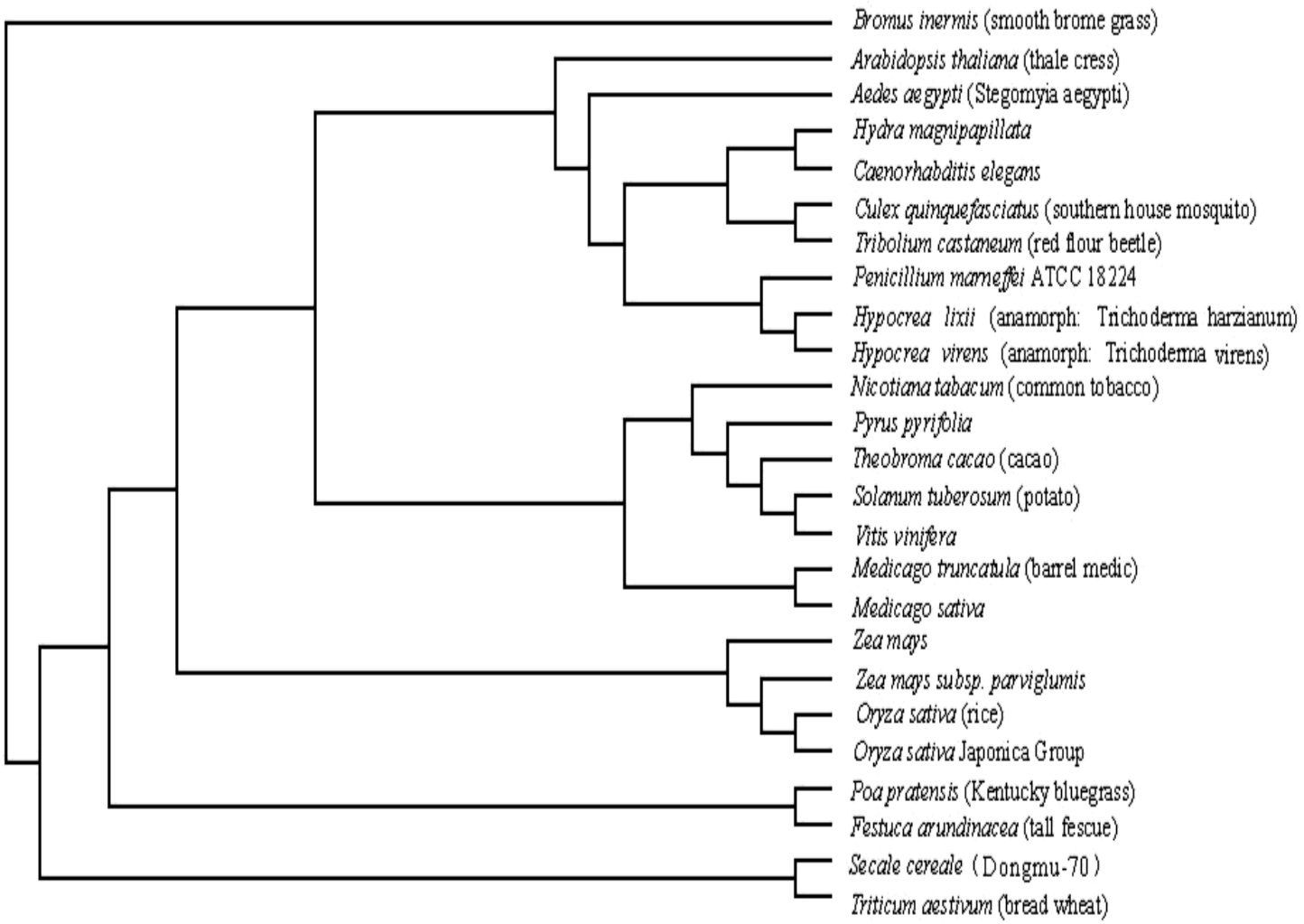

Figure 2. The evolution dendrogram of amino acid sequences encoded by the *EAPP* genes.

