Cloning of dehydrin coding sequences from *Brassica juncea* and *Brassica napus* and their low temperature-inducible expression in germinating seeds

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Abstract

A novel subclass of dehydrin genes, homologous to the *Raphanus sativus* late embryogenesis-abundant (LEA) protein (RsLEA2) and the *Arabidopsis thaliana* dehydrin, was isolated from *Brassica juncea* and *Brassica napus*, here designated *BjDHN1* and *BnDHN1*, respectively. The cDNA of *BjDHN1* and *BnDHN1* genes share 100% nucleotide identity. The encoded protein is predicted to consist of 183 amino acid residues (molecular mass of 19.2 kDa and pI of 7.0). It shares 85.3% and 65.4% amino acid sequence identity with the RsLEA2 and *Arabidopsis* dehydrins, respectively. This *Brassica* dehydrin also features a “Y3SK2” plant dehydrin structure. Expression analysis indicated that the *Brassica* dehydrin gene is expressed at the late stages of developing siliques, suggesting that the gene expression may be inducible by water-deficit. Analysis of gene expression also indicated that in germinating seeds the gene expression was inducible by low temperature. Seed germination under low temperature was compared between *B. juncea* and *B. napus*. The results showed that *B. juncea* seeds germinated faster than *B. napus* seeds. Expression of *Brassica* dehydrin gene was also examined as a function of seed germination under low temperature.

Keywords: *B. juncea*; *B. napus*; Dehydrin; Gene expression; Low temperature; Seed germination

1. Introduction

Dehydrins have been identified as one of the five major classes of late embryogenesis-abundant (LEA) proteins from many plant species. More specifically, dehydrins are the D-11 family of the super LEA protein family [2]. Plant dehydrins are encoded by multigene families and many distinct subclasses have been observed [3,4]. It is well known that the expression of dehydrin genes can be induced by environmental stresses, such as dehydration or low temperature [2–4]. Although the fundamental biochemical and physiological roles of the dehydrin proteins remain to be elucidated, the involvement of dehydrins in low temperature tolerance seems to be clear. For example, it has been found that the abundance of W COR410, an acidic dehydrin, is positively related to freezing tolerance of wheat seedlings, which provides a potential marker for freezing tolerance of different wheat varieties [5,6,8]. Other reports indicated that there is a correlation between the 35 kDa cowpea dehydrin and the chilling tolerance during cowpea seedling emergence [9,10]. The study showed that allelic variation of the dehydrin gene locus co-segregates with the chilling tolerance trait [10]. These studies suggested that exploration of dehydrin genes might provide useful markers for low temperature tolerance in other crops as well.

A whole spectrum of cold-responsive genes has been isolated from plants. Although many of these genes encode proteins with known functions, most of them encode either newly discovered proteins such as the *Arabidopsis* COR6.6, COR15 and COR78 or homologs of LEA proteins such as
the Arabidopsis COR47 [17]. Brassica homologues of the Arabidopsis gene family COR6.6 and COR15 were isolated [17], however, no isolation of Brassica homologues of the Arabidopsis dehydrin gene (COR47) has been reported.

Through extensive breeding efforts, the original Brassica juncea, which contains unfavorable traits including high erucic acid, high glucosinolate and low oleic acid has been converted to the new canola quality B. juncea [11,13,14]. The canola quality B. juncea not only possesses oil and meal qualities identical to B. napus and B. rapa but also is more heat- and drought-tolerant. It, therefore, provides an alternative canola crop species to the canola industry. In this study, we report the cloning of dehydrin genes from both B. napus and B. juncea. We also investigated whether the isolated Brassica dehydrin gene is involved in cold tolerance as in the case of cowpea during seedling emergence. Analysis of gene expression indicated that the Brassica dehydrin gene isolated in the current study may be involved in low temperature tolerance only during seed germination.

