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Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana* (L.) Heynh.

Kerstin Nordin, Tiina Vahala and E. Tapio Palva*
Department of Molecular Genetics, Uppsala Genetic Center, Swedish University of Agricultural Sciences, Box 7003, S-75007 Uppsala, Sweden (author for correspondence)*

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Abstract

Plant cold acclimation is correlated to expression of low-temperature-induced (*lti*) genes. By using a previously characterized *lti* cDNA clone as a probe we isolated a genomic fragment that carried two closely located *lti* genes of *Arabidopsis thaliana*. The genes were structurally related with the coding regions interrupted by three similarly located short introns and were transcribed in the same direction. The nucleotide sequences of the two genes, *lti78* and *lti65*, predict novel hydrophilic polypeptides with molecular weights of 77856 and 64510, respectively, *lti78* corresponding to the cDNA probe. Of the 710 amino acids of LTI78 and 600 amino acids of LTI65, 346 amino acids were identical between the polypeptides, which suggests that the genes may have a common origin.

Both *lti78* and *lti65* were induced by low temperature, exogenous abscisic acid (ABA) and drought, but the responsiveness of the genes to these stimuli was markedly different. Both the levels and the temporal pattern of expression differed between the genes. Expression of *lti78* was mainly responsive to low temperature, that of *lti65* to drought and ABA. In contrast to the induction of *lti78*, which follows separate signal pathways during low-temperature, ABA and drought treatment, the drought induction of *lti65* is ABA-dependent and the low-temperature induction appears to be coupled to the ABA biosynthetic pathway. This differential expression of two related genes may indicate that they have somewhat different roles in the stress response.

Introduction

Several plant species are able to cold-acclimate, i.e. they can increase their tolerance to extracellular freezing. This cold acclimation process and the resulting increase in frost hardiness is induced by an exposure of the plant to low but non-freezing temperatures [18, 31]. The response is relatively rapid, e.g. the freezing tolerance of *Arabidopsis thaliana* can be increased from the −4 °C of an

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X67670 (*lti65*) and X67671 (*lti78*).

unacclimated plant to about −10 °C by a five-day exposure to low temperature (4 °C) [7, 17]. Apart from the exposure to low temperature, increased freezing tolerance can also be achieved by drought treatment [4] and by exogenous abscisic acid (ABA) [3, 22, 23], indicating a close relationship between freezing and drought stress and involvement of ABA in mediating these stress responses. In nature, lethal freezing injury appears to be connected to cellular dehydration, as the formation of ice crystals during extracellular freezing causes a movement of water out of the cells [18].

The cold adaptation process that takes place during low-temperature exposure is accompanied by a variety of metabolic changes. These include cold-induced alterations in the protein synthesis pattern as well as changes in gene expression (for reviews [10, 35]). The changes in gene expression are detectable already during the first hours of cold acclimation and the synthesis of novel proteins starts during the first day at 4 °C [11, 15, 17, 26]. Recently several low-temperature-induced genes have been cloned and analysed and information about the amino acid sequences of the proteins encoded by some of these genes has been obtained [2, 8, 15, 16, 22, 26, 27]. However, the functions of the corresponding proteins are still unknown, and their possible role in the cold acclimation process has not been demonstrated. Still, the finding that proteins encoded by two cold-induced genes (*kin1* and *kin2* or *cor6.6*) in *A. thaliana* [8, 15, 16] show similarity to antifreeze proteins from arctic fish suggests that at least some of the proteins appearing during the cold treatment may have a direct function in freeze protection. Some of the other low-temperature-induced polypeptides share limited [8] or extended [21] similarity with the drought-induced RAB / LEA / DEHYDRIN (responsive to ABA / late embryogenesis-abundant / dehydration-induced) class of polypeptides [5, 33], in accordance with the suggested relatedness of the freezing and drought stress responses. The observation that most of the low-temperature-induced genes isolated so far indeed respond to all three stimuli, low temperature, drought and elevated ABA concentration [11, 15, 16, 21, 23, 26], supports this concept of relatedness between the stress responses.

We have previously isolated and characterized a cDNA clone corresponding to a low-temperature-induced gene *lti140* of *A. thaliana* [26]. The expression of this gene is induced by low temperature, exogenous ABA and drought, the induction by low temperature being clearly the most prominent. The responses to these different stimuli seem to follow separate signal pathways, as the low-temperature-induced expression is completely and the drought induction partly independent of ABA [26].

Analysis of a genomic clone carrying the *lti140* gene presented in this paper revealed the presence of two closely linked and structurally related genes, one of which was *lti140* (renamed *lti78*). The second gene was shown to encode a polypeptide similar to but not identical with the protein encoded by *lti78*. In this paper we report the sequences and the structure of these two genes and their gene products. Furthermore, we show that although both of the genes respond to the same stimuli (low temperature, ABA and drought), the responsiveness of the genes to these stimuli is markedly different, as are the signal pathways employed.

