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CbLEA, a Novel *LEA* Gene from *Chorispora bungeana*, Confers Cold Tolerance in Transgenic Tobacco

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A novel late embryogenesis abundant (LEA) gene (AY804193), named *CbLEA*, has now been isolated from *Chorispora bungeana*. This rare alpine subnival plant can survive sudden snowstorms and low temperatures. The full-length *CbLEA* is 842 bp, with an open reading frame encoding 169 amino acids. The putative molecular weight of *Cb*LEA protein is 17.9 kDa, with an estimated *pI* of 6.45. To investigate the functioning of this *Cb*LEA protein in cold-stress tolerance, *CbLEA* was introduced into tobacco under the control of the CaMV35S promoter. Second-generation (R_1) transgenic tobacco plants exhibited significantly increased tolerance to cold. These transgenics maintained lower malondialdehyde (MDA) contents and electrolyte leakage (EL) but their relative water content (RWC) was significantly higher compared with non-transgenic plants under chilling stress. Further experimental results showed that non-transgenic plants had severe freezing damage after exposure to -2°C for 1 h, whereas the transgenics suffered only slight injury under the same conditions. Moreover, survival was longer in the latter genotype at that temperature. The extent of increased cold tolerance was positive correlated with the level of *Cb*LEA protein accumulation, and was also reflected by the delayed development of damage symptoms. This indicates that *CbLEA* is an excellent stress tolerance gene, and holds considerable potential as a new molecular tool for engineering improved plant genetics.

Keywords: chilling stress, *Chorispora bungeana*, cold tolerance, freezing stress, *LEA* gene, transgenic tobacco

The development and survival of plants is constantly challenged by changes in their environment. To tolerate these adversities, plants elicit complex physiological and molecular responses. Many adaptive mechanisms are a consequence of stress perception, and are likely mediated through the stress-induced expression of specific genes, including an important group of late embryogenesis abundant (LEA) genes. These were first identified during the maturation and desiccation phases of seed development in cotton (Dure et al., 1981). LEA proteins are classified into seven groups based on their amino acid sequence homology (Dure, 1993; Ramanjulu and Bartels, 2002). They presumably protect specific cellular structures or ameliorate the effect of drought stress by sequestering ions and maintaining minimum cellular-water requirements (Baker et al., 1988; Dure et al., 1989). In recent years, genetic engineering has been achieved in order to enhance plant tolerance to drought, salinity, and other stresses (Jun et al., 2005; Kim et al., 2007). Overexpression of the *LEA* gene in transgenic rice, Chinese cabbage (*Brassica campestris*), wheat, and tobacco increases tolerance against both water and salt stresses (Xu et al., 1996; Sivamani et al., 2000; Rohila et al., 2002; Babu et al., 2004; Park et al., 2005). Likewise, *LE25*, a Group 5 LEA protein from tomato, improves resistance to high salinity and freezing when expressed in *Saccharomyces cerevisiae* (Imai et al., 1996).

Chorispora bungeana is a representative alpine subnival herbaceous plant that shows great resistance to abiotic stresses, including low temperatures, thin air, high radiation, and strong winds. It inhabits the freeze-thaw tundra along glacial borders where almost all other flowering plants have great difficulty in growing (Ayi et al., 1998; An et al., 2000). Previous research showed that this species possesses no special morphological characteristics that would help it survive under such freezing environments. Therefore, it provides a valuable tool for the cloning and research of stress-related genes and the determination of mechanisms for conferring cold resistance.

Although *LEA* genes have been isolated and studied in many plants (Moons et al., 1997; Shih et al., 2004; Kim et al., 2005; Wang et al., 2006), no examinations have been made in its cloning, molecular characterization, or expression in any alpine subnival plant. It remains unclear whether expression of the *C. bungeana LEA* gene changes in response to abiotic stresses, and the functioning of the *C. bungeana* LEA protein in stress tolerance is another important, unsolved problem. Therefore, in this study, we isolated a novel *LEA* gene from *C. bungeana* and took a transgenic approach with tobacco to investigate the role of that protein in protecting plants against cold stress.

MATERIALS AND METHODS

Cloning of the *CbLEA* Gene from *C. bungeana*

Full-length *CbLEA* cDNA from *C. bungeana* was obtained by reverse transcription polymerase chain reaction (RT-PCR) and rapid amplification of cDNA ends (RACE). Using the DNAMAN program, we designed degenerate primers based on the highly conserved amino acid sequences of LEAs in *Arabidopsis thaliana* (**CAA63006**) and *Brassica napus* (**CAD59382**).

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Briefly, the first fragment of partial *CbLEA* was gained by using primer CbL-F (5'-CAARGCTACAAAGCTGGTGARACM-3') and CbL-R (5'-GCWCCCATWGCCATYTGYTTMA-3'). Afterward, 5' and 3' RACE were performed with a GeneRacerkit (Invitrogen, USA) to produce full-length *CbLEA* cDNA. The *CbLEA* gene was then sequenced from both strands on an ABI 377 Sequencer (Perkin–Elmer, USA). Amino acid sequences were analyzed and multiple alignments were performed with DNAMAN software, and the secondary structure was predicted via SOPMA (Walker and Croteau, 2000; Jaakola et al., 2001).