2. Results and discussion

2.1. Comparison of Brassica seed germination at low temperature

As part of our continued research efforts, we investigated the canola quality B. juncea seed vigor and made comparisons with B. napus by testing seed germination under cold temperature [18]. Seed germination is defined as a physiological process that commences when the quiescent dry seed begins to take up water and is completed when embryonic axis elongates. Therefore, what we observed in these germination tests is the actual penetration by the radicle of structures surrounding the embryo, which is often called the visible germination [1]. As show in Table 1, over 98% of B. juncea and B. napus seeds germinated after 3 d at 22 °C. However, germination rate was quite different for B. juncea and B. napus when seeds were germinated at 5 °C. In particular, both lines of B. juncea germinated much faster at 5 °C than did the two B. napus varieties. Specifically, after 5 d of germination at 5 °C, the germination rates were 55% and 63%, respectively, for the two B. juncea lines. However, under the same conditions, only 4% and 15% germinated for the two B. napus varieties. From these germination tests we conclude that B. juncea seeds are more cold-tolerant during germination under low temperature than B. napus seeds.

### Table 1

Comparison of germination rates between *B. napus* and *B. juncea* seeds under different temperatures. Q2 and EC excel are *B. napus* species commercially available; PC98-44 and PC-105 are canola *B. juncea* breeding lines proprietary to the Saskatchewan Wheat Pool. Germination rate was counted after seeds were germinated at 22 °C for 3 d or at 5 °C for 5 and 10 d. The average of two independent germination experiments was shown for each treatment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cultivar</th>
<th>Germination rate (%)</th>
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<tr>
<td></td>
<td></td>
<td>22 °C/3 d</td>
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<tr>
<td><em>B. napus</em></td>
<td>Q2</td>
<td>&gt;98</td>
</tr>
<tr>
<td></td>
<td>EC Excel</td>
<td>&gt;98</td>
</tr>
<tr>
<td><em>B. juncea</em></td>
<td>PC98-44</td>
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<td></td>
<td>PC-105</td>
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2.2. Cloning of Brassica dehydrin coding sequences

The mechanisms involved in plant cold tolerance are very complex and many genes are believed to be involved [17]. Our research interest was inspired by one particular report that a cowpea dehydrin gene is associated with chilling tolerance during cowpea seedling emergence under low temperature [10]. We questioned whether the expression of any dehydrin gene(s) from *B. juncea* and *B. napus* could also be correlated to cold tolerance during seed germination at low temperature in a similar way as in the case of cowpea seedlings. It was our hope that successful identification of a candidate gene or genes that are responsible for the differences in cold germination between *B. juncea* and *B. napus* could lead to the development of a genetic marker for future plant breeding.

To examine if any *Brassica* dehydrin gene expression is correlated to the cold tolerance during seed germination under low temperature, we decided to clone dehydrin gene(s) from both *B. juncea* and *B. napus* and compare their expression under cold temperature. Total RNAs isolated from germinating seeds (5 °C for 3 d) of both *B. juncea* and *B. napus* were used to perform reverse transcription polymerase chain reactions (RT-PCRs) in order to clone the cDNAs. Because there was no previously reported *B. juncea* or *B. napus* dehydrin gene(s), we designed degenerate primers P1 and P2 on the basis of the reported dehydrin genes from *Raphanus sativus* (RsLEA2, GenBank accession number X56280) and *Arabidopsis thaliana* (AtDHN, GenBank accession number X91920), which belong to the Brassicaceae family.

Cloning and sequencing of the RT-PCR products (partial cDNA, 528 bp) confirmed that one unique dehydrin gene was cloned from each of *B. juncea* and *B. napus*. Comparison with sequences of the *R. sativus* RsLEA2 gene and the *Arabidopsis* AtDHN gene indicated that the first ATG at the 5′ end of the cDNA is the translation initiation codon (Fig. 1A). The missing 3′ end of the cDNA (24 bp) was determined by genomic walking technique using a TOPO Walker Kit (Invitrogen) in order to obtain the full-length cDNA. Analysis of the genomic walking product showed that there is an in-frame stop codon, followed by a perfect polyadenylation signal. Therefore, the full-length cDNA for both dehydrin genes of *B. juncea* (designated BjDHN1) and *B. napus* (designated BnDHN1) are predicted to be 552 bp in size.