Materials and methods

Plant material and growth conditions

The following genotypes of *Arabidopsis thaliana* (L.) Heynh. were used: the wild types Columbia and Landsberg erecta, and the ABA mutants *aba-1* (defective in ABA biosynthesis) [12] and *abi-1* (ABA-insensitive) [13]. The growth conditions and various stress treatments were as described by Nordin *et al.* [26]. In short, low-temperature treatment was performed by transferring the plants to 4 °C day/2 °C night. For ABA treatment a stock solution of ABA was added to the growth medium to give a final concentration of 60 μM. Drought treatment was done by removing the lid of the tissue culture plates.

Screening of genomic library, cloning and sequencing

25000 clones from a λEMBL4 genomic library of *Arabidopsis thaliana* were blotted on nitrocellulose filters and screened using the previously isolated 1.2 kb partial cDNA fragment corresponding to a low-temperature-induced gene *lti140* [26] as a probe. The filters were hybridized at 65 °C in 6× SSC, 1% SDS, 5× Denhardt's solution and 0.1 mg/ml herring sperm DNA and washed twice for 30 min at 65 °C with 1× SSC and 0.1% SDS. Of the clones hybridizing to the probe, one with a 15 kb insert was chosen for further analysis. Restriction fragments and overlapping deletion products of the genomic insert were subcloned into pBluescript SK(+) and KS(+) vectors (Stratagene, La Jolla, CA) and sequenced by the chain termination method of Sanger *et al.* [32] using the Sequenase (USB, Cleveland, Ohio) sequencing system. Sequence data were analyzed using the PCGENE and Intelligenetics programs.

Primer extension

The transcription start of the two genes contained in the genomic clone was determined by primer extension analysis. For the *lti78* gene, 1 μg of poly(A)$^+$ RNA from low-temperature-treated and control plants was hybridized to an oligonucleotide (5′-CAAAGATTTTTTTCTTTCCAATAGAAGTAA-3′) corresponding to the complementary strand of the bases +48 to +77 of the gene. For the *lti65* gene, the oligonucleotide (5′-CAAAGCTGTGTTTTCTCTTTTTCAAGTGAA-3′) corresponding to the complementary strand of the bases +52 to +81 was hybridized to 5 μg of poly(A)$^+$ RNA from ABA-treated and control plants. The oligonucleotides were purchased from Operon Technologies, Inc. (Alameda, CA) and used in an unpurified form. The extension reactions were performed using the first-strand synthesis reaction from a cDNA synthesis kit (Amersham, Buckinghamshire, UK). The elongation products were separated on an 8% polyacrylamide gel alongside with DNA sequencing reactions performed using the same primers as for the primer extension reactions and compared to sequences produced from the 5′ ends of the respective genes.

First-strand cDNA synthesis and polymerase chain reactions

To amplify cDNA corresponding to the 5′ part of *lti78*, the first strand of the cDNA was first synthesized from 1 μg of poly(A)$^+$ RNA from low-temperature-treated plants with a reverse transcriptase reaction and reagents from a cDNA synthesis kit (Amersham). Part of the resulting single-stranded DNA was amplified in a PCR reaction using an oligonucleotide (5′-TGGACCATGGATCAAACAGAGGAAC-3′) corresponding to bases +76 to +100 as the 5′-end primer and an oligonucleotide (5′-CTCATGACTTCTCACCGGAA-3′) corresponding to the complementary strand of the bases +1542 to +1561 as the 3′-end primer. Two bases in the 5′-end primer differ from the sequence in the gene, because an *Nco* I restriction site was created in the primer to facilitate the cloning of the PCR product. The reaction was performed with 40 cycles, each consisting of 30 s at 93 °C, 30 s at 50 °C and 3 min at 72 °C.

The cDNA corresponding to *lti65* was synthesized as the *lti78* cDNA, except that poly(A)$^+$ RNA from ABA-treated plants was used as a template in the first-strand synthesis. The 5′ part of the cDNA was amplified by PCR using an oligonucleotide (5′-TGGACCATGGAGTCACAGTTGACAC-3′) corresponding to bases +80 to +104 as the 5′-end primer and an oligonucleotide (5′-AATGATACAGGCTCAAT-3′), corresponding to the complementary strand of the bases +648 to +664, as the 3′-end primer. An *Nco* I site was created in this 5′-end primer, too. The reaction was performed with 25 cycles, consisting of 20 s at 93 °C, 30 s at 45 °C and 45 s at 72 °C.

RNA extraction and northern analysis

Total RNA was isolated from shoots of *A. thaliana* plants according to Logeman *et al.* [20] or, alternatively, Verwoerd *et al.* [36]. Poly(A)$^+$ RNA used for primer extension and cDNA synthesis was purified on an oligo-dT cellulose column. For northern analysis, total RNA (10 μg per lane) was separated on denaturing formaldehyde-agarose gels and blotted onto nylon filters which were prehybridized at 65 °C in 5× SSPE (1× SSPE = 180 mM NaCl, 1 mM NaH_2PO_4, 1 mM EDTA pH 7.4), 5× Denhardt's solution, 0.2% SDS and 0.5 mg/ml denatured herring sperm DNA. Hybridizations were performed overnight in the same solution with an addition of a probe labelled with α^{32}P-dATP by a multiprime reaction. The probe used to detect the *lti78* transcript was the previously isolated cDNA fragment [26] corresponding to bases + 1414 to + 2618 of the *lti78* gene. The probe homologous to the *lti65* transcript was a 450 bp fragment corresponding to bases + 1612 to + 2065 of the *lti65* gene. After hybridization the filters were washed twice for 30 min with 0.5× SSC, 0.5% SDS at 65 °C.