single, which may be related to proteins of disease duration of chitinase. After 6 h of 4℃ low temperature induction, expression of *EAPP* in Dongmu-70 rye increased obviously and reached its peak after 24 h. The same tendency of increase was shown in leaves, stems and roots, but the highest was in the leaves. Low temperature, short photoperiod, relatively low humidity and other climatic factors, characterized by winter, can stimulate the insect's *AFP* expression (Fei et al., 2000). Expression and activity of anti-freeze proteins differ in different growing periods and on different tissues. *EAPP* is expressed in the intercellular space of molecular leaf epidermis, roots, stems interior raw net of mesophyll, golgi apparatus, vesicle adjacent to the membrane, the thickened point of the secondary walls of protoxylem vessels and cell wall of epidermis of winter rye (Antikainen et al., 1996). Yeh et al. (2000) pointed out that chitinase in rye only indirectly accumulates part of the growing period during cold training and do not accumulate directly under low temperature and short period of sunshine. The experiment showed that *EAPP* gene of Dongmu-70 rye was induced by low temperature, and was expressed differently in tissues of roots, stems and leaves. Whether the expression was influenced by short period of sunshine or whether it was accumulated during growing, needs further proving.

The conventional domain, in sequences of anti-freeze proteins, plays an important role in its complete construction and anti-freeze function. Each variety of anti-freeze proteins has different number of repeated fractions composed of 12 to 13 amino acids, which showed positive relativeness to its anti-freeze quality (Liu et al., 2005). Anti-freeze protein of *Tenebrio molitor* consists of 7, 8, 10, 12 repeated units TCTXSXXCXXAX with rich disulfide bond (Graham et al., 1997; Graether et al., 2004). *EAPP* described in the paper was made of 11 repeated fractions, which illustrates its better anti-freeze activity. There is a certain resemblance in the structures of disease duration proteins and intercellular message transcription proteins, and the common sequence