Analysis of the nucleotide sequence indicated that the cDNAs of BjDHN1 and BnDHN1 are 100% identical and encode polypeptides of 183 amino acid residues. Alignment with the *R. sativus* RsLEA2 and the *Arabidopsis* AtDHN indicated that the Brassica dehydrin shares 85.3% and 65.4%
amino acid sequence identities, respectively (Fig. 1A). The results of a TBLASTN homology search using BjDHN1 as a protein query (http://www.ncbi.nlm.nih.gov/BLAST) indicated that the *R. sativus* RsLEA2 and the *Arabidopsis* AtDHN are the only two close matches that share significant sequence similarity with BjDHN1, which clearly distinguishes this subclass of dehydrins from other plant dehydrins. Based on the “YSK” nomenclature scheme developed by Close [4], consensus sequence analysis indicated that BjDHN1 and BnDHN1 possess a “Y3SK2” structure. In the previous report, RsLEA2 (GenBank accession number X56280) and AtDHN (GenBank accession number X91920) have been described as “Y3SK2” and “Y2SK2,” respectively [4].

To determine the structure of the *BjDHN1* gene, genomic sequence was obtained from clones isolated by genomic PCR using gene specific primers (P3 and P4) designed after the *BjDHN1* full-length cDNA. A 931 bp fragment was amplified, cloned and sequenced, which includes the 552 bp full-length *BjDHN1*cDNA and a 379 bp intron. Therefore, the *BjDHN1* gene contains a single intron in its coding sequence as does the *AtDHN* gene (GenBank accession number X91920; Fig. 1B).

### 2.3. Expression analysis of Brassica dehydrin genes

*BjDHN1* gene expression was determined by RT-PCR with the gene specific primers (P3 and P4), which amplify the 552 bp full-length cDNA. Expression of *Brassica* actin gene was used as a control. For organ-specific expression of *BjDHN1*, *B. juncea* plants were grown in the greenhouse for about 12 weeks and then flower buds, cauline leaves and root tissue were also collected. Individual flowers were tagged on the day of flower opening so that the developing siliques were collected at different stages based on the number of weeks.
after flowering. Total RNA was isolated from these tissues for analysis of gene expression by RT-PCR. As shown in Fig. 2A, the expression of \textit{BjDHN1} gene was not detectable in leaves, flowers and roots. In developing siliques, \textit{BjDHN1} gene expression was not detectable until siliques reached a late developmental stage (stage 4). There is a dramatic color change in siliques during development (from light green to dark green then to milky yellow). At stage 4, both silique walls and the seeds began to look milky yellow. It will take about 6 weeks for stage 4 developing siliques to reach full maturity (day seeds).

To examine if \textit{BjDHN1} gene expression could be inducible by cold temperature, total RNAs isolated from leaves and developing siliques that were treated under different temperatures prior to gene expression analysis by RT-PCR. For this purpose, plants were grown in a greenhouse to a proper stage (~12 weeks after seeding). One set of plants was transferred to the lab bench (22 °C) and was kept in darkness for 24 h before isolation of total RNA. The other set of plants was transferred to a refrigerator (5 °C) and was kept in darkness for 24 h before isolation of total RNA from cauline leaves and developing siliques. As shown in Fig. 2B, the \textit{BjDHN1} gene expression was not detectable in leaf (L) and stage 3 siliques (S3) neither under room temperature (22 °C) nor after low temperature treatment (5 °C). The expression level of \textit{BjDHN1} gene in stage 4 siliques (S4) remained virtually unchanged after low temperature treatment. Previous studies indicated that the expression of cold-regulated \textit{Arabidopsis} dehydrin gene \textit{COR47} could be detected in leaves only after a 12-h exposure to low temperature [12,16]. Our data indicated that the expression of the \textit{BjDHN1} gene is not low temperature-inducible neither in leaves nor in developing siliques. Similar results were obtained when the expression of \textit{BnDHN1} gene were analyzed using \textit{B. napus} plants to perform the same experiments (data not shown).