*Cb*LEA Expression in *Escherichia coli* and Production of Polyclonal Antibodies against *Cb*LEA Protein

*Cb*LEA protein was expressed in *E. coli* with the pET28a expression vector (Novagen, USA). The *CbLEA* cDNA coding region was amplified using ULTma DNA polymerase (Promega, USA) and gene-specific primers containing either the *NcoI* (5'-GCGCCATGGCGTCTCATCAAGAACAG-3') or the *SalI* (5'-GCGGTCGACTCACTTCCTCTGTGTCGCA-3') restriction sites. This amplified *CbLEA* fragment was digested with *NcoI*/*SalI* and ligated into the corresponding sites of pET28a. Integration of the cloned insert was confirmed by automated DNA-sequencing. The resulting pET28a-*CbLEA* was introduced into *E. coli* BL21 (DE3) *plysS* cells and grown in an LB medium under kanamycin selection (50 μg mL^{-1}) at 37°C with vigorous agitation (300 rpm). Recombinant *Cb*LEA protein expression was induced with IPTG. Preparative SDS-PAGE was used to separate the proteins, and the band corresponding to the *Cb*LEA protein was cut from the gel. After freeze-drying, the gel pieces were pulverized and injected into two rabbits. The antiserum obtained was tested by *Cb*LEA protein and shown to be specific for this protein.

Tobacco Transformation

The *CbLEA* cDNA coding region was cloned into pBI121 by replacing the *gus* gene, thereby creating plasmid pBI-*CbLEA*, in which the cauliflower mosaic virus (CaMV) 35S promoter drives expression of *CbLEA* cDNA. Plasmid pBI-*CbLEA* was introduced into *Agrobacterium tumefaciens* LBA4404 by direct transformation (Hofgen and Willmitzer, 1988). Leaf discs of tobacco (*Nicotiana tabacum* cv. Xanthi) were inoculated for 10 min with *A. tumefaciens* that had grown to saturation in an M9 medium (Maniatis et al., 1982). The inoculated discs were then incubated for 2 d on an MS medium (Murashige and Skoog, 1962) at 25°C under a 16-h photoperiod. Afterward, these discs were placed on a shoot-induction medium (MS salts plus 0.01 mg L^{-1} NAA and 2.0 mg L^{-1} 6-BA) that contained 100 mg L^{-1} kanamycin and 200 mg L^{-1} carbenicillin. Young shoots were then transferred to a rooting medium (half-strength MS with 100 mg L^{-1} kanamycin and 200 mg L^{-1} carbenicillin). After two weeks of hardening, the putative transgenic plants were transferred to pots in the greenhouse.

Confirmation of Transgenic Plants by PCR, Real-Time Quantitative RT-PCR, and Western Blot Analyses

We confirmed the presence of these transferred genes into regenerated tobacco plants through polymerase chain reaction (PCR), real-time quantitative RT-PCR, and western blot analysis. For PCR, total plant DNA was isolated via the CTAB method from transgenic and non-transgenic plants (Doyle and Doyle, 1990). Two primers CbL-1: 5'-ATGGCGTCTCATCAAGAACAG-3' and CbL-2: 5'-TCACTTCCTCTGTGTCGCA-3' were used for *CbLEA* gene amplification. The probes amplified 510-bp fragments. This reaction comprised 30 cycles of 40 s at 94°C for denaturing, 40 s at 56°C for annealing, and 1 min at 72°C for extension. The PCR products were analyzed by electrophoresis on a 1% agarose gel. For real-time quantitative RT-PCR, total RNA was extracted from transgenic and non-transgenic plants using TRIzol reagent (Invitrogen), and was reverse-transcribed with oligo(dT) primers, using M-MLV Reverse Transcriptase (MBI) in a 20 μL volume. The primers CbL-3: 5'-GGAGGCTTTGGGAACGATGAG-3' and CbL-4: 5'-GAGTTGTTTGTGCGGCTTGAG-3' were used for amplification of the *CbLEA* gene. Primers for the *GAPDH* gene (AJ133422), which served as an internal reference, included G1: 5'-GGAAAGTCCTACCAGCATTG-3' and G2: 5'-ATCTATTGTCTCCCACGAAG-3'. Real-time RT-PCR was repeated three times for each sample, utilizing SYBR Premix Ex Taq (TaKaRa, Japan) on a Multicolor Real-Time PCR Detection System (Bio-Rad, USA) according to the manufacturer's recommendations. Protein extracts were prepared by grinding the leaves of transgenic and non-transgenic plants in liquid nitrogen, and homogenizing those tissues in an extraction buffer containing 10 mM NaCl, 10 mM Tris-HCl (pH 8.3), and 25 mg mL^{-1} PMSF. The homogenates were centrifuged at 13000 rpm for 20 min at room temperature. Protein concentrations were determined based on the method of Bradford (1976). For the western blot analysis, equal quantities of proteins were separated on a 15% (w/v) SDS-PAGE gel and transferred electrophoretically for 1 h at 100 V to a 0.45-μm nitrocellulose membrane (Hybond-C; Amersham Pharmacia Biotech, USA), without SDS in the transfer buffer. Immunoblotting was performed as described previously (Danyluk et al., 1998), with the anti-*Cb*LEA antibody diluted at 1:1000 and the secondary antibody diluted to 1:5000.

