Plant Molecular Biology **23**: 871–879, 1993.

Molecular analysis and spatial expression pattern of a low-temperature-specific barley gene, *blt101*

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Received 27 April 1993; accepted in revised form 8 September 1993

Key words: cold, low temperature, barley, gene expression, cDNA, shoot meristem

Abstract

A cDNA clone of the previously unreported low-temperature-induced gene *blt101* was isolated after a differential screen of a cDNA library prepared from low-temperature (6 °C day/2 °C night) grown barley shoot meristems. Southern blot analysis of barley ditelosomic addition lines was used to assign this single-copy gene to the long arm of chromosome 4.

Analysis of steady-state levels of *blt101* mRNA showed the induction of this transcript in shoot meristems upon transfer of barley (cv. Igri) plants from control (20 °C/15 °C) to low (6 °C/2 °C) temperature treatment. Further, the high level of this transcript is maintained at low temperatures but is reduced on transfer from low to control temperatures. The gene is not induced by drought or by foliar application of ABA. Analysis of segregating doubled haploid lines shows that there is no specific association of this gene with either spring/winter growth habit or frost hardiness. Examination of the spatial expression pattern revealed ubiquitous expression of *blt101* in low-temperature (6 °C/2 °C) grown barley shoot meristems, mature leaves and roots.

Introduction

A large number of temperate plant species possess the ability to alter their subsequent freezing tolerance during exposure to positive low temperatures. This process is known as 'cold acclimation' and is thought to involve changes in a range of biochemical and physiological processes within the plant cell such that the plant becomes hardened to low temperature and frost injury is reduced [9].

In overwintering crop plants, tolerance to low temperature is an important agronomic character. In addition, many cereals require a low temperature treatment to trigger flower development (vernalisation) [25]. The temperature cereal *Hordeum vulgare* L. (barley) shows altered growth and frost hardiness following exposure to low temperate [14] and winter cultivars exhibit vernalisation requirement. Studies have been performed on a range of cereals to analyse the genetics of vernalisation requirement and frost

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number Z25537.

hardiness. Recent reports have shown that it is possible to dissect the genetical components of both characters [26]. Analysis of chromosomal substitution lines [23] implicates at least two loci on chromosome 5A of wheat (*Triticum aestivum*) in cold hardening and cytogenetic studies [29] on the group 6 chromosomes in wheat have revealed that at least one gene (dominant allele) on the short arm of chromosome 6D (cv. Chinese Spring) prevents chilling injury. In addition, Doll *et al.* [4] have used doubled haploid lines to show that in barley, vernalisation requirement is controlled by two pairs of alleles, with additional modifying genes determining the length of cold treatment required for vernalisation.

In many plant species, changes in gene expression (mRNA levels) and polypeptide profiles occur in response to positive low temperature treatment [9, 12]. However the precise role, if any, that such changes in gene expression play in the cold acclimation process have not yet been determined. It has been reported that several cold-regulated genes are responsive to other stresses such as drought [5, 10, 16, 20]. In addition, many of these genes are also abscisic acid (ABA) responsive [10, 13, 16, 20]. Chen *et al.* [2] reported that endogenous ABA levels increase in some plants in response to low temperature or drought treatment and existing reports suggest that most cold-induced genes are also induced by ABA and drought. It has been suggested that ABA is involved in the acclimation of plants to low temperature. However, the demonstration that certain cold-responsive genes are not induced by ABA implies that for these genes, the low-temperature response is not dependent on ABA. Mohapatra *et al.* [21] have reported three alfalfa clones that are induced by low temperature alone. Furthermore, Gilmour and Thomashow [7] and Nordin *et al.* [22] have used ABA-deficient and ABA-insensitive mutants of *Arabidopsis thaliana* to demonstrate that low temperature and ABA regulate the expression of certain cold-regulated genes through separate pathways. Similarly, Heino *et al.* [11] have shown that the mechanism of low-temperature induction of some cold-induced polypeptides is independent of the mechanism of ABA induction. Of the two previously reported low temperature barley genes, *blt4* and *blt14*, the former is also induced by drought and ABA [13] while the latter is not [31].

This paper is the first report of a winter barley (cv. Igri) gene (*blt101*) which is induced to high steady-state levels in mature leaves, roots and shoot meristems by low-temperature treatments but is not similarly induced by drought or ABA. The chromosomal location, genomic organisation and sequence of this gene have been determined. Furthermore, segregating doubled haploid barley lines have been used in conjunction with various expression studies to analyse *blt101* gene function.