Results

Isolation and structure of the low-temperature-induced genes lti78 *and* lti65

A genomic library of *A. thaliana* in λEMBL4 was screened with a cDNA fragment corresponding to a low-temperature-induced gene in *A. thaliana*, previously named *lti140* [26]. Out of the 25000 plaques screened, four clones hybridizing to the probe were found. One of these partly overlapping clones, containing an insert of 15 kb, was subjected to further analysis: 7.5 kb of this genomic fragment was subcloned and sequenced. The insert proved to contain two distinct but related genes separated by 1.7 kb (Fig. 1), the downstream gene corresponding to the *lti140* cDNA. The nucleotide and the deduced amino acid sequences of the two genes are shown in Figs. 2 and 3. The predicted molecular weight for the 710 amino acid polypeptide encoded by the downstream gene was 77856 and that for the 600 amino acid polypeptide encoded by the upstream gene was 64510. The genes were therefore named *lti78* (previously *lti140*) and *lti65*, respectively.

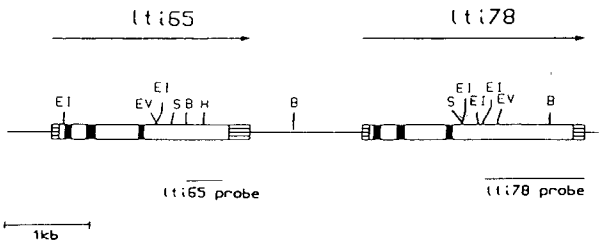

Fig. 1. Physical organization of the *lti78* and *lti65* genes. The open reading frames are shown with open bars, the introns with black bars and the 5′- and 3′-untranslated regions with striped bars. The restriction sites shown are *Bgl* II (B), *Hin*d III (H), *Eco* RI (EI), *Eco* RV (EV) and *Sal* I (S). The location of the DNA fragments used as probes in northern blot hybridizations are indicated as lines below the DNA fragment.

The coding regions of *lti78* and *lti65* were interrupted by three introns (Figs. 1, 2 and 3). The exact location of the introns was determined by sequence comparison of genomic and cDNA clones and by comparing the sequences at the putative splice sites to published consensus sequences at intron splice sites. The cDNA for *lti78* was synthesized by reverse transcription of low-temperature-induced poly(A)$^+$ RNA and the cDNA for *lti65* using poly(A)$^+$ RNA derived from ABA-treated plants, amplified by PCR and

Fig. 2. The DNA sequence and deduced amino acid sequence of the *lti78* gene. The DNA sequence of *lti78* is shown from the transcription start site at position 1 to base 2618 which corresponds to the last nucleotide before the poly(A) tail in the corresponding cDNA clone. The putative polyadenylation signal (AATAAA) is underlined. Oligonucleotides used as primers in primer extension analysis and PCR are indicated with arrows under the DNA sequence. The nucleotide sequence and the predicted amino acid sequence of *lti78* differs slightly from the previously reported cDNA sequence [26]. The differences are found at the nucleotide

positions 1886–1888 of the *lti78* sequence, where a codon CTA (coding for leucin) is found, instead of the codon CAT (coding for histidine) of the cDNA sequence, and in the amino acid position 561 of LTI78, where there is a serine instead of the alanine residue, which was shown in the cDNA sequence. These differences are due to errors made previously when sequencing and analyzing the cDNA.