Chilling- and Freezing-Stress Treatments

Seeds of transgenic (R_1) and non-transgenic plants were planted on MS media supplemented with and without kanamycin (100 μg mL^{-1}), respectively. After 16 d, kanamycin-positive green seedlings and non-transgenic green seedlings were placed on MS media for another 12 d. To induce chilling stress, seedlings were transferred to a cold chamber maintained at 2°C for 7 d. For our freezing-stress treatments, these 28-d-old seedlings were divided into six groups. One was for the controls, which remained at 25°C. The other groups were directly exposed to 0, -1, -2, -3, or -4°C.

Malondialdehyde (MDA) Content

The leaves of transgenic and non-transgenic tobacco plants (50 to 100 mg) were homogenized in 3 mL of 10% trichloroacetic acid (TCA), and were centrifuged at 12000 rpm for 10 min. Afterward, 2 mL of 0.6% thiobarbituric acid (TBA) in 10% TCA was added to 2 mL of the supernatant. The mixture was heated in boiling water for 15 min, then quickly cooled in an ice bath. Following centrifugation at 12000 rpm for 10 min, absorbance of the supernatant was

determined spectrophotometrically at 450 and 532 nm. The concentration of malondialdehyde was calculated as: MDA content ($\mu mol\ L^{-1}$) = 6.45 A_{532} – 0.56 A_{450}.

Relative Water Content

Leaf water status was assessed by measuring the relative water content (RWC) and then applying the formula of Weatherley (1950): RWC= (FW-DW)/(TW-DW) × 100, where FW is the fresh weight, DW is the dry weight, and TW is the turgid weight of the tissue after it was soaked in water for 4 h at room temperature.

Electrolyte Conductivity

Leaves were rinsed with distilled deionized water to remove possible ion contamination on their surfaces. Six leaf discs (5-mm diam.) were punched and placed in 20 × 150 mm glass test tubes containing 10 mL of distilled deionized water. These tubes were then shaken at 300 rpm for 4 h in a slanted position. Afterward, the solution was monitored with a conductivity detector (DDS SJ-308A; China). The solutions were then boiled for 20 min to completely lyse the plant cell walls. Electrolyte conductivities of the boiled solutions were recorded as the absolute conductivity. Finally, the percentage of electrolyte leakage (EL) was calculated by dividing the initial conductivity by the absolute conductivity.

