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Phylogenetic analysis and seasonal cold acclimation-associated expression of early light-induced protein genes of *Rhododendron catawbiense*

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The early light-induced proteins (ELIPs) are nuclear-encoded, light stress-induced proteins located in thylakoid membranes and related to light-harvesting Chl *a*/*b*-binding proteins. Recent evidence from physiological and genetic (mutant) studies supports a photoprotective function for ELIPs, particularly when green tissues are exposed to high light intensities at suboptimal temperatures. Broad-leaved evergreens belonging to genus *Rhododendron* are often exposed to a combination of low temperatures and high light in their natural habitat as the understory plants in deciduous forests and, therefore, are expected to employ photoprotective strategies during overwintering phase. Here we report analysis and characterization of previously identified ELIP expressed sequence tags (ESTs) from winter-collected *Rhododendron catawbiense* leaves. 5′ or 3′ rapid amplification of complementary DNA ends (RACEs) coupled with bioinformatic analyses were used to identify seven unique *ELIPs* from the 40 ESTs and were designated as *RcELIP1*–*RcELIP7*. Phylogenetic analysis revealed separate clustering of *ELIP* homologs from lower plants, monocots and eudicots (including RcELIPs) and further indicated an evolutionary divergence of ELIPs among angiosperms and gymnosperms. To gain insights into the cold acclimation (CA) physiology of rhododendrons, relative and absolute quantitative expression of *RcELIP*s was examined during seasonal CA of *R. catawbiense* leaves using real time reverse transcriptase–polymerase chain reaction. All seven *RcELIP*s were distinctly upregulated during the CA. It is postulated that RcELIPs expression constitutes an adaptive response to cold and high light in winter-adapted rhododendron leaves and perhaps plays a key role in the protection of photosynthetic apparatus from these stresses.

Introduction

Rhododendrons are among the most important woody landscape plants. Broad-leaved members of this group belong to genus *Rhododendron*, the largest in the heath family (Ericaceae), and comprising approximately 1000 species. Among them, over 800 species are distributed throughout the Northern Hemisphere, ranging from tropical to polar climates and varying widely in their

Abbreviations – CA, cold acclimation; ELIP, early light-induced protein; EST, expressed sequence tag; LT_{50}, leaf freezing tolerance; NA, non-acclimation; PCR, polymerase chain reaction; pI, isoelectronic point; RACE, rapid amplification of complementary DNA end; RT, reverse transcriptase; UTR, untranslated region.

cold hardiness (Leach 1961, Sakai et al. 1986). Woody perennials growing in temperate zones survive harsh winters through cold acclimation (CA), a phenomenon where plants increase their freezing tolerance in response to decreasing day length, low non-freezing temperatures and finally the subfreezing temperatures through the fall and winter (Sakai and Larcher 1987, Weiser 1970). CA is a complex process involving a number of physiological and biochemical changes (Wisniewski et al. 2003), including the changes in gene expression, plant cell wall composition, membrane structure and function and primary and secondary metabolism (Guy 1990, Kaplan et al. 2004, Thomashow 1999).

Our laboratory recently conducted a comparative expressed sequence tag (EST) study using the cDNA libraries prepared from non-acclimated (NA; summer collected) and cold-acclimated (CA; winter-collected) leaves of *Rhododendron catawbiense* (L.) to identify CA-responsive genes in this hardy species; leaf freezing tolerance (LT_{50}) of NA and CA plants were −7°C and −52°C, respectively (Wei et al. 2005). Results from this study indicated that cDNAs encoding early light-induced protein (ELIP) homologs were the most abundant class in the CA library (40 cDNAs randomly picked from 423 ESTs), while no ELIP cDNA was detected among 439 ESTs from the NA library (Wei et al. 2005). Northern blot results, based on one of the *ELIP* transcripts, confirmed that this gene was indeed upregulated in CA leaves of *Rhododendron* (Wei et al. 2005). This was the first report of the upregulation of ELIP genes in winter-acclimated leaves of an overwintering, broad-leaved evergreen.

ELIPs are thylakoid proteins that belong to the Chl *a*/*b*-binding family (Adamska et al. 2001) and are widely distributed among plant species. The mature forms are localized in the stroma lamellae, where they are anchored to thylakoid membranes via three transmembrane domains (Adamska and Kloppstech 1991). Based on the observations that a partially purified ELIP fraction from pea contained Chl *a* and lutein, Adamska et al. (1999) proposed that ELIPs perhaps have a protective function within the thylakoids by binding free chlorophylls released during photoinhibition in high light. Hutin et al. (2003) later demonstrated that the *chaos* mutant of *Arabidopsis*, which is unable to accumulate ELIPs during light stress, suffers extensive photooxidative damage when exposed to chilling and high light. ELIP mutants (*Arabidopsis*) have also been shown to be more sensitive to light stress than the wild-type counterparts (Casazza et al. 2005). Overexpressing *ELIP2* in *Arabidopsis* plants resulted in a downregulation of Chl synthesis pathway, which indicated that ELIPs might work as Chl sensors that modulate Chl synthesis to prevent accumulation of free Chl, and hence prevent photooxidative stress (Tzvetkova-Chevolleau et al. 2007). All these reports suggest that ELIPs play a protective role in plants exposed to high levels of light.