Materials and methods

Plant material

Barley (cv. Igri) plants were grown from seed and harvested as described by Dunn *et al.* [6]. Once harvested, plant material was immediately frozen in liquid nitrogen and stored at −70 °C until required for experimental analysis.

Abscisic acid (ABA)-treated plants were grown as described by Hughes *et al.* [13] and sprayed with either 10 μM ABA on day 0 or 100 μM ABA each day for 7 days. Plant material was harvested on days 1, 3 and 7 following the initial treatment. Control plants were sprayed with water only. Drought-stressed plants were grown as described by Dunn *et al.* [6].

The pattern of induction of *blt101* was investigated using plants grown at 20 °C day/15 °C night for 3 weeks and then transferred to 6 °C day/2 °C night. Total RNA was extracted from shoot meristematic tissue 24, 72 and 168 h after transfer. The pattern of reduction of the transcript was examined using plants acclimated to 6 °C/2 °C prior to being transferred to 20 °C/15 °C. Total RNA was extracted 5.5, 35.5 and 100 h after transfer. Day length and light intensity were maintained as in Dunn *et al.* [5].

DNA was extracted from plants which had been grown at 20 °C/15 °C for a total of 14 days.

RNA extraction and cDNA library construction and screening

Total RNA was extracted from 4 g plant material (80 plants) according to Hughes and Pearce [14] and details of cDNA library construction and screening are given in Dunn *et al.* [5, 6].

cDNA subcloning and sequencing

Restriction analysis of λ BLT101 was carried out as in Dunn *et al.* [6]. Subsequently, pBLT101 insert DNA was subcloned into the *Eco* RI site of the plasmid vector pIC19H [19] and the sequencing vector M13mp18 [30]. cDNA sequences were determined in both directions using Sequenase Version 2.0 (United States Biochemical Corporation) and *Taq* Dye Primer Cycle Sequencing (Applied Biosystems) and synthetic oligonucleotides. Analysis of the DNA and amino acid sequences was performed using DNAsis and Prosis software (Pharmacia).

DNA extraction

DNA was extracted from 0.5 g leaf sheath base material (10 plants) as Dellaporta *et al.* [3]. For the purpose of gene copy number analysis, the method of Graham [8] was used omitting the addition of 2% (w/v) SDS (stage 6).

Northern and Southern blot analysis

Northern and Southern blot analysis was carried out as described in Dunn *et al.* [5] using Hybond N (Amersham International) nylon membranes. Probe preparation and autoradiographic analysis were performed as in Dunn *et al.* [6].

Results

Sequence analysis

blt101 was isolated following a differential screen of a cDNA library prepared in λgt10 from low temperature (6°C/2°C) grown winter barley (cv. Igri) shoot meristems. Dot blot homology tests were used to test for homology between this clone and previously reported genes [5, 6].

Northern blot analysis of total RNA extracted from low-temperature-grown shoot meristems indicated that the *blt101* transcript size is approximately 500 bases. The pBLT101 insert was subcloned into the *Eco* RI site of pIC19H [19] for *Taq* Dye Primer (ABI) sequencing. In order to confirm the cDNA sequence obtained, the pBLT101 insert was also subcloned into the sequencing vector M13mp18 [30] for sequencing by the Sequenase (USB) method. The *blt101* nucleotide sequence from both methods was identical; in addition, another independently isolated cDNA clone had an identical coding sequence and differed only in the length of the non-coding regions. A genomic clone for *blt101* has also been isolated and sequenced in both directions. This genomic clone contains the cDNA sequence shown in Fig. 1 which is interrupted by a single 119 bp intron positioned after base number 125 in Fig. 1. Analysis of *blt101* cDNA sequence indicates that this encodes a short polypeptide of 54 amino acids with a molecular mass of 5.9 kDa.

No significant homology has been found between the *blt101* cDNA or amino acid sequences and entries in GenBank R72·0, PIR R32·0, EMBL R31·0 and SWISS-PROT R22·0 databases. Consequently, it is believed that *blt101* is a unique barley gene isolated due to upregulated steady-state mRNA levels in shoot meristems grown at low temperature. The hydrophobicity plot of the deduced polypeptide [17] suggests that this polypeptide is predominantly hydrophobic with a 20 amino acid N-terminal peptide which has consensus features found in signals present in extracellular proteins [26].