We further examined the gene expression patterns of \textit{BjDHN1} and \textit{BnDHN1} in germinating seeds under low temperature. As shown in Fig. 3A, there was no detectable expression for both \textit{BjDHN1} and \textit{BnDHN1} genes in the dry seeds of \textit{B. napus} and \textit{B. juncea}. These data seem to be contrary to the results that the expression of \textit{BjDHN1} was detectable at stage 4 of the developing siliques. However, it would normally take about an additional 6 weeks for the seeds of stage 4 developing siliques to reach full maturity (dry seeds). When seeds

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Fig. 2. Expression of \textit{BjDHN1} gene in \textit{B. juncea} line PC98-44 determined by RT-PCR. A, \textit{BjDHN1} gene expression in different tissues. L, leaves; F, flowers; R, roots; S1–S4, siliques collected at 1–4 weeks after flowering, respectively. B, Comparison of \textit{BjDHN1} gene expression under different temperatures. Leaves (L) and developing siliques (S3 and S4) kept for 24 h at 22 °C or at 5 °C before isolation of total RNA. The expected RT-PCR products (552 bp) of \textit{BjDHN1} (\text{DHN}) and \textit{B. juncea} actin gene (\text{ACTIN}) are marked by arrows. The gels were stained with ethidium bromide.

Fig. 3. Expression of \textit{BjDHN1} and \textit{BnDHN1} genes in dry and germinating seeds determined by RT-PCR. A, Dry seeds of \textit{B. napus} cultivar Q2 (1) and cultivar EC Excel (2); dry seed of \textit{B. juncea} line PC98-44 (3) and line PC-105 (4). B, Germinating seeds of \textit{B. napus} variety Q2 (n) and of \textit{B. juncea} line PC98-44 (j) that were germinated at 22 °C for 1 d (n1 and j1) and 3 d (n3 and j3). C, Germinating seeds of \textit{B. napus} variety Q2 (n) and of \textit{B. juncea} line PC98-44 (j) that were germinated at 5 °C for 1 d (n1 and j1), 2 d (n2 and j2), 3 d (n3 and j3), 5 d (n5 and j5), 8 d (n8 and j8) and 10 d (n10 and j10). Arrows mark the expected RT-PCR products (552 bp) of \textit{BjDHN1} and \textit{BnDHN1} (\text{DHN}) and the \textit{Brassica} actin genes (\text{ACTIN}). The gels were stained with ethidium bromide. Lane P is a PCR product amplified from the plasmid pDKL3 that contains the \textit{BjDHN1} cDNA.
germinated 22 °C for up to 3 d, there was no detectable expression for both BjDHN1 and BnDHN1 genes (Fig. 3B). Germination rates were >98% for both B. napus and B. juncea varieties after 3 d at 22 °C (Table 1). However, when seeds were germinated at 5 °C the expression of both BjDHN1 and BnDHN1 genes was detected (Fig. 3C). Specifically, after germination for 24 h at 5 °C, expression of BjDHN1 and BnDHN1 genes was detected and the expression remained detectable for up to 5 d under low temperature, which indicated that the expression of BjDHN1 and BnDHN1 genes is inducible by low temperature in germinating seeds. The results, together with the fact that expression of BjDHN1 and BnDHN1 genes was not detected in dry seeds (Fig. 3A) and the fact that the expression was not induced by low temperature in leaves nor in developing siliques (Fig. 2B), suggest that the genes are developmentally regulated within seeds.

