

Cloning and expression pattern of a dehydrin-like *BDN1* gene from drought-tolerant *Boea crassifolia* Hemsl.

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Abstract A 500-bp cDNA fragment was amplified via RT-PCR from drought-induced total RNA of the drought-tolerant *B. crassifolia* Hemsl. using primers based on the sequence of published dehydrin conserved region. By using 5' RACE, full-length coding region (1 148 bp) of *BDN1* gene was produced. It is a new member of the dehydrin gene family. Southern analysis indicated that *BDN1* is present in the *B. crassifolia* genome as a single-copy gene. Northern analysis revealed that its expression is inducible by drought and cold stresses as well as ABA application.

Keywords: drought-tolerant plant, *Boea crassifolia* Hemsl., dehydrin, 5' RACE, *BDN1* gene.

Resurrection plants are a group of extreme drought-tolerant plants that are capable of enduring various cellular dehydration-related stresses, such as freeze, heat and drought, at their vegetative stages. The drought-tolerant mechanisms of the resurrection plants have been actively studied in recent years. Bartels and his co-workers^[1] detected the presence of dehydrin in the leaf tissues of resurrection plants. Dehydrins are a group of drought-inducible proteins in higher plants^[2]. Such proteins are commonly present in seeds at the late stage of seed development. Dehydrin molecule contains one to several conserved, 15-amino acid, lysine-rich domains of EKKGIMDKIKEKLPG^[3]. This group of proteins is strongly hydrophilic and heat-stable.

Several plant species unique to China were found with resurrection plant-like characteristics^[4]. Upon watering, these plants could recover and resume growth even after losing 80%—90% of tissue water. *Boea crassifolia* is a highly desiccation-tolerant species found in China, although it may not fit exactly to the definition of “resurrection plant”. A small, fragile, *in vitro* derived *B. crassifolia* plantlet with two to four expanded leaves started to show a sign of wilt only after 11 d under 50% RH. By this time the tissue lost about 70% of its water content. Larger plants would be able to tolerate an even longer period of drought conditions.

We have chosen *B. crassifolia* to study the mechanism of drought tolerance. In this study a dehydrin-like protein gene was cloned from this species. Its possible function was proposed based on the amino acid sequence and its expression pattern was revealed with Northern analysis. Research into the details of its gene-expression may facilitate our understanding of plant stress-tolerant mechanisms.

1 Materials and methods

(i) Enzymes and reagents. TRIzol was from MBI fermentas, and TaqDNA polymerase was from Sangon. Reverse transcriptase and 5' RACE kit were from Gibco-BRL. DIG DNA Labeling and Detection Kit were from Roche. Restriction endonucleases and pGEM-T Easy vector were from Promega. Glass milk DNA recovery kit was from Pingyuan and the charge modified nylon membrane was from Sigma.

(ii) Plant material and induction treatments. *Boea crassifolia* Hemsl. plants were collected from the field in Guangxi (a gift from Prof. Li Zhenyu of Botanical Institute, the Chinese Academy of Sciences (CAS)). After surface disinfection, the plants were *in vitro* propagated on MS medium. Young plantlets at 4—6-leaf stage were used in experiments.

For all experiments, except the Northern hybridization, total RNA was extracted from leaves which were subjected to drought-stress induction by exposing the *in vitro* plantlets to dry air in a greenhouse for 24 h (about 35% of the tissue water were lost). For Northern hybridizations, total RNA was extracted from leaves which were subjected to the following induction treatments: full-turgor

control, drought-stressed, cold-stressed and ABA applied. Drought-stress induction was carried out by subjecting the *in vitro* plantlets in wide-mouth culture flasks to the open air overnight. Cold-stress induction was carried out by incubating the *in vitro* plantlets at 4°C for 24 h. ABA induction was carried out by spraying the *in vitro* plantlets 4 times in 12 h with 10 µmol/L ABA.