RESULTS

Cloning, Sequencing and Characterization of *CbLEA*

The full-length *CbLEA* cDNA of *C. bungeana* comprised

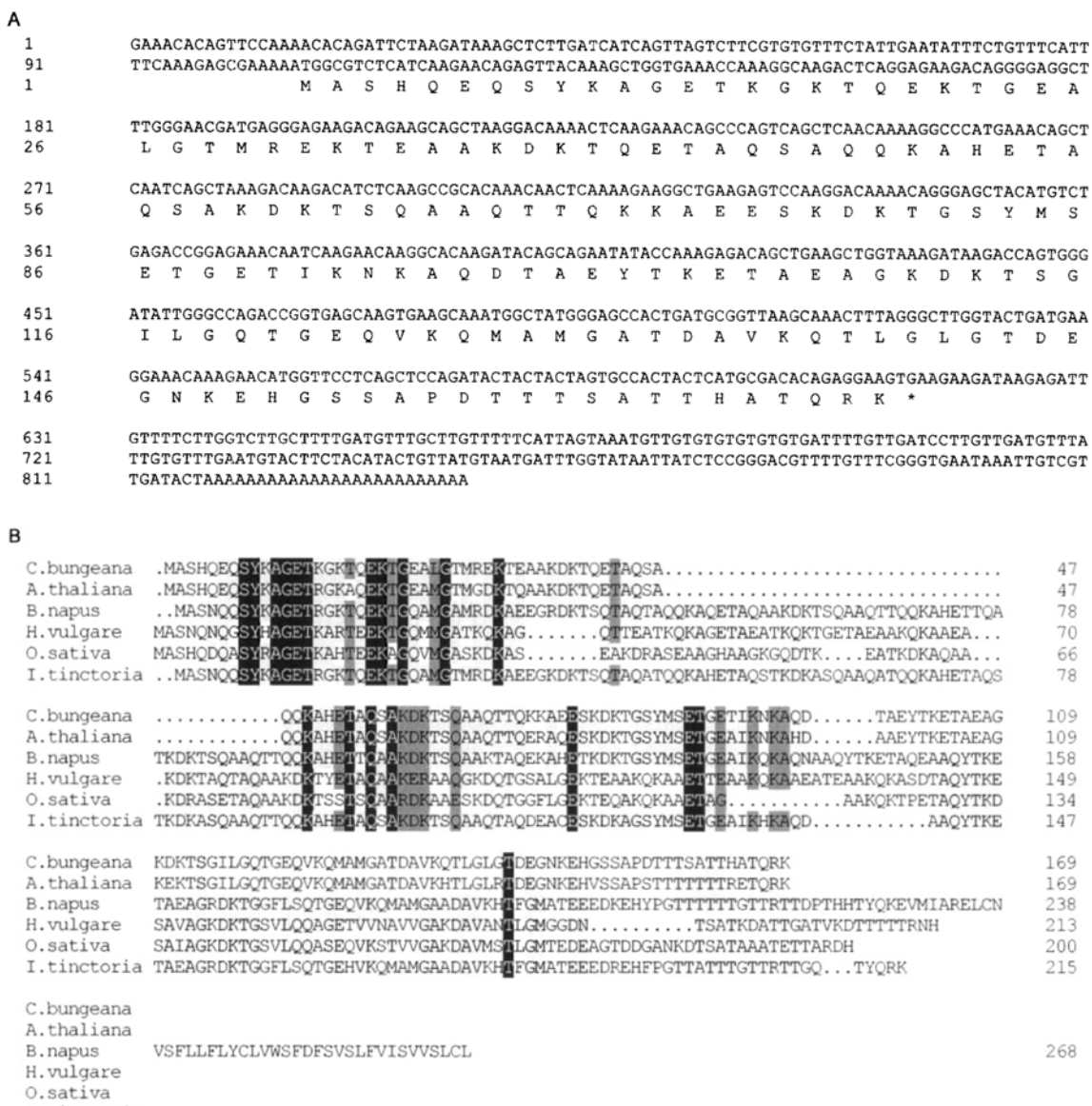

Figure 1. (**A**) Full-length cDNA sequence and deduced amino acid sequence of *CbLEA*. (**B**) Multiple alignment of LEA proteins from different plants. Identities in amino acid sequences between *C. bungeana* CbLEA protein and those of *Oryza sativa*, *A. thaliana*, *B. napus*, *Hordeum vulgare*, and *Isatis tinctoria* were 41.87, 87.57, 42.38, 35.84, and 52.78%, respectively. GenBank accession numbers for these LEA proteins are AAV67892 (*C. bungeana*), AAD02421 (*O. sativa*), CAA63006 (*A. thaliana*), CAD59382 (*B. napus*), S08313 (*H. vulgare*), and AAW59567 (*I. tinctoria*).

842 b, with a 510 b open reading frame (ORF), 105 b in the 5-terminal untranslated region (UTR), and 227 b in the 3-UTR, with a poly(A) tail (Fig. 1A). The ORF encoded a polypeptide of 169 amino acids, with a calculated molecular weight of 17.9 kDa and an estimated *pI* of 6.45. Its cDNA and deduced amino acid sequences have been submitted to the NCBI GenBank with Accession Number **AY804193**. Our BlastP search indicated that the *C. bungeana* LEA protein was most similar to LEA **CAA63006** in *A. thaliana*, showing an amino acid sequence identity of 87.57%. Multiple alignment of LEA proteins from different species revealed that the *Cb*LEA protein shared low similarity with all others except *A. thaliana* (Fig. 1B). This implies that *CbLEA* and the *LEA* of *Arabidopsis* genes are most closely related. The predicted secondary structure for the *Cb*LEA protein (Fig. 2) was highly hydrophilic and contained an 83.43% alpha helix and a 16.57% random coil.

Confirmation of Transgenic Plants by PCR, Real-Time Quantitative RT-PCR, and Western Blot Analyses

Tobacco leaf discs infected with *A. tumefaciens* containing the *CbLEA* gene were selectively cultured on a kanamycin medium. After the plants generated from 15 independent lines were grown for 30 to 40 d, their transgenic status was verified by PCR using *CbLEA*-specific primers. Of these, 12 lines were confirmed to have the expected 510-bp amplified product, whereas the non-transgenic plants showed no bands (Fig. 3).

Expression of *CbLEA* in the leaves of these true transformants was analyzed by real-time quantitative RT-PCR. Relative mRNA expression was calculated according to the formula: relative expression quantity = amount of *CbLEA*/amount of *GAPDH*. Here, we selected 7 from the 12 transgenic lines to continue our experiments. All the tested transgenic plants were confirmed to express the *CbLEA* gene, while the non-transgenics did not (Fig. 4). Moreover, the level of expression varied by line, with plants from Lines 4 and 9 showing the highest levels of transcript compared with only weak expression in Lines 5 and 12. The other three lines (1, 2, and 14) had intermediate levels of expression.