After germination for 5 d at 5 °C, the expression level of BjDHN1 became undetectable, whereas the expression of BnDHN1 remained detectable for up to 10 d at 5 °C (Fig. 3B). Seed germination data indicated that B. napus seeds germinated much slower than did B. juncea seeds (Table 1). The low temperature-inducible expression indicated that BjDHN1 and BnDHN1 gene products may be involved in coping with the low temperature stress in germinating seeds. The fact that BnDHN1 gene expression was detectable for a longer period of time than was BjDHN1 gene expression at low temperature may simply be due to the fact that B. napus seeds germinated more slowly. The fact that the low temperature-inducible expression of BjDHN1 and BnDHN1 genes occurs exclusively in germinating seeds suggests that this class of Brassica dehydrins may play a role in coping with low temperature stress only in germinating seeds. It has been suggested previously that plant dehydrins play a role in stabilizing membranes and proteins [4,6]. Our data are consistent with the concept that the mechanism underlying plant cold tolerance is much more complex and multiple genes are involved [17]. The present study indicates that although the expression of Brassica dehydrin genes (BjDHN1 and BnDHN1) are inducible by low temperature during seed germination, the exact physiological function of these gene products remains to be elucidated.

The finding that BjDHN1 and BnDHN1 are 100% identical may simply reflect the fact that allotetraploid B. juncea (A and B genomes) and B. napus (A and C genomes) share one common diploid progenitor B. rapa (A genome). It is noteworthy that the cDNAs of both R. sativus RsLEA2 and the Arabidopsis AtDHN1 were isolated from dry seed [15]. However, our study shows that the expression levels of BjDHN1 and BnDHN1 in dry Brassica seeds were not detectable. To our knowledge, this is the first description of a plant dehydrin gene whose expression is low temperature-inducible only in germinating seeds. In future studies, isolation of the 5′ upstream region would help understand the mechanisms underlying the regulation of the Brassica dehydrin gene expression.

3. Conclusion

Brassica homologues of the R. sativus dehydrin gene (RsLEA2) and the A. thaliana dehydrin gene (AtDHN1) were isolated from B. juncea (BjDHN1) and Brassica napus (BnDHN1). BjDHN1 and BnDHN1 share 100% identity in nucleotide in their coding sequences. Expression of both BjDHN1 and BnDHN1 can be low temperature-inducible, which occur exclusively in germinating seeds but not in other tested tissues such as leaves and developing siliques. Although B. juncea seeds germinate faster than B. napus seeds under low temperature, no difference was found in the level of low temperature-inducible expression of BjDHN1 and BnDHN1.

4. Methods

4.1. Seed germination

Two lines of B. napus L. (Q2 and EC Excel) and two lines of B. juncea (L.) Czern. (PC98-44 and PC-105) were used for germination test under controlled temperatures. For this purpose, 100 seeds from each line (seeds were not sterilized) were placed on sterilized wet Whatman paper, which was placed in a steel container and germinated at either 5 °C or 22 °C under darkness. For germination at 5 °C, the wet Whatman paper was pre-chilled at 5 °C for at least 6 h before use. Visible germination were observed and scored. Germination rate was counted daily up to 3 d for seeds germinated at 22 °C and up to 10 d for seeds germinated at 5 °C. Germinating seeds were also collected for RNA isolation for the purpose of cloning the dehydrin coding sequences and analyzing expression.

4.2. Plant materials

B. napus (Q2) and B. juncea (PC98-44) seeds were planted in soil and grown in an environmentally controlled greenhouse under the growth conditions of 16 h of 25 °C with light and 8 h of 20 °C without light. For analysis of gene expression in various tissues, cauline leave, flower bud and root were collected from ~12-week-old plants. Flowers were individually tagged so that the age of siliques was recorded. The siliques were collected at four different stages: 1 week after flowering (stage 1; light green siliques with very little seeds inside), 2 weeks after flowering (stage 2; light green siliques with seeds inside), 3 weeks after flowering (stage 3; dark green siliques walls and green seeds) and 4 weeks after flowering (stage 4; milky yellow siliques walls and milky yellow seeds). Collected tissues were immediately frozen using liquid nitrogen and stored at −70 °C freezer until use for total RNA isolation. For cold treatment, plants were grown in a greenhouse under the same conditions described above to a proper stage (~12 weeks after seeding). One set of plants were transferred to the lab bench (22 °C) and kept under darkness.