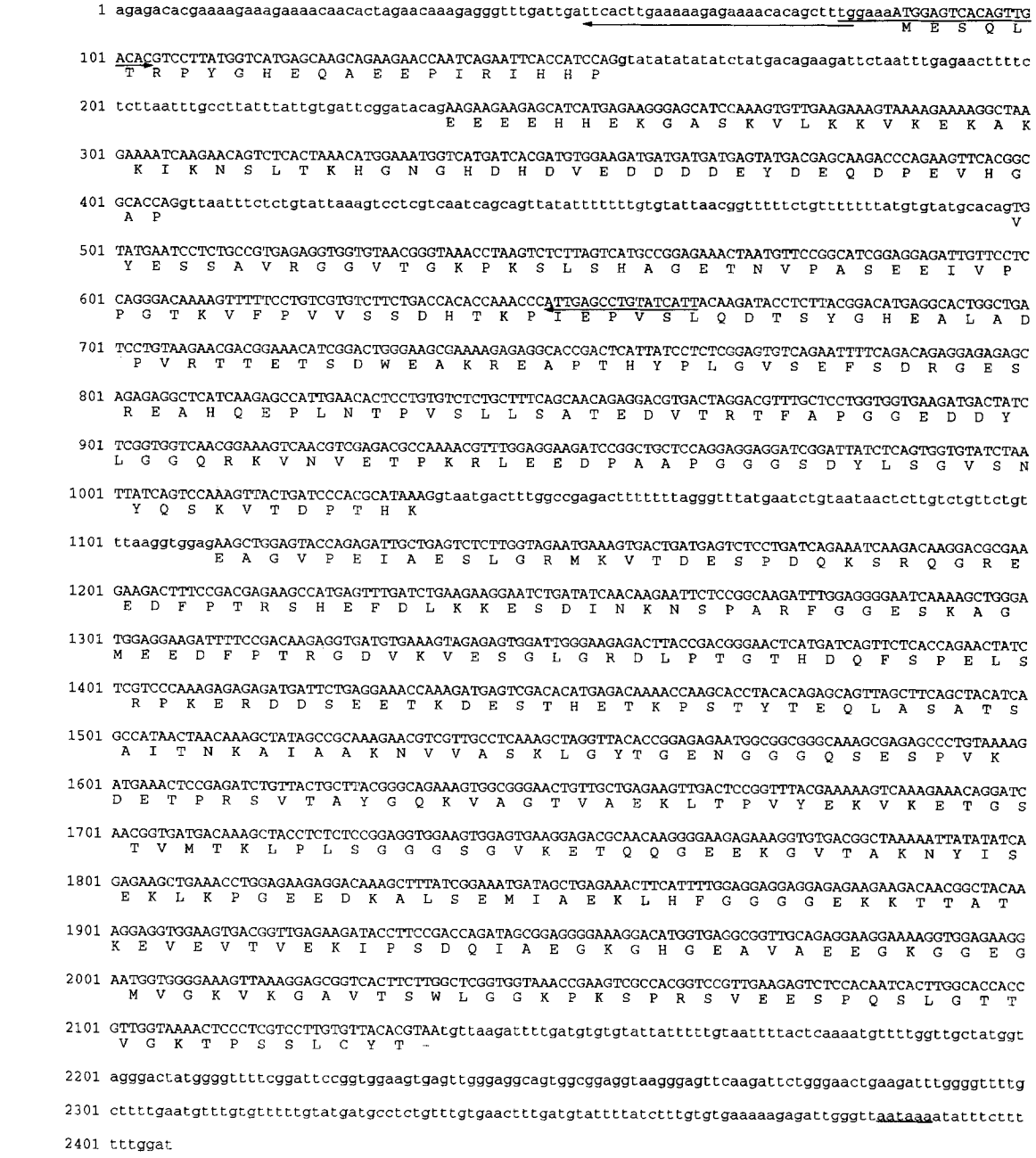

Fig. 3. DNA sequence and deduced amino acid sequence of the *lti65* gene. The DNA sequence of *lti65* is shown from the transcription start site at position 1 to base 2407, the presumed end of transcription. This is located at the same distance from the putative polyadenylation signal (AATAAA, underlined) as in *lti78*. Oligonucleotides used as primers in primer extension analysis and PCR are indicated with arrows under the DNA sequence.

sequenced. The *lti78* cDNAs, including the previously obtained cDNA clone, corresponded to the whole gene but for *lti65* we could only obtain a cDNA corresponding to the genomic fragment containing the first two introns. As the PCR amplification of the remaining part of the cDNA was

not successful, the localization of the third intron was only deduced from splice site consensus and by comparison with the location of the third intron of *lti78*.

A putative translation initiation codon (ATG) was found within the first exon of both genes. The transcription start sites were mapped by primer extension analysis to 81 bp and 85 bp upstream of the putative initiation codons of the genes *lti78* and *lti65*, respectively (Figs. 2, 3 and 4). A putative polyadenylation signal was found in the 3′-untranslated regions of both genes, 116 and 238 bp after the stop codon for *lti78* and *lti65*, respectively. The estimated sizes of the transcripts obtained using the criteria above were 2351 bases for the *lti78* gene and 2171 bases for the *lti65* gene, not including the poly(A) tails. This estimate corresponded well to the observed sizes of these transcripts (cf. Fig. 6).

Both genes contain the sequence TATAAA, a putative TATA box, located between positions −33 and −28 in *lti78* and between positions −34 and −29 in *lti65* (Fig. 4). Analysis of the 5′ upstream regions of these genes (Fig. 4) showed the presence of sequences with homology to known regulatory sequences identified in other plant genes. Both *lti78* and *lti65* contained a sequence TACGTGGC, similar to the 'ABRE' (ABA-responsive element) found in other ABA-inducible genes [9, 25]. The upstream region of *lti65* also contained another ABRE-like sequence, GACGTGGC.