(iii) Primers. Gene-specific primers were synthesized by Sangon (China) and Institute of Microbiology, CAS. For RT-PCR, the upstream was 43217 (5' TGGTACCGGA(A/G)AA(G/C)AT(T/C/A)AA(G/C)GA(A/G)AA3') and the down stream was 43218 (5' GCGGCCGCT₍₁₈₎3'). For 5' RACE, the upstreams were 46121 (5' GGCCCGACGTTCGCATG3') and 46122 (5' GGCCCGACGTTCGCATGAATTC₍₁₂₎3') and the down streams were 43513 (5' TTGGATTCTCAGTGGCATGC3'), 43514 (5' TACTTCTCGGTC TTCTTG3') and k1494 (5' CGTACTTCTCAACTG GTACAG 3'). For cloning of the *BDN1* gene coding region, the upstream was k1495 (5' TTCATGGCCGATTACCA ACATCAC 3') and the down stream was 43513 (5' TTGGATTCTCAGTGGCATGC3').

(iv) RT-PCR reaction. Using TRIzol kit, total RNA from the drought-stress induced leaves was isolated and digested with DNaseI to remove DNA residual. The primer 43218 was used to reverse-transcript the total RNAs and synthesize the first-strand cDNAs. They were used as the templates for RT-PCR amplification using primers 43217 and 43218. Primer 43217 was designed based on the conserved region of published dehydrin gene. The amplification protocol consisted of an initial denaturation cycle of 5 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 53°C and 20 s at 72°C, then by a final step of 10 min extension at 72°C.

(v) 5' RACE. The first-strand cDNA was synthesized using gene specific primer 43513. Two 5' RACE procedures were performed. The first followed the protocol by Kimura et al.^[5] using primers 46121, 46122, 43513 and 43514 which were designed according to the partial sequence of 3' *BDN1* gene cDNA obtained with the method described in sec. (iv). The second was carried out according to Gibco-BRL 5' RACE kit protocol using primers 43513, 43514, k1494 and primers AAP and AUAP from the kit. The relative locations of the primers on the dehydrin-like gene *BDN1* of *B. crassifolia* are shown in fig. 1.

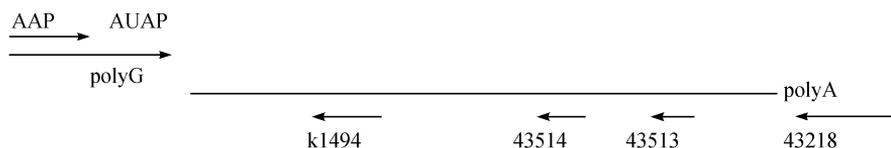


Fig. 1. The relative locations of the primers on the dehydrin-like gene *BDN1* of *B. crassifolia*.

(vi) *BDN1* gene coding-region cloning. The same RT-PCR amplification as in sec. (iv) was carried out except that the primers used were k1495 and 43513 and annealing temperature was 55°C.

(vii) DNA cloning and sequencing. The amplified DNA segments were collected with glass-milk, inserted into vector pGEM-T Easy and introduced into *E. coli* DH5α. Sequencing was carried out with the automatic sequencer at Institute of Microbiology, CAS.

(viii) Southern analysis. Genomic DNA was isolated from *B. crassifolia* using SDS procedure and restricted with *EcoR* I, *BamH* I and *Hind*III. After electrophoresis in 1% agarose gel, the segments were transferred onto charge-modified nylon membrane. Using DIG-labeled *BDN1* gene coding-region as the probe, Southern hybridization was carried out according to the protocol of the DIG DNA Labeling and Detection Kit.

(ix) Northern analysis. TRIzol RNA kit was used to isolate total RNA from the full-turgor control, drought-stress induced, cold-stress induced and ABA induced plantlets (see sec. (ii)). 10 µg total RNA from each of the above treatment was subjected to denaturing agarose gel electrophoresis and transferred onto the charge-modified nylon membrane. Northern hybridization was carried out according to the protocol of the DIG DNA Labeling and Detection Kit using DIG-labeled *BDN1* gene

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coding-region DNA as the probe.