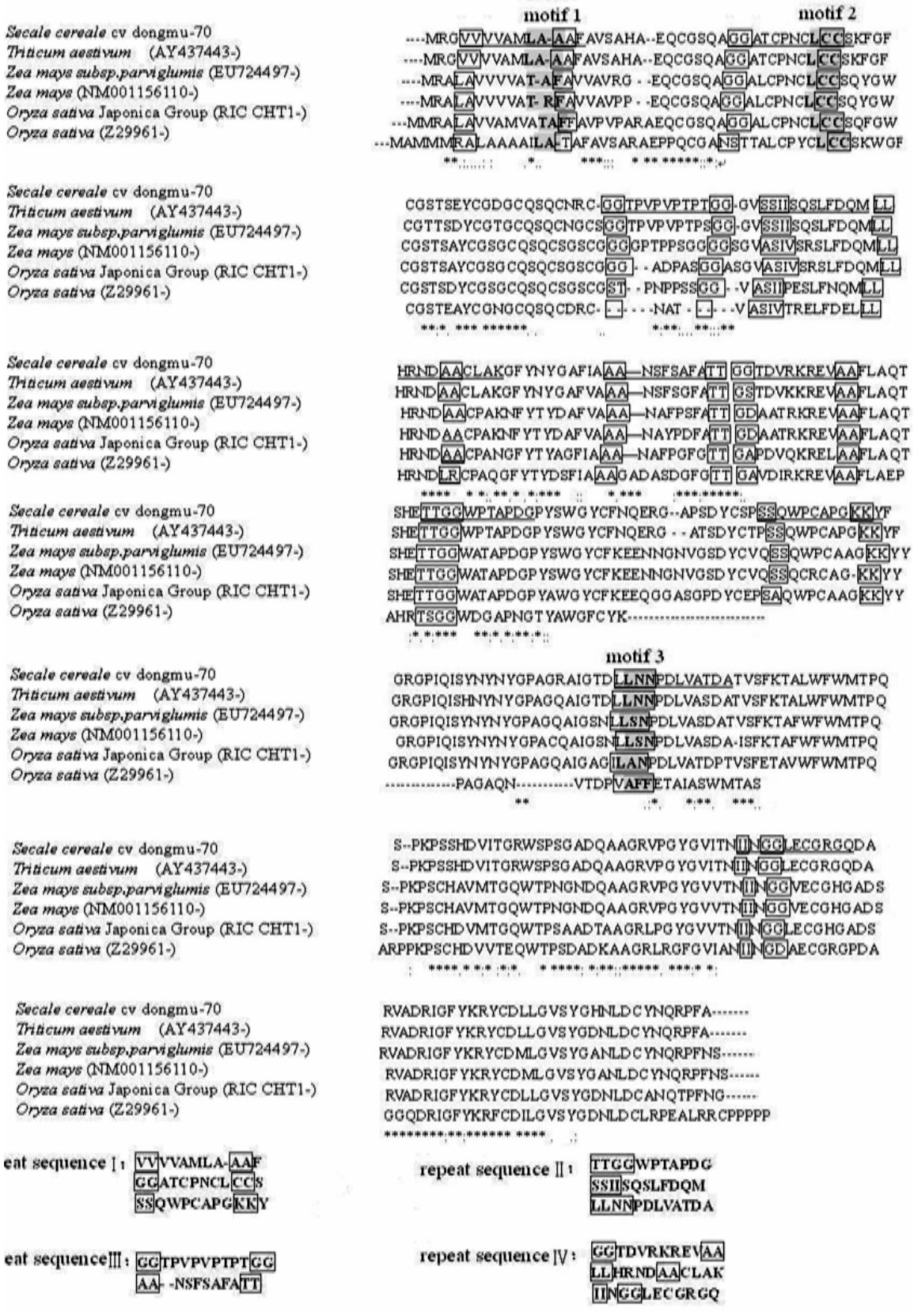

Figure 3. Multiple alignment of the deduced amino acid sequences of *EAPP* proteins from different higher plants. The gray region marks the conserved amino-terminal “LRR” motif. The underlined regions mark structural motifs conserved in eukaryotic *EAPPs*.

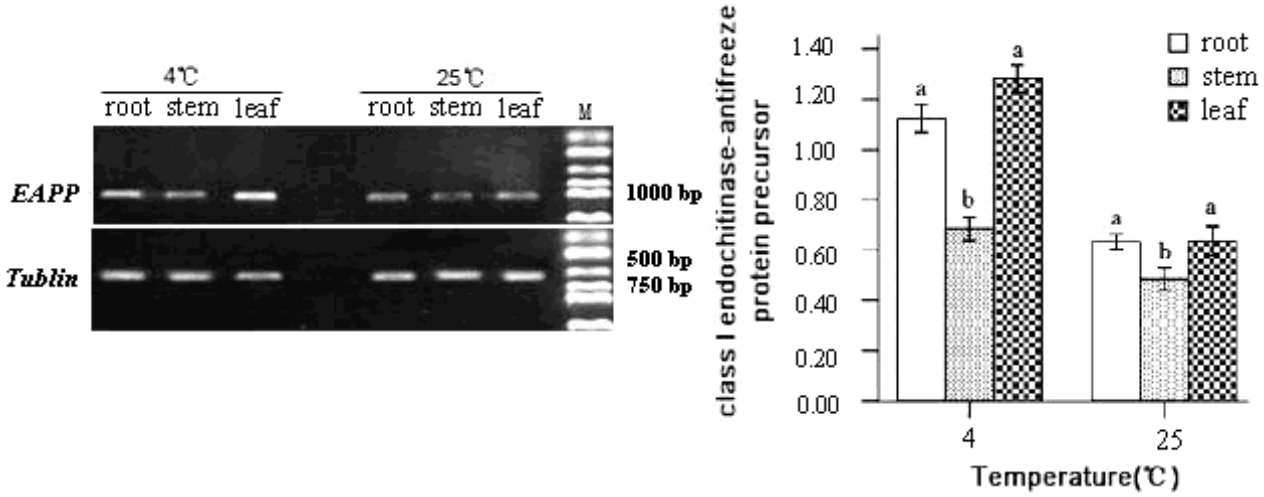

Figure 4. RT-PCR of *EAPP* gene under different times of low temperature in root, stem and leaf of Dongmu-70 rye.