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for 24 h before collecting cauline leaves and developing siliques (Stage 3 and Stage 4) for isolation of total RNA. The other set of plants were transferred to a refrigerator (5 °C) and kept under darkness for 24 h before collecting cauline leaves and developing siliques (Stage 3 and Stage 4) for isolation of total RNA.

4.3. Cloning of cDNA sequences

Partial cDNA fragments were amplified by RT-PCR using total RNAs isolated from B. juncea and B. napus seeds germinated at 5 °C for 3 d. Degenerate primers, P1 (5′-ATGGCNGAYTNNARRGAYGAR-3′) and P2 (5′-NCCNGGNAGYTTNCTTYTNTF-3′), were designed on the basis of conserved regions of dehydrin genes from R. sativus (RsLEA2, GenBank accession number X56280) and A. thaliana (AtDHN, GenBank accession number X91920). For the first strand cDNA synthesis, 1 µg of total RNA was primed with the gene specific primer P2 in a total volume of 20 µl in the presence of 200 units of Superscript™ II reverse transcriptase (Invitrogen) according to the manufacturer’s protocols. For PCR, 2 µl of the reverse transcription mixture was used as DNA template and the amplification was performed for 4 cycles of 94 °C for 1 min, 60 °C for 1 min and 72 °C/ for 1.5 min with primers P1 and P2. The RT-PCR-produced single 528-bp amplification band appeared on agarose gel for both B. juncea and B. napus. After cloning of the amplified fragment into a PCR 4-TOPO TA cloning vector (Invitrogen), the insert sequence was analyzed from four randomly selected colonies each from B. juncea and B. napus using the PRISM DyeDeoxy™ Terminator Cycle Kit and a 377 DNA Sequencer (Applied Biosystems). Sequence analysis was performed with the Lasergene DNA software kit (DNASTAR Inc.). The analysis of sequence indicated that the cloning products include the putative initiation codon at the 5′ end but lack the 3′ end of the cDNA. The missing 3′ end (24 bp) of the cDNA was determined with 3 overlapping gene specific primers (P5, 5′-GTGAAGGAGGACG-ATGGACAG-3′; P6, 5′-CGATGCGAACGGAGAAGAGAAA-3′; and P7, 5′-GAGAAAGAAGGCCATGAAGACATA-3′) using a TOPO Walker Kit according to the manufacturer’s protocol (Invitrogen). Gene specific primers, P3 (5′-ATGGCCGAT-TTGAAGACGAA-3′) and P4 (5′-TCACGGGTGTGTTGTGTTGGG-3′), designed based on the BjDHN1 full-length cDNA was used for RT-PCR to generate a full-length cDNA fragment, which was cloned into a pCR 4-TOPO vector resulting the plasmid pDKL3. The final assembled full-length cDNA is 552 bp in size.

4.4. Cloning of genomic DNA sequences

Genomic PCR was performed using 100 ng DNA isolated from B. juncea rosette leaf tissue (line PC98-44) using the gene specific primers, P3 and P4. Cycling conditions were identical to those used for RT-PCR. Cloning and analysis of the insert sequence were carried out using the same protocols described above.

4.5. Analysis of gene expression

For analysis of gene expression, gene specific primers, P3 and P4, were used for RT-PCR, which were performed using total RNA isolated from various tissues. Brassica actin genes were used as controls for analysis of gene expression. PCR primers (Bactin5′, 5′-TTGGCATCACATTTCTACA-3′; and Bactin3′, 5′-CAACGGAATCTCTCAGCTCC-3′) were designed based on highly conserved regions of actin genes of B. napus, B. oleracea and Arabidopsis. RT-PCR was performed in a similar way as for dehydrin genes. Identity of the actin gene was confirmed by sequence analysis.

4.6. Isolation of DNA and RNA

Genomic DNA was isolated following the protocols described previously [7]. Total RNA was isolated from various tissues using TRIzol reagent (Invitrogen) according to the manufacture’s protocols.

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References