Chromosomal location

The chromosomal location of *blt101* was determined using ditelosomic additions of barley chromosomes into a wheat background [15]. DNA samples, extracted from the barley parent (cv.

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1    T GAA AGC AAG GAG AAA GAA AGA AAC CAG ACT CAA GTG AAG CAA ACC ATG GGC TCT GCA ACA GTC TTG GAG GTG ATC     76
1                                                          M  G  S  A  T  V  L  E  V  I      10

77  CTC GCC ATC ATT CTG CCA CCG GTC GGC GTC TTC CTG CGC TAC AAA CTC GGT GTG GAG TTC TGG ATC TGT CTC TTG CTG    154
11  L  A  I  I  L  P  P  V  G  V  F  L  R  Y  K  L  G  V  E  F  W  I  C  L  L  L      36

155 ACC ATT CTG GGG TAC ATA CCG GGG ATC ATC TAC GCG GTG TAT GTG CTG GTG GTT TAA GCA ACA GCC TCT GCT GCA GGG    232
37  T  I  L  G  Y  I  P  G  I  I  Y  A  V  Y  V  L  V  V  *                                              55

233 TCC GGC GTT TGG ACG AGC GAG TCG CTG TGC AAG ATC AAC TGA TGC TCA TGC TCT ATG GGT TAA GAG AGA ATG TAT GCG    310

311 TGT ATG TAT GGG TTA AGA GAG TAT GTG TAT CGA TGT ATC TAT TGA ATA TTG TGG ATT TCA TTT TTT TTT CTG GAA CTC    388

389 GGT ATT GTT GAT CCT GTG ATG TCA CGA GTG TAC CTA GCT AGC TCG AGG GCA TTT GTA GTA GAG CAT TCG TCG GCC ACA    466

467 TTG TTT GTG ATC TTA AAT TTA AAG TAT TGG ACG TCG AAA AAA AAA AAA AAA AAA A                                  521
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Fig. 1. cDNA and derived amino acid sequence of *blt101*.

Betzes), the wheat parent (cv. Chinese Spring) and thirteen of the possible fourteen addition lines, were digested with *Eco* RI and probed with the insert of pBLT101. Figure 2 shows the results of washing the filter at moderate stringency, i.e. 0.1 × SSPE (0.18 M NaCl, 10 mM sodium phosphate pH 7.7, 1 mM EDTA) 0.1% SDS at 56 °C. The pBLT101 insert hybridizes to four *Eco* RI restriction fragments in cv. Chinese Spring revealing sequence homology with sequences in the