The upstream regions of the two genes showed only limited sequence homology, except for a stretch of 39 bp between positions −303 and −265 in the *lti78* gene and between positions −267 and −229 in the *lti65* gene, and some shorter stretches spread throughout the sequence (Fig. 4). A sequence ACCGACA, located at the 3′ end of the 39 bp homologous region, is repeated three times with slight modifications in the *lti78* promoter region. In contrast, only one repeat of

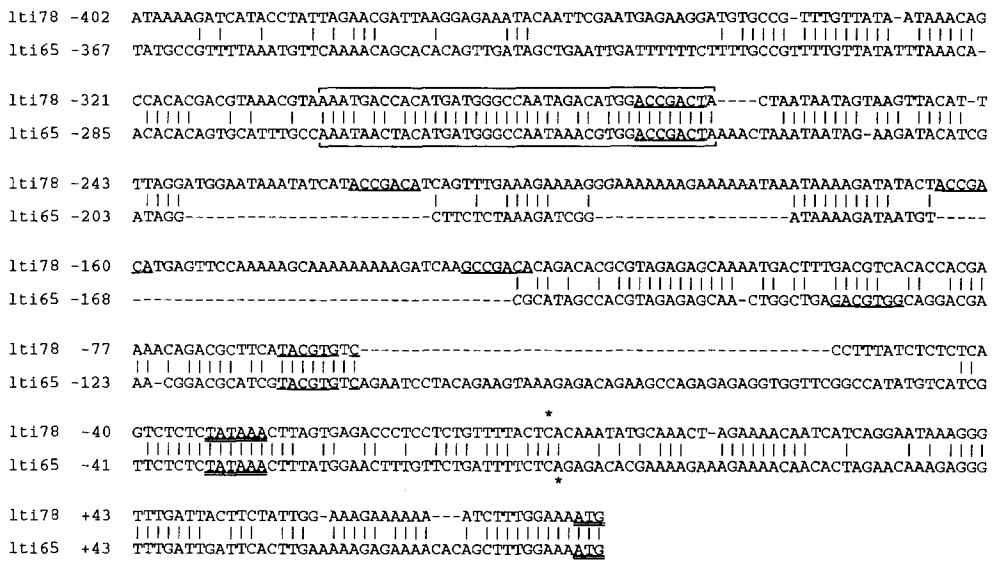

Fig. 4. Alignment of the 5′ upstream sequences of *lti78* and *lti65*. 483 bases of the 5′-untranslated region of *lti78* were aligned with 452 bases of the corresponding region of *lti65*. Lines indicate identical nucleotides in the two promoter regions. Transcription start sites are marked with asterisks and the initiation ATGs and putative TATA boxes (TATAAA) are underlined with double lines. The stretch of 39 nucleotides with high homology between the two promoters is marked with brackets. The repeated sequence ACCGACA and the ABRE-like sequences (TACGTGGC and GACGTGG) are underlined with single lines. – indicates a gap inserted in the sequence for better alignment.

this sequence is present in the upstream region of *lti65*.

Similarity of LTI65 and LTI78 polypeptides

The coding region of *lti78*, encodes a polypeptide of 710 amino acids with a predicted molecular weight of 77856. The smaller gene, *lti65*, encodes a 600 amino acid polypeptide giving a predicted molecular weight of 64510. Both polypeptides are hydrophilic and lack membrane spanning regions, N-terminal signal peptides and other known targeting signals, which suggests a cytoplasmic location for these proteins. The pI is low for both polypeptides, 4.2 for LTI78 and 4.9 for LTI65.

Amino acid sequence comparison of the polypeptides encoded by *lti65* and *lti78* showed that 346 amino acids were identical and 41 amino acids were similar between the two proteins (Fig. 5), indicating that the genes are closely related. Highest degree of sequence similarity was found in the N-terminal and C-terminal regions, whereas other parts of the polypeptides differed more, resulting in several gaps in the amino acid sequence alignment (Fig. 5). Apart from the similarity between LTI65 and LTI78, amino acid similarity searches in the PIR31 and Swiss-Prot 21 protein sequence data banks did not reveal significant similarity to any other proteins.

The LTI78 polypeptide contains several repeats, some of which are also present in LTI65 (Fig. 5). A sequence with the consensus D/NFPTRSHEFDLKTES is found three times in LTI78 but only once in LTI65. The C-terminal part of both proteins contains five imperfect repeats of the sequence VAEKL. LTI78 also contains an imperfect repeat of the sequence PvGFGdESGAELEK at amino acids 303–316 and