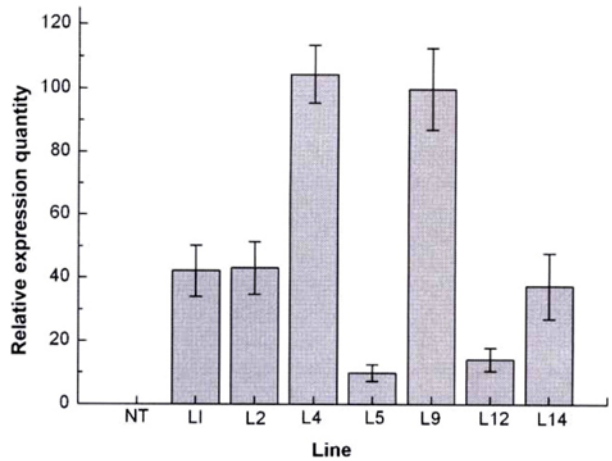

Figure 4. Real-time quantitative RT-PCR analysis of *CbLEA* expression in transgenic lines. NT, non-transgenic plants; 1, 2, 4, 5, 9, 12, and 14, transgenic lines. Each value represents mean of measurements from three individual plants; error bars indicate SE.

Accumulation of *Cb*LEA protein in transgenic leaves was analyzed by western blots (Fig. 5), with non-transformed plants serving as the negative control. All seven transgenic lines showed a hybridization signal, while the negative control did not. These results indicated that *CbLEA* had been integrated into the genome, and they demonstrated the accumulation of *Cb*LEA protein in transgenic plant leaves. Again, the pattern of this accumulation in different lines was similar to that found with *CbLEA* expression.

Chilling Test

To investigate whether constitutive expression of the *CbLEA* gene in tobacco might provide protection against

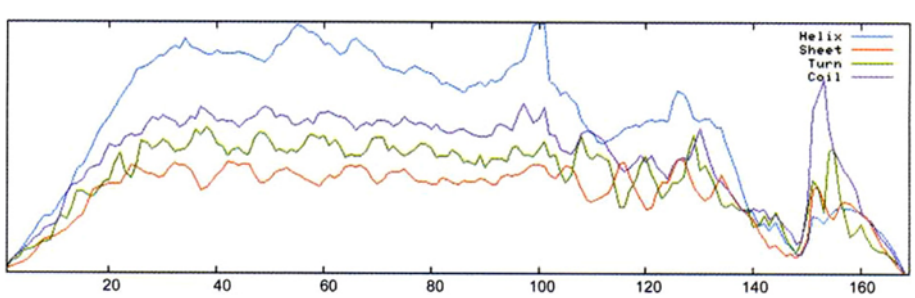

Figure 2. Prediction of secondary structure for *Cb*LEA. Helix, sheet, turn, and coil are respectively indicated by blue, red, green, and peach lines.

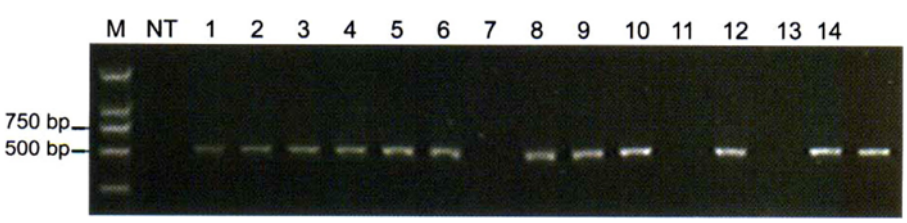

Figure 3. Detection of transformant from R_0 regenerated tobacco plants by PCR, using *CbLEA*-specific primers. M, DL2000 Marker; NT, non-transgenic plants; 1-15, transgenic lines.

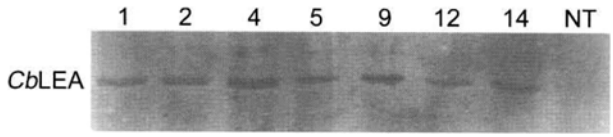

Figure 5. Detection of transformant from R_0 regenerated tobacco plants by western blotting. NT, non-transgenic plants; 1, 2, 4, 5, 9, 12, and 14, transgenic lines.

chilling damage, were measured MDA contents, RWC, and EL in three transgenic lines and one non-transgenic line before and after the stress treatments. Under normal conditions, no differences were calculated in those three parameters among genotypes. However, MDA contents did increase in all plant types after 7 d at 2°C, although this rise was less in the transgenics (Fig. 6). Among the three transformed lines, MDA content was smallest in Line 4, and was 28.4% (P <0.05) lower than that measured in the non-transgenic plants after chilling stress. These results demonstrate that the *CbLEA* gene product functions in protecting the antioxidation system, and plays a role in reducing oxidative damage in transgenic tobacco.