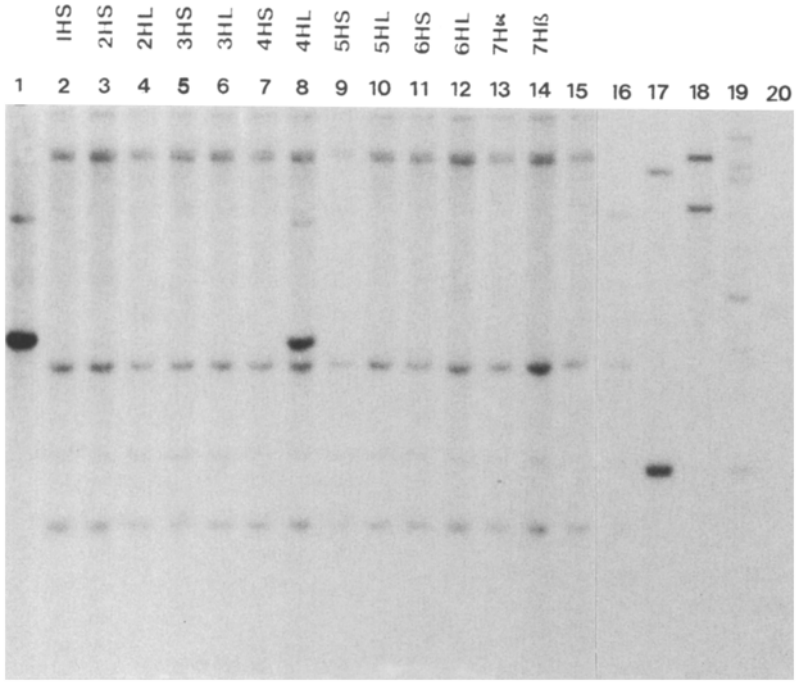

Fig. 2. Chromosomal location of *blt101*. Southern blot analysis of barley ditelosomic addition lines compared with the barley parent (cv. Betzes, lane 1) and the background wheat parent (cv. Chinese Spring, lane 15). Lanes 2–14, barley ditelosomic addition lines (1HS, *Hordeum vulgare* L. chromosome 1, short arm; 2HL, *H. vulgare* chromosome 2, long arm, etc.). Lanes 16–18, progenitors and relatives of the hexaploid wheat genome: lane 16, *Triticum dicoccoides*; lane 17, *Aegilops speltoides*; lane 18, *A. squarrosa*; Lane 19, rye (*Secale cereale*); lane 20, oats (*Avena sativa*). DNA digested with *Eco* RI, probed with *blt101* and washed at moderate stringency (0.1 × SSPE, 0.1% SDS at 56 °C).

wheat parent genome. In barley cv. Betzes there is one strongly hybridizing band of 6.9 kb and a minor band of 13.0 kb both of which are visible in the barley chromosome addition line corresponding to the long arm of chromosome 4.

Figure 2 also shows hybridization of *blt101* to sequences in a range of cereal species. *Triticum dicoccoides*, *Aegilops speltoides* and *A. squarrosa* are all related to, or precursors of, the hexaploid genome of *Triticum aestivum*. Sequence homology is also revealed between the pBLT101 insert and *Eco* RI fragments in rye (*Secale cereale*) and oats (*Avena sativa*).

Gene copy number reconstruction

Barley (cv. Igri) genomic DNA was digested with *Bam* HI, *Dra* I, *Eco* RI or *Hin*d III and electrophoresed alongside *Bam* HI-linearized pBLT101

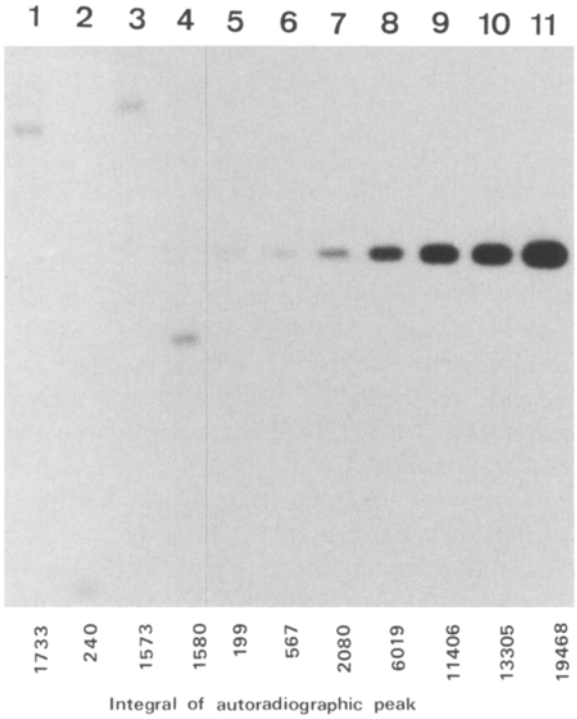

Fig. 3. Gene copy number reconstruction. Restriction enzyme digests of barley cv. Igri genomic DNA: lane 1, *Bam* HI; lane 2, *Dra* I; lane 3, *Eco* RI; lane 4, *Hin*d III. *Bam* HI-linearized pBLT101 equivalent to 0.5, 1, 2, 4, 8, 10 and 20 copies of *blt101* per haploid genome (lanes 5–11 respectively). The filter was probed with *blt101* and washed at low stringency (0.1 × SSPE, 0.1% SDS at 25 °C).

corresponding to 0.5, 1, 2, 4, 8, 10 and 20 gene copies per haploid genome in the equivalent quantity of genomic DNA. The filter was hybridized with a probe made from pBLT101 insert DNA and washed at low stringency (0.1 × SSPE, 0.1% SDS at 25 °C) (Fig. 3). Densitometric analysis was used to compare the intensity of the homologous restriction fragments with the calculated gene copy equivalents. Integrated peak values of the genomic restriction fragments and the gene copy number equivalents (Fig. 3) indicate that *blt101* is a single-copy gene in barley. This result contrasts with the other reported barley low-temperature genes *blt14* [5] and *blt4* [6] which hybridize with a number of major and minor restriction fragments in all examined digests of barley genomic DNA after a low-stringency wash and where both *blt14* and *blt4* are present as multigene families with at least two members up-regulated by low temperature.