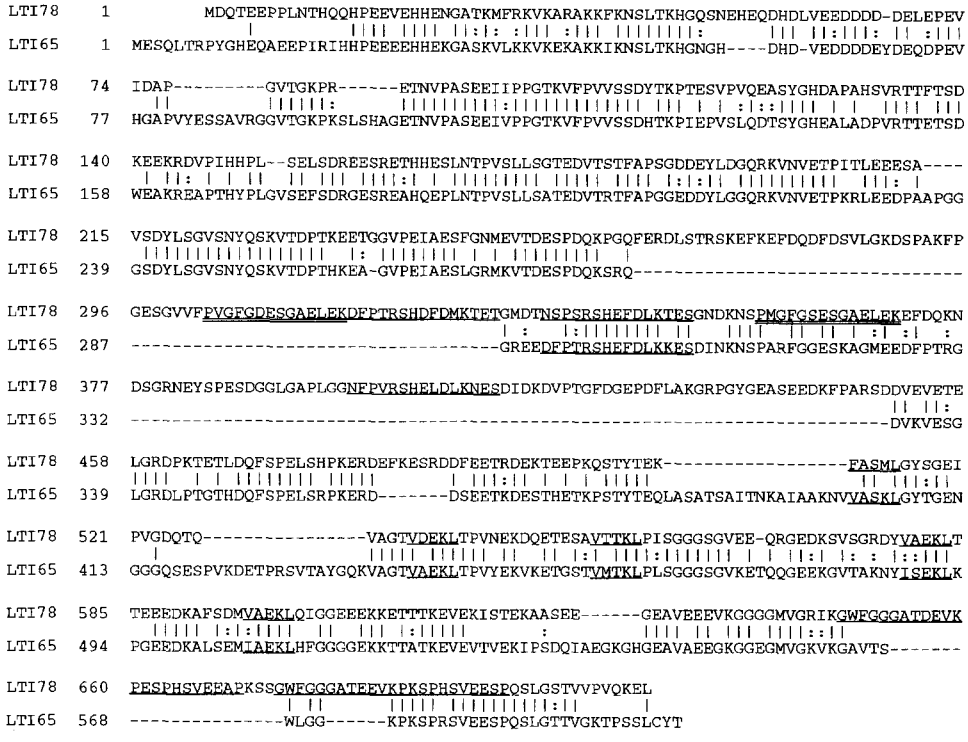

Fig. 5. Comparison of the amino acid sequences of the polypeptides encoded by *lti78* and *lti65*. A line indicates identical amino acids and a colon similar amino acids. The four different types of repeated amino acid sequences are underlined. – indicates a gap inserted in the sequence for better alignment.

357–370, and the sequence GWFGGGATdE-VKPeSPHSVEEaP is, slightly modified, present twice in the C-terminal end of the protein.

Differential induction of lti65 *and* lti78 *by low temperature, ABA and drought*

We have shown earlier that the expression of the *lti78* gene (previously called *lti140*) is strongly and rapidly induced by low temperature (4 °C), but can also be induced by exogenous ABA and by drought treatment, albeit to a lower level [26]. Northern blot analysis with gene specific probes was used to study whether the *lti65* gene could also be induced by these stimuli and to compare its expression pattern to that of the *lti78* gene. The sizes of the mRNAs hybridizing to the 3′-end fragments of the *lti78* and *lti65* genes used as probes (Fig. 1), were in accordance with the calculated sizes of transcripts (Fig. 6). The *lti65* probe crosshybridizes weakly to the *lti78* mRNA, which resulted in detection of both transcripts with this probe, whereas the *lti78* probe used did not enable the detection of the *lti65* transcript (Fig. 6).

Expression of both genes was clearly induced by low temperature, as well as by exogenous ABA and drought (Fig. 6). However, the responsiveness of *lti65* and *lti78* gene expression to these stimuli differed markedly. Comparison of the relative abundance of the *lti65* and *lti78* mRNAs following low-temperature, ABA or drought treatments suggested that *lti78* is mainly a low-temperature-induced and *lti65* a drought- and ABA-induced gene (Fig. 6). In addition to the different relative levels seen, also the time of appearance of these transcripts differed. Large amounts of the *lti78* transcript could be detected after four hours of low-temperature treatment, whereas detectable levels of *lti65* mRNA were not present until after 24 h at 4 °C and they never reached the level of the *lti78* transcript. Differences were also evident in the ABA-induced expression of these genes. Firstly, *lti65* was more strongly induced by ABA than by low temperature, in contrast to *lti78* where low-temperature