After treatment at 2°C for 7 d, relative water contents decreased in all genotypes. However, the RWC in transgenic Lines 4, 5, and 14 were 23.4 (P <0.05), 4.1, and 16.9% higher, respectively, than that measured in the non-transgenics (Fig. 7). These results suggest that the *CbLEA* gene product possibly prevents water loss and protects against the crystallization of cellular components.

Although electrolyte leakage was significantly increased in all plants after 7 d of treatment at 2°C (non-transgenics, 42.6%; transgenic Line 4, 26.4%; Line 5, 37.0%; Line 14, 30.4%), EL values from transgenic lines were lower than from the non-transgenic plants (Fig. 8). Our analysis of variance showed significant differences, i.e., among the transgenics, the EL for Line 4 was the least, being 38.1% (p <0.05) lower than that in the non-transgenic plants by Day 7. The implication was that the plasma membranes from plants of that line suffered only slight damage. This finding

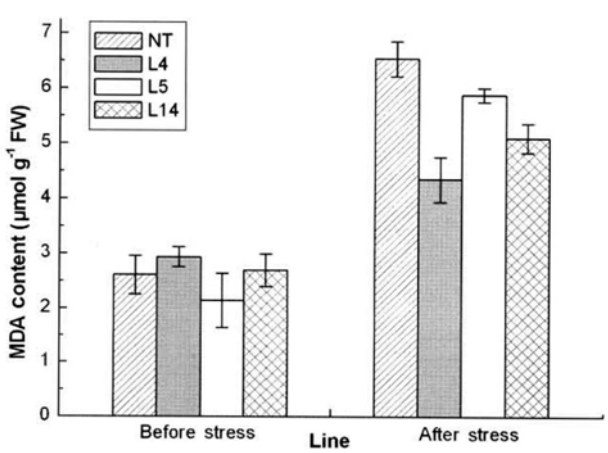

Figure 6. Malondialdehyde content analysis in transgenic and non-transgenic tobacco plants before and after chilling stress. NT, non-transgenic plants; L4, L5, and L14, transgenic lines. Each value represents mean of measurements from three individual plants; error bars indicate SE.

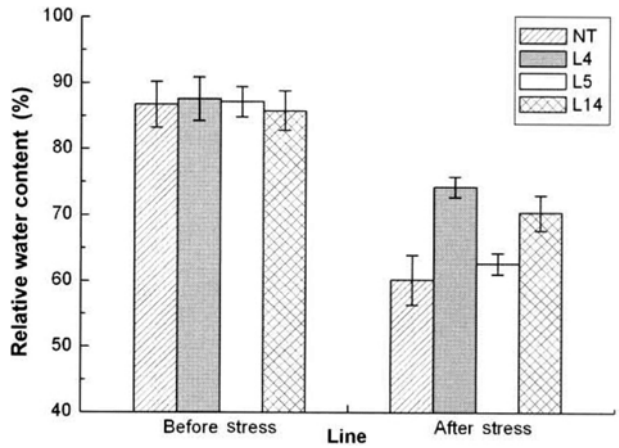

Figure 7. Relative water content analysis of transgenic and non-transgenic tobacco plants before and after chilling stress. NT, non-transgenic plants; L4, L5, and L14, transgenic lines. Each value represents mean of measurements from three individual plants; error bars indicate SE.

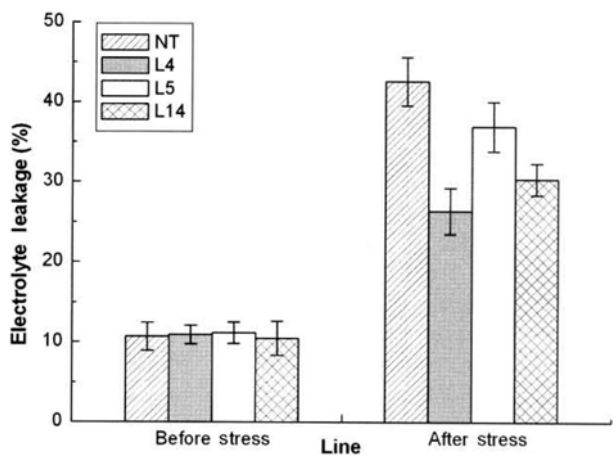

Figure 8. Electrolyte leakage analysis of transgenic and non-transgenic tobacco plants before and after chilling stress. NT, non-transgenic plants; L4, L5, and L14, transgenic lines. Each value represents mean of measurements from three individual plants; error bars indicate SE.

suggests that the *CbLEA* gene product functions in cell membrane protection, and serves to enhance the chilling tolerance of transgenic plants. Phenotypically, those transformed seedlings appeared healthier than the non-transgenic seedlings following chilling (Fig. 9). For example, in the transgenic seedlings, their fourth or fifth leaves had already emerged, while only three leaves were observed on the untransformed plants.