Cold transfer experiments

The pattern of induction of the *blt101* transcript was investigated using barley plants grown at 20 °C/15 °C before transfer to 6 °C/2 °C. In addition, *blt101* transcript reduction was examined using plants acclimated to 6 °C/2 °C before transfer back to 20 °C/15 °C. Equal amounts of total RNA, extracted from shoot meristematic tissue, were electrophoresed in a denaturing gel. After hybridization with *blt101*, analysis of steady-state levels of *blt101* mRNA (Fig. 4) reveals that the transcript is induced after only 24 h low-temperature treatment (lane 2) and reaches maximal levels by 168 h (7 days) cold treatment (lane 4) as compared with 3 weeks cold treatment (lane 6). Conversely, increasing the ambient temperature from 6 °C day/2 °C night results in a rapid decline in the *blt101* transcript steady-state levels. *blt101* expression is reduced to basal levels after only 100 h (4.2 days) (lane 10) at the control temperature regime. This data suggests that the *blt101* transcript exhibits a faster rate of decrease than increase under these experimental conditions.

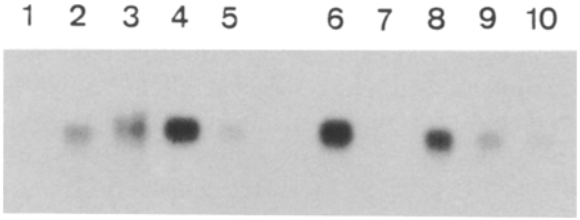

Fig. 4. Cold transfer experiments. Northern blot analysis of induction of the *blt101* transcript at acclimating temperatures and inhibition of the transcript at non-acclimating temperatures. Lanes 1–5, barley seedlings were grown at the control temperature (20 °C/15 °C) prior to transfer to low temperature (6 °C/2 °C): lane 1, control (20 °C/15 °C); lanes 2, 3 and 4, 24, 72 and 168 hours respectively after transfer to low temperature; lane 5, 168 h at control temperature. Lane 6, three weeks at low temperature; lane 7, control material. Lanes 8–10, seedlings were acclimated to 6 °C/2 °C prior to transfer to 20 °C/15 °C: lanes 8, 9 and 10, 5.5, 35.5 and 100 h respectively after transfer to control temperatures. The filter was probed with *blt101* and washed at moderate stringency (0.1 × SSPE, 0.1% SDS at 50 °C).

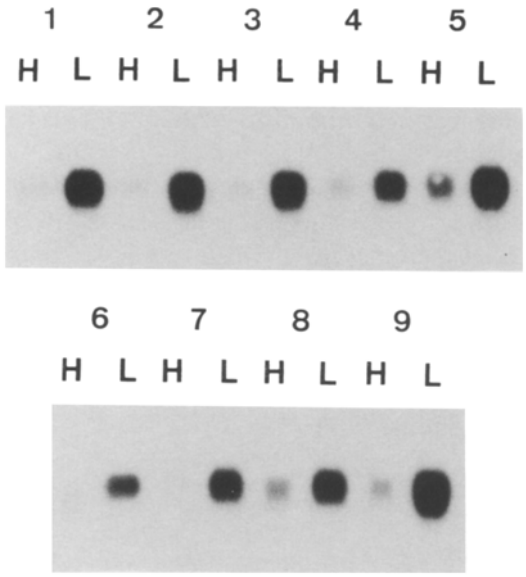

Fig. 5. Northern blot analysis of *blt101* expression in segregating doubled haploid lines of barley from the cross cv. Vogelsanger Gold (winter cultivar) × cv. Tron (spring cultivar). Lane 1, cv. Vogelsanger Gold (parent); lane 2, cv. Tron (parent); lanes 3 and 4, spring type, frost-sensitive lines; lanes 5 and 6, spring type, frost-hardy lines; lanes 7 and 8, winter type, frost-hardy lines; lane 9, cv. Igri. H, control temperature (20 °C/15 °C); L, low temperature (6 °C/2 °C). The filter was probed with *blt101* and washed at moderate stringency (0.1 × SSPE, 0.1% SDS at 42 °C).