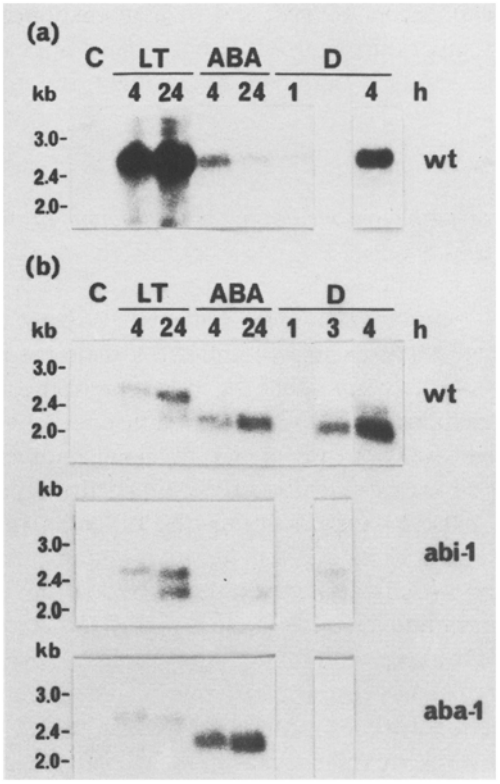

Fig. 6. Expression of the *lti78* and *lti65* genes in response to different stress treatments in wild-type plants and in ABA-insensitive and ABA-deficient mutants of *A. thaliana*. (a) Northern blot analysis of total RNA from wild-type (wt) plants using a 1.2 kb DNA fragment from the *lti78* gene (see Fig. 1) as a probe. The plants were exposed to low temperature (LT), ABA or drought (D) for the number of hours indicated. RNA from untreated control plants is analysed in the lane marked C. Molecular size markers are indicated in kilobases (kb). (b) Northern blot analysis of total RNA from wild-type (wt) plants, the ABA-insensitive mutant (*abi-1*) and the ABA-synthesis mutant (*aba-1*). The probe used was a 450 bp DNA fragment from the *lti65* gene (see Fig. 1). The plants were exposed to low temperature (LT), ABA or drought (D) for the numbers of hours indicated. RNA from untreated control plants is analyzed in the lane marked C. Molecular size markers are indicated in kilobases (kb).

induction was more prominent. Secondly, although the mRNAs of both genes were detectable after four hours in plants treated with ABA (Fig. 6), the *lti78* mRNA level declined upon prolonged ABA treatment, whereas *lti65* mRNA continued to accumulate to a higher level than that of *lti78* mRNA. In contrast to the differen-

tial expression of *lti65* and *lti78* in response to low temperature and ABA, both genes appeared to be similarly induced by drought treatment (Fig. 6).

Signal pathways mediating the induction of lti65 *and* lti78

We have previously demonstrated [26], by employing ABA-insensitive and ABA-deficient mutants of *A. thaliana*, that the induction of the *lti78* gene expression by low temperature, ABA and drought follows three separate signal pathways. In the present study, we determined the expression pattern of *lti65* during the different stress treatments in an ABA-insensitive mutant, *abi-1*, and in an ABA-deficient mutant, *aba-1* (Fig. 6b). Similar to the results obtained with *lti78* [26], the ABA inducibility of *lti65* was abolished in the *abi-1*, but not in the *aba-1* mutant in which the induction with exogenous ABA resulted in the same level of expression as in the wild-type plants (Fig. 6b). However, the low-temperature-induced accumulation of *lti65* mRNA was abolished in *aba-1*, but not in the *abi-1* mutant, in contrast to *lti78* which is low-temperature-inducible in both of these mutants. Maximal drought induction of *lti65* in the wild-type plants appeared during the first two to four hours of treatment. During this time, the drought-induced accumulation of the *lti65* transcript was completely blocked in both mutants (Fig. 6b) and a prolongation of the drought treatment to up to eight hours did not cause any induction in the mutants (data not shown). This expression pattern shows a marked difference to *lti78*, where the drought inducibility is only slightly reduced in the *abi-1* mutant [26] and equal to the wild type in the *aba-1* mutant. Consequently, the requirement for the presence of ABA in the cells and the sensitivity to ABA during low-temperature and drought-induced gene expression appears to differ between *lti65* and *lti78*. In conclusion, these data suggest that the signal pathways mediating the expression of the *lti65* gene are distinct from those utilized for the *lti78* gene.

Discussion

Two stress-induced genes of Arabidopsis thaliana *are structurally related*

Recent cloning and characterization of genes specifically expressed during plant cold acclimation is providing the basis for understanding the molecular mechanism of freezing tolerance. In this paper we present a characterization of two novel but related genes of *A. thaliana*, *lti78* and *lti65*, both of which are inducible by low temperature, ABA and drought. The genes are closely linked, show partial sequence homology and are physically organized in the same way, both having three introns of about 80 to 100 bases at almost identical locations. The polypeptides encoded by the genes are, however, of different length, 710 and 600 amino acids in LTI78 and LTI65, respectively. An alignment of the deduced amino acid sequences (Fig. 5), demonstrated the relatedness of these polypeptides, as 346 of the amino acids are identical.

The homology between *lti65* and *lti78* (Figs. 2, 3 and 4), suggests that the genes belong to the same gene family. This together with the close linkage and the same transcriptional orientation of the genes clearly indicates a common origin and that these genes have most likely arisen from the same ancestral gene via duplication and subsequently diverged through deletions and point mutations. There are several examples of similar clustering of members of a gene family in the genomes [28, 30, 34], including low-temperature or drought-induced genes [15, 24].

The *lti78* gene was shown to correspond to the previously isolated cDNA clone [26]. This gene was previously called *lti140* based on the apparent molecular weight of the corresponding polypeptide obtained by *in vitro* translation of hybrid selected mRNA. However, as we demonstrate here the complete genomic sequence of the gene (renamed *lti78*) predicts a protein with a molecular weight of 77856, which is also in accordance with the size of the *lti78* transcript. The discrepancy between the deduced molecular weight of the protein and its migration in SDS-

polyacrylamide gel eletrophoresis may be due to some unusual secondary structure of the polypeptide, not disrupted by SDS denaturation. This could be reflected in the hydrophilicity and boiling stability of the polypeptide, characteristics shared by several proteins appearing at low temperature [19]. The *lti65* gene product has not been identified with certainty by gel electrophoresis and no information is available about the abundance of the protein in stressed cells.