2 Results and discussion

(i) Isolation and sequencing of the dehydrin-like gene segments from *B. crassifolia*. Through RT-PCR amplification and 1.2% agarose electrophoresis, two cDNA fragments of 500 and 200 bp were obtained. Both the fragments were inserted into the pGEM-T Easy vector and transformed into *E. coli*. The Xgal-IPTG positive clones were selected.

After sequencing, we found that there was no gene sequence in the EMBL database showing homology to the 200-bp segment (sequence not shown). However, the 500-bp segment showed homology to a maturation-related gene of *Fragaria vesca* in the database. We named this 500-bp segment *BDNI*. Its sequence is listed in fig. 2.

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GGTACCGGAGAAGATCAAGGAGAAAGCTACCCGGCGGCAAGAAGACCGAGGAAGTGTACGCACCGCCGCCACCGAAGAGG
ACGTGGCCGAGTACTCCACCGCGCCGGAGTGAGGGCAAGGAAAAGAAAGGTTTCTTGATAAGATAAAGGAGAAGCTTCC
AGGCTACCATCCCAAGGCTGAAGGAAGAAAAGGAGAAGGAAAAAGAGAGGAAGCATGCCACTGAGAATCCAATTACTTTT
ATTGATTTGTGGCTTTCCTTCTTTGATTATAATAATACATATATATACATAATATTATTATTATTTGTAATTTGAAGTGTCTGGG
TCATATGATTGTGTGTTGTTGGGTGGCCCTTTGATTCTTTGCTTATTTTTATTTCATTCTGGGGGATGATTTATCTTGGTTT
GAAGTTTCTATGATTGAATTTTTTAATTAAGTGCCTTTGTGTATGAATATATATATATTTGGTTAACAAAAAAAAAAAAA
AAAAAGCGGCCGC
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Fig. 2. cDNA 3' end of dehydration-like gene *BDNI* of *Boea crassifolia* Hemsel. The three possible polyadenylation signal sequences are shown in bold letters; the stop codon is boxed; and the primers are underlined.

It possesses an open-reading frame which contains two lysine-rich, 15-amino acid, conserved dehydrin-specific domains and three possible polyadenylation signal sequences^[6].

(ii) Cloning and sequence analysis of 5' end of *BDNI* gene cDNA. From the first 5' RACE amplification, a 200-bp specific product was obtained. After cloning, the sequence analysis revealed that this 200-bp segment (shown in fig. 3) was the partial sequence of the 5' end of *BDNI* gene cDNA.

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CGGCCCGACGTCGATGAATTCCTCCCCCCCCCAAAAAAAAAAAAAAAAAAGCGGCCGCAAAGAAGAAGAAAAAGAGGGTTTA
AAAGATAAGGTCAAGGAAAAATAACGGAGACAAGAAAGAAGAAGCAGCAGAAACCAAGTGTGAAGAAACATCTGTAC
CAGTTGAGAAGTACGATGAAATTCATACCCTCGAGCCGGAGGAGAAGAAAGGGTTCTTAGATAAGATCAAGGAGAAGCTAC
CCGGCGGCAAGAAGACCGAGGAAGTGTA
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Fig. 3. Partial sequence of the 5' end of *BDNI* gene cDNA. Primers 43121, 43218 and 43514 are shown by underlined arrow, underlined dots and underline, respectively.

It overlaps the 500-bp *BDNI* gene segment obtained in sec. 2 (i) by 69-bp segment. Since this 200-bp segment contains no start codon ATG and includes the segment complementary to primer 43218 which was used to amplify the first-strain cDNA, it might result from the amplification of the polyC tail attached to 43218 primer which failed to be removed during glass milk purification.