Analysis of gene function

Analysis of the expression of *blt101* as measured by steady-state mRNA levels was made in shoot meristems in a series of doubled haploid barley lines [4] (Fig. 5). The series were produced by crossing the frost-hardy winter cultivar Vogelsanger Gold with the frost-sensitive spring cultivar Tron. The doubled haploid lines show segregation for the characters frost hardiness/frost sensitivity and winter/spring habit. Total RNA was extracted from each of the selected doubled haploid lines, each parental cultivar and cv. Igri then electrophoresed in equal amounts in a denaturing gel. The filter was probed with *blt101* and washed at moderate stringency (0.1 × SSPE, 0.1% SDS at 42 °C). Examination of the steady state levels of *blt101* mRNA reveals that this gene is induced to a similar extent in all of the doubled haploid lines, both parents and cv. Igri following low temperature (6 °C/2 °C) treatment. This result indicates that there is no specific association of *blt101* with either spring/winter growth habit or frost hardiness in this cross. A similar investigation was performed with doubled haploid lines from a cross between the frost-sensitive spring cultivar Tystofte Prentice and Vogelsanger Gold; analysis of steady-state levels of *blt101* mRNA (results not shown) gave the same results.

A study of the induction of *blt101* by ABA was undertaken. Total RNA was extracted from meristematic tissue of barley plants which had been grown at 20 °C/15 °C but which had been sprayed with 10 μm ABA and harvested 1, 3 or 7 days after the initial ABA treatment. Autoradiographic analysis of filters washed at moderate stringency (0.1 × SSPE, 0.1% SDS at 50 °C) reveals no signal indicating that the *blt101* transcript is not induced by either of these ABA treatments (data not shown). A similar result was obtained from material which had been treated with 100 μm ABA. These same filters had previously been used to demonstrate ABA induction of *blt4* [13]. Northern blot analysis of *blt101* expression in shoot meristems of drought-stressed

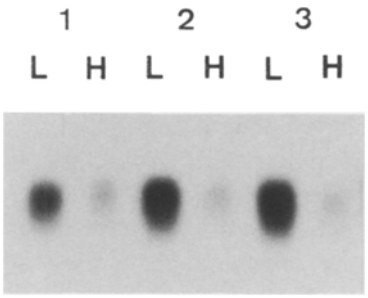

Fig. 6. Spatial expression pattern. Northern blot analysis of *blt101* in barley shoot meristems (lane 1), mature leaves (lane 2) and roots (lane 3) after one week of low temperature treatment. H, control temperature (20 °C/15 °C); L, low temperature (6 °C/2 °C). The filter was probed with *blt101* and washed at moderate stringency (0.1 × SSPE, 0.1% SDS at 42 °C).

plants shows that the gene is not induced by this stress (data not shown).

Spatial expression pattern

A study of the low-temperature-induced expression of *blt101* in various barley tissues was undertaken. Total RNA was extracted from mature leaves, roots and shoot meristematic tissue grown at both 20 °C/15 °C and 6 °C/2 °C, and electrophoresed in a denaturing gel. Figure 6 shows the result of hybridizing the Northern blot filter with *blt101* and washing at moderate stringency (0.1 × SSPE, 0.1% SDS at 42 °C). As measured by steady state mRNA levels, *blt101* shows ubiquitous expression, after low-temperature treatment, in all three tissue types studied. For comparison, further hybridization of the same filter reveals that *blt14* and *blt4* are regulated by low temperature in leaves and shoot meristems, but *blt14* is constitutively expressed in the roots while *blt4* shows no expression in this organ [13].

Discussion

Plants are immobile organisms and must alter their metabolism to enable them to withstand a wide range of environmental stresses. Cold stress elicits a response, the complexity of which is exemplified by the number of different cold-responsive genes which have been isolated by several groups from various species. Many of these cold-responsive genes are also induced by other stresses such as drought [5, 10, 16, 20], whilst only a small number have been found to be low temperature-specific [21, 31]. This paper is the first report of a single-copy winter barley (cv. Igri) gene (*blt101*) which is induced in all tested tissue types by low temperature but not by drought or ABA.

Conventional genetic crosses have been used to examine the possible cosegregation of *blt101* expression with recognised phenotypic characters. The success of this method depends upon the choice of suitable lines which, when crossed, give segregation of the genetical characters under investigation. In this study, barley doubled haploid lines [4] were used in which segregation occurred for vernalisation requirement and frost hardiness. However, for the two crosses examined *blt101* did not cosegregate with either of these characters indicating that the genetic basis of the difference between Vogelsanger Gold versus Tron or Tystofte Prentice does not involve *blt101*. Since several genes are involved in the determination of frost hardiness in cereals [26] a function for *blt101* in the determination of frost hardiness cannot be excluded on the basis of these results.