Freezing Test

To determine whether the introduction of *CbLEA* into tobacco protected cells under freezing stress, we performed two freezing tests. Cellular damage due to freezing-induced membrane lesions was estimated by measuring EL from the leaves of treated plants. In the first test, tissues were exposed for 1 h to 0, -1, -2, -3, or -4°C. EL values rose in all plants after 1 h, although the extent of these increases differed significantly (P <0.05) between the one non-transgenic and the three transgenic lines at each temperature (Fig. 10). For

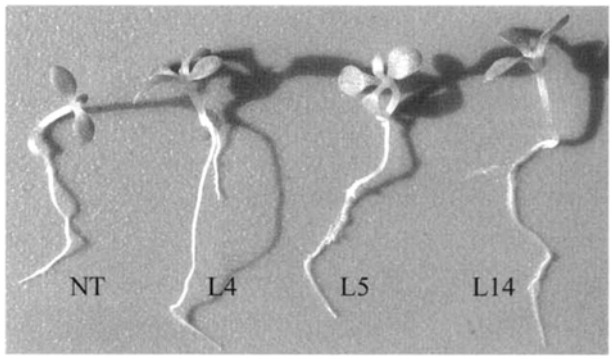

Figure 9. 28-D-old transgenic and non-transgenic seedlings were treated at 2°C for 7 d. NT, non-transgenic plants; L4, L5, and L14, transgenic lines.

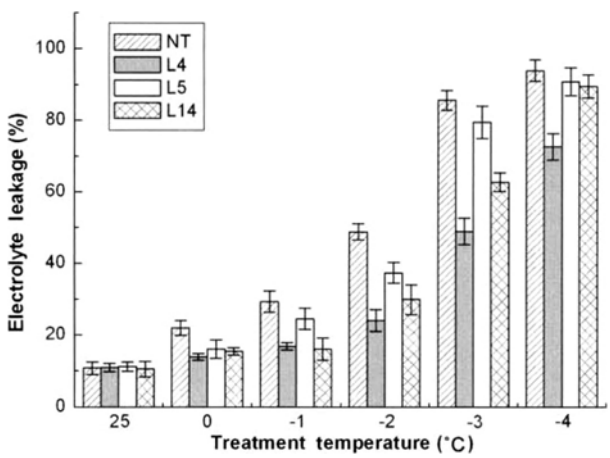

Figure 10. Electrolyte leakage analysis of transgenic and non-transgenic tobacco plants exposed to various temperatures for 1 h. NT, non-transgenic; L4, L5, and L14, transgenic lines. Each value represents mean of measurements from three individual plants; error bars indicate SE.

example, at -2°C, electrolyte leakage from transgenic plants increased to 48.8%, compared with 24.1, 37.3, and 29.9% for transgenic Lines 4, 5, and 14, respectively. Likewise, at -3°C, the EL of Lines 4, 5, and 14 were 42.8 ($P < 0.01$), 7.6, and 26.8% ($P < 0.01$) lower, respectively, than the value determined for the non-transgenic plants. These results show that the non-transgenics exhibited severe freezing damage when exposed to -2°C for 1 h, and that their mortality rate was 100% at -3°C. In contrast, the transgenics were just slightly harmed at -2°C, and plants of Lines 4 and 14 were only partially damaged at -3°C.

In the second test, plants were held at the constant freezing temperature of -2°C for various periods (Fig. 11). At all time points, the transgenic plants had less leakage than the non-transgenics. EL values for Lines 4 and 14 did not exhibit significant increases until after exposure for 4 h at -2°C. However, the non-transgenic plant ELs rose significantly after just 1 h at that temperature. This indicates that those two transgenic lines were only slightly damaged during the first 3 h of freezing treatment, and were able to survive longer than the non-transgenic plants at -2°C. Similar differences in degrees of freezing tolerance were observed in transgenic Line 5, which survived for 1 h.

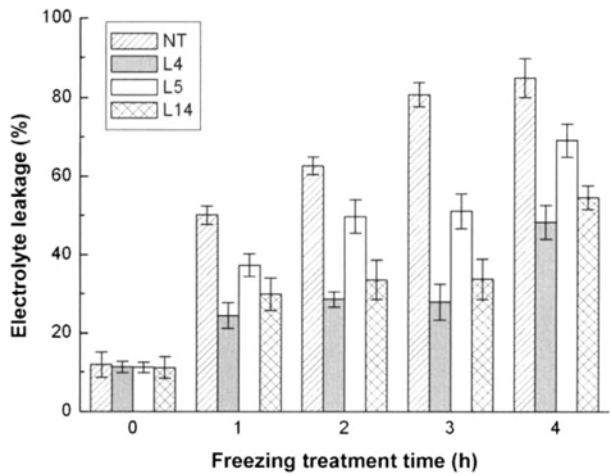

Figure 11. Electrolyte leakage analysis of transgenic and non-transgenic tobacco plants exposed to freezing at -2°C for various times. NT, non-transgenic; L4, L5, and L14, transgenic lines. Each value represents mean of measurements from three individual plants; error bars indicate SE.