The second 5' RACE amplification was followed by nested-PCR amplification using gene-specific primers 43514 and AAP. Multiple bands were obtained. In spite of repeated changing reaction conditions, no specific band appeared. A second nested-PCR amplification was carried out using primer k1494 and AUAP. The primer k1494 was designed based on the partial 5' end sequence of the *BDNI* gene cDNA obtained in the first 5' RACE amplification. A specific band resulted. This band was inserted into the pGEM-T Easy vector and transformed into *E. coli*. The sequence of the Xgal-IPTG positive clone is listed in fig. 4.

Its first ATG start codon is 114 bp down-stream to the 5' end, surrounded by sequence with characteristic to that of plant initiator^[6], that is 5' NNNNANA/UN U/AANNNNANN AUGGCU3'. It results in the largest possible coding region when it is used as an initiator. We, therefore, believe that this sequence is the full-size cDNA of *B. crassifolia* dehydrin gene, a new member of the dehydrin

family. It has been registered on the GenBank as number AF190474.

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GGCCACGCGTCTGACTAGTACGGGGGGGGGGGGGATTCGAAATCAAAGCTTCCACTTTTACAGCGACTCAGCACTGATCTC
GATTATCATACATCTGTGTAGATTCAATTTCTGGGTTTTTATCACAAAGTTAAGGAGATTCAAGAATGGCCGATTACCAACA
TCACGAGCTGAAGAGCGAGGAGCCATACGCTCCACCCCGTTAAGATTGAGGAAATTGATCAGCCGGTGGAGGCCCTCCGA
TCGTGGGCTGTTGATTTATCGGGAAGAAAAAGGATGAGGAGGAAGAGAAGAAGTGTGACGAGGGTAAGTTGCATCTGA
ATTTGACGACAAAAGTTTCAGATTTGTGACGAGAAGAAGGAAGAGCCAAATTTGAGGTTTATGAAGATCTAAACTTGAGGT
TTCTGAAGAGCCAAAGGAGGAGGAAAAGAAGCAGCAAAGCTTGCTCGAAAAGCTACACCGAAGCAACAGCTCCAGCAGT
TCTTCTAGCGATGAAGAAGAAGTAGAGGAAGGAGGTGAAAAGAAGAAGAAAAAGAAGGGTTAAAAAGATAAGGTCAAGG
AAAAAATAACCGGAGACAAGAAAGAAGAAGCAGCAGAAACCAAGTGTGAAGAAACATCTGTACCAGTTGAGAAGTACG

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Fig. 4. 5' end sequence of the 5' end of *BDN1* gene cDNA. The start codon is shown in bold letters. Primers AUAP, K1494 are shown by underlined arrow and underline, respectively.

(iii) Structural analysis of the dehydrin-like *BDN1* gene from *B. crassifolia*. The full-length cDNA sequence and its coded amino acid sequence of the *B. crassifolia* dehydrin-like *BDN1* gene obtained from sec. 2(i) and (ii) are shown in fig. 5. It is 1148 bp in length and contains a 753-bp coding region for a 251-amino acids protein. The non-coding region at the 5' end is 114 bp long and that at the 3' end is 281 bp long. There are 3 possible polyadenylation signals at the 3' end non-coding region. They are ATTTA, AATAA and ATTAAA. The polypeptide derived from its coding region contains the following dehydrin characteristics: one serine-rich domain (rectangular framed) and two lysine-rich domains^[3]. Amino acid sequence analysis indicated that the *BDN1* protein readily forms α -helical secondary structure and is highly hydrophilic.