The relationship between a drought and a low-temperature response is expected since both can be dehydrative stresses. Most cold-induced genes which have been cloned are also responsive to drought stress and ABA treatment [10, 13, 16, 20] therefore *blt101* can be distinguished from these genes on the basis of this result. Recently it has been reported by Zhang *et al.* [31] that the cold induced barley gene *blt14* is not induced by drought or ABA. However, dot blot tests and sequence homology analysis reveal no homology between *blt14* and *blt101*. Similarly, Mohapatra *et al.* [21] reported three alfalfa clones (pSM784, pSM2201, pSM2358) which are induced by low temperature but not by drought or ABA. However, no sequence information has been published for these clones, therefore their homology with *blt101* cannot be tested.

It has been shown that *blt101* is expressed in low-temperature-treated roots, mature leaves and shoot meristems. This result has provided some information regarding the biochemical function of *blt101* because it eliminates an involvement in any process which is tissue specific such as photosynthesis. It also provides further confirmation that *blt101* and *blt14* are different. Cattivelli and Bartels [1] have analysed the spatial expression patterns of five barley low-temperature-induced clones (pT59, pV60, pAO29, pAO86, pAF93). On the basis of the reported transcript sizes and spatial expression patterns, none of the five clones appears to be the same as *blt101*. Furthermore, *blt101* can be distinguished from pT59 and pAO86 on the basis of published sequence data. Cattivelli and Bartels [1] also looked for sequence homology in three other cereal species, namely wheat, rye and oats. Of the unsequenced clones, pV60 hybridizes with sequences in wheat and rye, pAO29 hybridizes only with sequences in wheat and pAF93 hybridizes with neither wheat, rye nor oats. The finding that *blt101* has homologues in wheat, rye and particularly oats further distinguishes this gene from pV60, pAO29 and pAF93.

blt101 is a single-copy gene residing on barley chromosome 4 which is homologous with chromosomes 4A, 4B and 4D in wheat. Wheat chromosomal substitution lines [18, 24, 27] have been used to demonstrate the importance of chromosome 4 (particularly 4B and 4D) in the determination of frost hardiness in wheat. Furthermore, Veisz and Sutka [27] showed that in wheat substitution lines the level of frost hardiness attained is dependent not only on the presence of different chromosomes but also on the duration of hardening, indicating that either the genes for frost resistance exhibit different levels of expression during phases of the hardening process or that gene product accumulation is necessary for maximal hardening. In conjunction with sequence data, cytogenetical information such as this can provide a tool to investigate the biochemical functions of genes located on equivalent chromosomes in other cereal species. However, none of the wheat chromosome 4 genes has been cloned, therefore homology with *blt101* cannot be tested.

Since *blt101* is a single-copy gene whose transcript is often undetectable in plants grown at moderate temperature (20 °C day/15 °C night) it can be inferred that it encodes a gene product specifically required in low-temperature-grown cells and does not encode a 'cold' isoform of a protein functioning in both temperature regimes. Further, the maintenance of high steady-state *blt101* levels during low temperature growth in all parts of the plant suggests that the gene product is continuously required to maintain cell function at low temperatures. The small size (54 amino acids) and hydrophobic nature of the predicted *blt101* gene product is confirmed by all of the DNA sequence data. The possible extracellular location of the *blt101* gene product, predicted by sequence analysis, is a feature of the two other barley shoot meristem low-temperature genes which have been cloned [13, 31]. Both of these genes (*blt4* and *blt14*) have hydrophobic N-terminal peptides with structural features found in consensus signal peptides of extracellular proteins. A role for extracellular proteins has not been discussed in relation to physiological studies of frost tolerance and it is interesting that three of the low-temperature meristematic barley genes which have been cloned are small and are predicted to have this location. Further studies on the function and low-temperature control of expression of *blt101* are in progress.

Acknowledgements

The authors would like to acknowledge Vince Lea (PBI Cambridge Ltd.) for assistance with the chromosomal location work and Dr Roger S. Pearce (University of Newcastle upon Tyne) for help with growth of the plants.

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