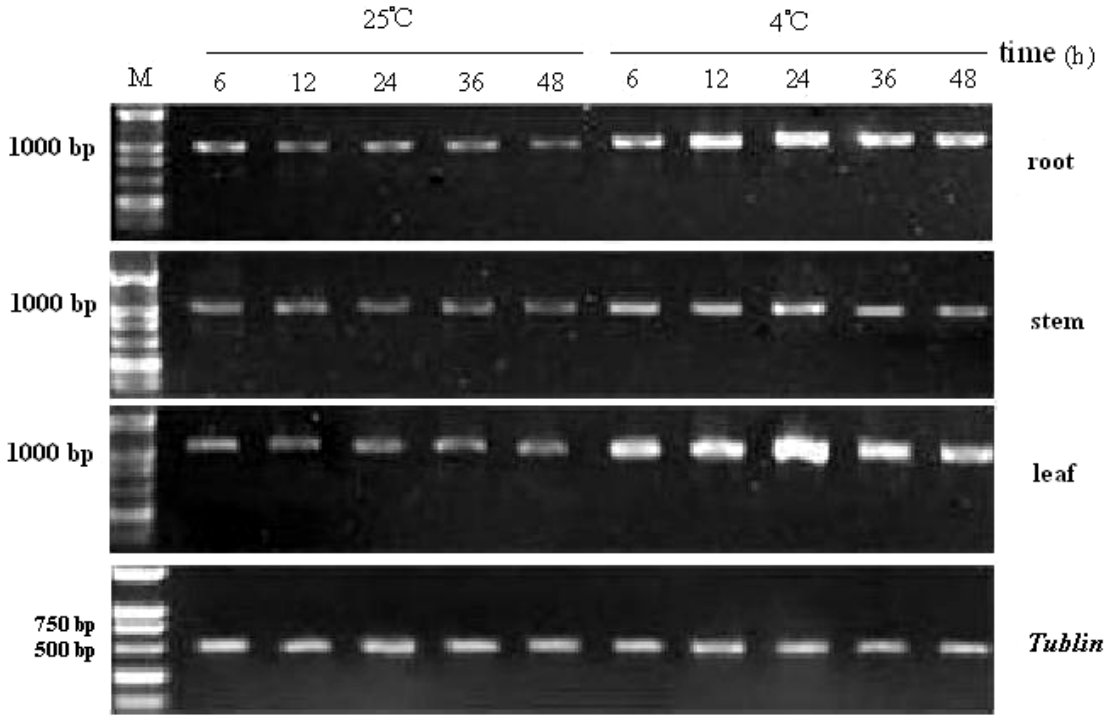

Figure 5. RT-PCR of *EAPP* and *Tubulin* gene under different times of low temperature in the root, stem and leaf of Dongmu-70 rye.

consists of 24 leucine rich repeat (LRR) (Dong et al., 1980) of amino acids residues. LRR is made of dozens (15 to 40) of incomplete *LRR* structures considered as the identifying vector of the receptor. A resemblance is found in the structures of protein R's LRR and intercellular message transcription proteins (Clark et al., 1997). These rich disease duration proteins of LRR cannot only be expressed by some pathogens, such as *OsRLK1* of LRR of rice, but can be expressed under low temperature and salting (Lee et al., 2004). However, the pepper's *CALRR1* of LRR is expressed with high density of salt, *ABA* and non-species stress (Jung et al., 2004). Meyer et al. (1999) found that LRR comprised 74% of the anti-freeze proteins of carrot. The LRR basic sequence of plant specific proteins is composed of 23 to 25 amino acids and it maintains a conventional sequence, in which each LRR unit consists of 1 β-sheet and α-spiral linked by one ring (Zhang et al., 1998). There exist lots of pairs of

cysteine and island structure, closely related to the adjacent area and receptor basis, and its mutation would influence the receptor's binding capacity greatly (He et al., 2000). Zhang et al. (2004) and others replaced asparagine residue with glutamate, while the thermal hysteresis of carrots' anti-freeze proteins reduced by 7%. If asparagines residue is replaced with threonine, the thermal hysteresis of the carrots' anti-freeze proteins is increased by 28%, because the increasing number of icing locus proved that the change of LRR and the components of its amino acids are related to the anti-freeze protein's activity. The sequence of amino acid in *EAPP*, analyzed in the paper, consisted of three anti-freeze *LRR* structures. Among the six plants that were compared in the experiment, only one anti-freeze protein had the same structure as that of LRR. LRR of the other two differs extremely among different species, which may explain why the anti-freeze quality of Dongmu-70 rye is different from that of other species. Besides, *EAPP* exists mostly in poaceae plants and has high homology with two forage legumes. As such, this provides a new approach to explore the source of anti-freeze quality of *EAPP* in rye.

ACKNOWLEDGEMENTS

This work was supported by grants from Shaanxi Natural Science Research Fund (2009k01-19), Key Projects in the National Science & Technology Pillar Program (2011BAD17B05) and Special Fund for Agro-scientific Research in the Public Interest (200903060).

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