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ATTCGAAATCAAAGCTTCCACTTTTACAGCGACTCAGCACTGATCTCGATTATCATA 57
CATCTGTGTTAGATTTCAATTTCTGGGTTTTTATCACAAAGTTAAGGAGATTCAAGA 114
ATGGCCGATTACCAACATCAGAGCTGAAGAGGAGGCCATACGCTCCACCCCGTTAAGATTGAGGAAATTTGATCAGCCGGTGGAG 204
M A D Y Q H H E L K S E E P Y A P T P V K I E E I D Q P V E 30
GCCTCCGATCGTGGGCTGTTGATTTATCGGGAAGAAAAAGGATGAGGAGGAAGAGAAGAAGTGTGACGAGGGTAAGTTGCATCTGAA 294
A S D R G L F D F I G K K K D E E E E K K C D E G K F A S E 60
TTTGACGACAAAAGTTTCAGATTTGTGACGAGAAGAAGGAAGAGCCAAATTTGAGGTTTATGAAGATCTAAACTTGAGGTTTCTGAAGAG 384
F D D K V Q I C D E K K E E P K F E V Y E D P K L E V S E E 90
CCAAAGGAGGAGGAAAAGAACACGAAAGCTTGCTCGAAAAGCTACACCGAAGCAACAGCTCCAGCAGTTCTTCTAGCGATGAAGAAGAA 474
P K E E E K K H E S L L E K L H R S N S S S S S S D E E E 120
GTAGAGGAAGGAGGTGAAAGAAGAAGAAAAAGAAGGGTTTAAAAAGATAAGGTCAAGGAAAAATAACCGGAGACAGAAGAAGAAGCA 534
V E E G G E K K K K K G L K D K V K E K I T G D K K E E A 150
GCAGAAACCAAGTGTGAAGAAACATCTGTACCAGTTGAGAAGTACGATGAAATTCATACCCCTCGAGCCGGAGGAGAAGAAAGGTTCTTA 654
A E T K C E E T S V P V E K Y D E I H T L E P E E K K G F L 180
GATAAGATCAAGGAGAAGCTACCCGGCGGAAGAAGACCGAGGAAAGTGTACGACCGCCGCCACCGAAGAGGAGCTGGCCGAGTACTCCA 744
D K I K E K L P G G K K T E E V Y A P P P P K R T W P S T P 210
CCGCGCCGAGTGAAGGGAAGGAAAAGAAGGTTTCTTGGATAAGATAAGGAGAAGCTCCAGGCTACCATCCAAAGGCTGAGGAAGAA 834
P R R S E G K E K K G F L D K I K E K L P G Y H P K A E E E 240
AAGGAGAAGGAAAAAGAGAGAAGCATGCCACTGAGAATCCAATTTACTTTTATTTGATTTGTGGCTTCTTCTTTGATTTATAATAATA 924
K E K E K R E E A C H * 251
CATATATCATACAAATTTATTAATTTGTAATTTGAAGTGTCTGGGTGATGATGTTGTGTTGTTGGGTGGCCCTTTTGATTTCTT 1014
TGCTTATTTTATTTTCAATCTGGGGATGATTTATCTGGTTTGAAGTTTCTATGATTAATTTTAAATTAAGTGCTTTGTGAT 1104
GAATATATATATATTTGGTTTAAACAAAAAATAAAAAAAAAAAAAA 1148

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Fig. 5. Full-length cDNA sequence and its coding amino acid sequence of the *B. crassifolia* dehydrin-like *BDN1* gene. One Ser-rich region and two Lys-rich regions are indicated with box and underlined, respectively.

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Based on EMBL database, the BDN1 protein possesses the highest degree (52%) of homology with the LTI45^[7] gene of Arabidopsis. The LTI45 gene is a water-stress inducible protein gene responding cold and drought stresses.

(iv) Cloning of *BDN1* gene coding sequence. Based on the above complete *BDN1* gene cDNA sequence, primer k1495 was designed. Using primers k1495 and 43513, the *BDN1* gene coding sequence was cloned via RT-PCR from drought-induced total RNAs. Its length is about 700 bp, which corresponds with the estimated length.

The same primers were used to amplify the *BDN1* gene from the genomic DNA template isolated from *B. crassifolia*. A specific band having the same length as that of the *BDN1* gene cDNA was produced. Sequence analysis indicated that this band had an identical sequence as that of the *BDN1* gene cDNA. Thus, *BDN1* gene is likely with no intron.