Regulatory sequences in the 5′ flanking regions of lti78 *and* lti65

The 5′ regulatory sequences of *lti78* and *lti65* have diverged more than the coding regions and may reflect the differential expression patterns of the genes (see below). However, we could identify similar sequence motifs that could be essential for the regulation of expression of both genes. The sequence TACGTGTC which is similar to the ABRE box identified in several ABA-responsive genes [9, 21, 25] exists in the upstream region of both genes, but the distance from the transcription start site is different in the two genes and it also remains to be demonstrated that the sequence is an ABA-responsive element in the *lti78* and *lti65* genes. So far, no specific promoter elements involved in low-temperature regulation of any gene have been identified. Comparison of the *lti78* and *lti65* promoter sequences suggests the presence of a putative, low-temperature-response element with the consensus sequence ACCGACA. This motif is found in four copies in the *lti78* upstream region and once in *lti65*. The different number of copies of this element in the two promoters might explain the fact that *lti78* is much more strongly low-temperature-induced than *lti65*. The same ACCGACA sequence can also be found in the promoter regions of other low-temperature-responsive genes from *A. thaliana*, *rab18* [21], *kin1* and *kin2* [14]. No promoter sequence directly involved in drought induction can be suggested, and a presence of such an element in *lti65* is not sure as the drought response appears to be mediated by ABA (see below).

In addition to the ABA-response element (ABRE) found in promoters of ABA-inducible genes with the consensus sequence (T/C/G)ACGTGGC, related regulatory elements have been found in promoters of several genes. These include the binding sites for different transacting factors, like the G-box factor (CACGTG), AP-1 (TGACTCA), CRE (TGACGTCA), GCN4 (TGACTCA) and ASF-1 (TGACGT(A/C)A) (reviewed by Williams *et al.* [37] and Ziff [38]). The upstream regions of *lti78* and *lti65* contain similar sequences, such as the sequences GACGTAAACGTAA, TGACGTCA and TACGTGTC in the *lti78* promoter and the sequences AACGTGGA, CACGTA, GACGTGGC and TACGTGTC in the *lti65* promoter. The question therefore remains, whether some of these sequences function as specific regulatory elements involved in ABA induction or whether they are binding sites for more general transcription factors.

The lti78 *and* lti65 *genes are differentially expressed by low temperature, ABA and drought*

The expression patterns of *lti78* and *lti65* showed marked differences. Although the expression of both *lti78* and *lti65* was induced by low temperature, drought and ABA, both the responsiveness to these stimuli and the signal pathways employed were different. The results indicate that *lti78* is clearly a low-temperature-induced gene which can also respond to drought and ABA, whereas *lti65* is primarily a drought- and ABA-induced gene with some response to low temperature.

The differential responsiveness of these genes to the stress stimuli was manifested both in the respective expression levels as well as the temporal pattern of expression. Maximal expression of *lti65* required a longer exposure to low temperature or ABA than that of *lti78*. Furthermore, the *lti65* transcript level did not show the same decline as the *lti78* transcript upon prolonged exposure to ABA. These differences in the expression levels may reflect differences both in the regula-

tory elements of the respective promoters and in the stability of the mRNA during the stress.

A remarkable feature in the regulation of the two related genes was that the induction of *lti65* gene expression does not seem to follow the same signal pathways as that of *lti78*. Studies with ABA-deficient (*aba-1*) and ABA-insensitive (*abi-1*) mutants have shown that the low-temperature induction of *lti78* is completely and the drought induction at least partly independent of ABA [26]. This type of pattern has later been confirmed by Gilmour and Thomashow [6] for *lti78* (*cor160* according to their nomenclature) and seems to hold for two other cold-regulated genes as well. In contrast to the expression pattern of *lti78* and other similarly expressed genes, *lti65* was not responsive to low temperature in the ABA-deficient mutant indicating involvement of ABA metabolism in this signal pathway. However, low-temperature induction was normal in the ABA-insensitive mutant, suggesting that a metabolite in the ABA synthesis pathway, rather than ABA itself, is required for mediating the low-temperature response. The expression of *lti65* in response to drought stress was shown to be ABA-mediated. In contrast to *lti78*, the drought-induced accumulation of *lti65* mRNA was completely blocked in both the ABA-deficient and the ABA-insensitive mutant. This ABA-mediated signal pathway is reminiscent of that observed with other drought-responsive genes, e.g. *rab18* of *A. thaliana* [21] and drought-induced genes of tomato and maize [1, 29]. However, the expression pattern of *lti65* is not identical to that of the *rab18* gene where both the low-temperature and drought responses are strictly ABA-mediated. In conclusion, the expression pattern of *lti65* and the signal pathways utilized for mediating the stress responses differ from both previously characterized categories of low-temperature and drought-responsive genes (exemplified by *lti78* and *rab18*, respectively).

This differential expression of two related genes may indicate that they have somewhat different roles in the stress response, e.g. in freeze-induced dehydration tolerance. However, as the LTI78 and LTI65 proteins show no significant sequence similarity to known proteins, any definite function of the proteins in plant cold acclimation cannot yet be suggested.

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