DISCUSSION

C. bungeana is a typical alpine subnival plant that survives under frequently fluctuating and freezing temperatures (Fu et al., 2006). Blooming can occur even under acutely low temperatures at elevations greater than 3800 m (Ayi et al., 1998; An et al., 2000). Therefore, this species is valuable for research into the mechanism for cold-hardiness as well as stress-related genetic cloning. Numerous plant genes are induced after exposure to various abiotic stresses (Seki et al., 2001). Members of one such family, containing the *LEA* genes, are frequently expressed in vegetative tissues in response to environmental stresses (Liang et al., 2004). *LEA* genes have been cloned from many plants (Raynal et al., 1990; Moons et al., 1997; Shih et al., 2004; Kim et al., 2005), but their cloning and molecular characterization have not previously been reported in any cryophyte. With this current study, we have now become the first group to isolate a *LEA* gene from *C. bungeana*. Our analysis showed that the *Cb*LEA protein shares only a low level of amino acid sequence similarity with any other LEA proteins, except from *A. thaliana*, in the NR database, thereby indicating that it is a novel *LEA* gene.

Previous studies have demonstrated that overexpression of LEA proteins in transgenic rice, wheat, tobacco, and Chinese cabbage enhances their resistance to drought and salt stresses (Xu et al., 1996; Sivamani et al., 2000; Park et al., 2005; Wang et al., 2006). Moreover, the LE25 protein or hiC6 protein, expressed in yeast, confers improved resistance to high salinity and freezing (Imai et al., 1996; Honjoh et al., 1999). However, no earlier studies have reported that such overexpression in transgenic plants can increase cold tolerance. Like other LEA proteins, the *Cb*LEA protein is highly hydrophilic, and this property may contribute to the increased stress tolerance in transgenic plants with other such proteins (Thomashow, 1998; Hasegawa et al., 2000). Here, we used the parameter of *Cb*LEA protein accumulations as our basis for choosing three transgenic lines. These

were tested to learn whether overexpression of that protein could increase their tolerance against cold stress, with the following trends noted: Line 5, low expression; Line 14, intermediate; and Line 4, high. Under low-temperature conditions, the transgenic R_1 plants maintained the highest RWC (Fig. 7), and had lower values for MDA (Fig. 6) and EL (Fig. 8), compared with the non-transgenics. This indicated that all the plants from these transformed lines exhibited significantly greater tolerance to chilling. However, our results differed from those of Kim et al. (2005), who observed no significant enhancement. This contrast may have been associated with the use of different genetic sources and protein structures. *CbLEA* was isolated from *C. bungeana*, which grows in the freeze-thaw tundra in glacial borders where survival of most other flowering plants is more difficult. Therefore, the *Cb*LEA protein may effectively protect cells against cold stress in that particular species. At the same time, this protein is highly hydrophilic, a property that may contribute to the plant's capability to bind water, guarding its cellular structures under deficits triggered by such stress.

Using a freezing test, we found that tolerance in transgenic plants was also significantly improved. The death rate for non-transgenic cells was approximately 50% after 1 h of exposure at -2°C. In comparison, the transgenics suffered only slight damage under the same conditions (Fig. 10). Values for LT_{50} (i.e., the temperature at which 50% of the cells are killed) were significantly altered in the transformed plants, declining to -3°C. Moreover, the transgenics survived longer than did the non-transgenic plants under constant treatment at -2°C (Fig. 11). These results indicate that *CbLEA* is an excellent stress-tolerance gene, and that its introduction can protect cell membranes and decrease their potential for damage under chilling and freezing stresses. Such an *in vivo* protective role also suggests that *CbLEA* has considerable usefulness as a new molecular tool in the engineering of plant stress tolerance.

When we compared the R_1 plants from different transgenic lines, those in Line 4 were found to accumulate the highest level of *Cb*LEA protein, and they also performed better under chilling stress than those from Lines 5 and 14, as shown by their highest RWC and lowest MDA and EL values. Furthermore, electrolyte leakage from plants of Line 4 was only 48.3% after 4 h of treatment at -2°C. In contrast, transgenic Line 5 had the lowest protein accumulation and RWC values, but the highest levels of MDA and EL, with 49.7% leakage after 2 h of exposure to -2°C. Thus, the enhancement of stress tolerance in R_1 plants seems to be correlated with the accumulation of the *Cb*LEA protein. This conclusion is also in accordance with those of Xu et al. (1996) and Kim et al. (2005).

In summary, we are the first to report the cloning of full-length *LEA* cDNA from *C. bungeana*. We have now demonstrated that the *CbLEA* gene can enhance cold tolerance in transgenic plants. The discovery of this previously unknown member of the *LEA* gene family will enable researchers to explore the unique cold-hardiness mechanism in *C. bungeana*, and also provide a new gene for genetic engineering to improve stress tolerance in plants.

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