(v) Southern analysis of the *BDN1* gene. Fig. 6 shows that one hybrid band was shown when Southern hybridization was performed with *EcoR* I or *BamH* I restricted *B. crassifolia* genomic DNA using *BDN1* gene coding region as the probe. While two bands presented when *Hind* III restricted DNA was used. Since *BDN1* gene contains only one *Hind* III site and no *EcoR* I or *BamH* I site, *BDN1* must be present as a single-copy gene in the *B. crassifolia* genome.

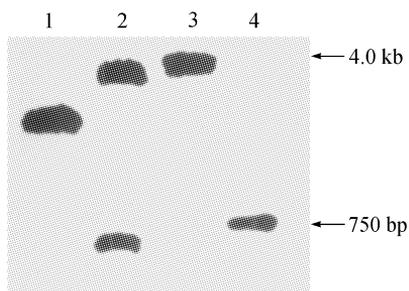


Fig. 6. Southern analysis of *BDN1* gene. *B. crassifolia* genomic DNA was cut by *EcoR* I (lane 1), *Hind* III (lane 2) and *BamH* I (lane 3) using coding region of the gene as the probe. Lane 4 was the positive control of non-cut coding region of the *BDN1* gene using the same probe.

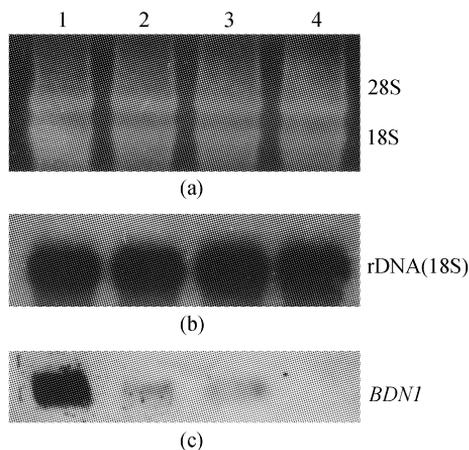


Fig. 7. Northern analysis of *BDN1* gene. Total RNAs (a) of the *B. crassifolia* were hybridized with the 18S rDNA (b) and *BDN1* gene coding region (c) as the probes. 1, Extracted from a cold-stressed plantlet; 2, from a drought-stressed plantlet; 3, from an ABA-applied plantlet; 4, from the leave of a full-turgored plantlet.

(vi) Northern analysis of the *BDN1* gene. Northern analysis revealed that the *BDN1* gene did not express from the full-turgor control plant (fig. 7). It was inducible under drought and cold stresses as well as ABA application. Expression was the strongest under cold stress. Since the expression via ABA induction was much weaker than that from cold induction, it is possible that the signal transduction pathway used by cold stress may not include ABA signal transduction pathway^[8]. Its organ-specific expression patterns will be analyzed in the near future.

In conclusion, we have cloned a new gene member of the dehydrin gene family from *B. crassifolia* and named it *BDN1* (*Boea* dehydrin 1). It belongs to the group SK₂ dehydrin proteins^[2] and contains the typical dehydrin characteristic of a serine-rich domain and two conserved, 15-amino acid, lysine-rich domains. Its secondary structure is rich in hydrophilic α -helix. It is present in the *B. crassifolia* genome as a single-copy gene. Its expression is inducible by drought and cold stresses as well as by ABA application. Besides *dehydrins*, *rd29A*, which is induced by the same stresses as *dehydrins*, has also been studied extensively^[9]. The *trans*-factor for the expression of *rd29A* has been isolated recently^[10]. Transgenic *Arabidopsis* with this factor showed drought-tolerance^[11]. Further studying its gene-expression mechanisms, including its positive and negative control factors, will facilitate our

understanding of plant stress-tolerance mechanisms and developing the strategy in crop improvements.

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