Rhododendron leaves are likely often exposed to a combination of freezing temperatures and high light in their natural habitat as understory evergreen plants in the deciduous forests. Downregulation of the photosynthetic metabolism in overwintering leaves, as noted in our earlier study based on the gene expression data (Wei et al. 2005), could potentially result in the light energy harvested by the leaves to be in excess of what can be processed by photosystems, thus making these plants particularly vulnerable to photoinhibition and photooxidative damage. We had, therefore, hypothesized that the overwintering leaves of rhododendrons should be equipped with a light-stress tolerance or avoidance mechanism and that ELIPs may be one component of this stress response (Wei et al. 2005, 2006).

In this paper, we report analysis and characterization of previously identified (Wei et al. 2005) ELIP ESTs from *R. catawbiense*. 5′ or 3′ rapid amplification of complementary DNA ends (RACEs) and bioinformatic analysis were used to identify seven unique ELIP genes from the 40 ESTs and were designated *RcELIP1*–RcELIP*7*. Because of the remarkably high sequence similarity among the gene members, *RcELIP*s were not classified into subfamilies. Phylogenetic analysis revealed separate clustering of *ELIP* homologs from lower plants, monocots and eudicots (that included *RcELIP*s). To gain insights into the CA physiology of rhododendron, relative expression of *RcELIP* members was examined during the seasonal CA of *R. catawbiense* leaves.

Materials and methods

Plant material, CA treatment, tissue collection and LT_{50} measurements

Leaf tissues for the earlier preparation of non-acclimated (NA) and CA cDNA libraries were collected from the field-grown plants of *R. catawbiense* Catalga plants being maintained at David G. Leach Research Station of The Holden Arboretum (for details, see Wei et al. 2005). NA and CA tissues were sampled during summer (July) and winter (January), respectively, and had a LT_{50} of −7°C and −52°C, respectively (Wei et al. 2005).

To study the seasonal expression profiles of *RcELIP*s, leaf tissues were also collected from the same plantings (at approximately monthly intervals except for the last sampling) during August, September, October, November, December and February. Because the highest and lowest average temperatures in Northern Ohio are in July–August and January–February, respectively, this sampling

period covers three seasons (summer→fall→winter). Relative LT_{50} of the monthly samples was evaluated using a laboratory freeze–thaw protocol and ion-leakage test as described by Lim et al. 1998. A portion of these monthly collections was immediately frozen in the liquid nitrogen and was stored at –80°C until RNA preparation.

Identification of ELIP EST clones

Putative *ELIPs* EST clones from CA cDNA libraries were identified by subjecting stringently edited (high-quality) sequences of unique transcripts as queries to the Protein Information Resource: Non-Redundant Reference protein database, release version. 1.42, and using Standalone BLASTX program, as described in our earlier study (Wei et al. 2005). To obtain the full lengths of several of the partial-length cDNA clones, 5′- or 3′-RACE was used as described in Sambrook and Russell (2001; chapter 8, protocol 9 and 10). The polymerase chain reaction (PCR) products were cloned into pCR2.1 TA vector (Invitrogen, Carlsbad, CA) and the DNA sequencing were carried out at the DNA Facility of Iowa State University.

Sequence alignments and phylogenetic analysis

All the putative RcELIP protein sequences were analyzed based on the translation of cDNA sequences. RcELIP protein sequences were aligned by CLUSTALX (Thompson et al. 1997) and subsequently adjusted manually. Molecular weight (MW) and isoelectronic point (pI) predictions for deduced ELIPs were carried out by Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html, Bjellqvist et al. 1993), chloroplast transit peptide was predicted by CHLOROP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP/, Emanuelsson et al. 1999), and hydrophobic-helic domains were predicted by TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

A data set that consisted of the deduced amino acid sequences from *RcELIP* cDNAs (EF527262–EF527268) and other 19 *ELIP* cDNAs represented by 13 additional taxa was subjected to phylogenetic analysis: *Arabidopsis thaliana* (AF134132 and U89014), *Craterostigma plantagineum* (X66598), *Triticum aestivum* (AB019617), *Glycine max* (U82810), *Pisum sativum* (X05979), *Hordeum vulgare* HV90 (X15692), HV60 (X15691) and HV58 (X15693), *Helianthus annuus* (CAA63338), *Onoclea sensibilis* (AAB25012), *Oryza sativa* (NP_001059030), *Tortula ruralis* (AY034890 and AY034891) and *Dunaliella bardawil* (P27516), *Vitis vinifera* (TC59319), *Pinus taeda* 1 (TC60139), *P. taeda* 2 (TC60140) and *P. taeda* 3 (TC67936); tentative consensus (TC)-based identifiers can be obtained from the TIGR Gene index Databases (http://www.tigr.org/tdb/tgi). Phylogenetic tree analysis was performed with CLUSTALX and MEGA3.1 (Kumar et al. 2004). Unrooted tree, relationship dendrogram was produced by using UPGMA algorithms of MEGAV. 3.1, and the phylogenetic tree was constructed by employing neighbor-joining methods of CLUSTALX and MEGAV. 3.1. Nomenclature based on dendrogram relationship and sequence identity.

Sample preparation for real time PCR

Total RNA was extracted from leaves according to the modified hot-borate method of Wilkins and Smart (1996). The RNA concentration was determined by spectrophotometry, and its integrity was assessed by electrophoresis in 1% (w/v) formaldehyde agarose gels (Sambrook et al. 1989). The residual genomic DNA in the extract was removed by several treatments with RNase-free DNase I (Invitrogen). First-strand cDNA was synthesized using approximately 2 μg of total RNA, 0.5 μg $Oligo(dT)_{18}$ and M-MLV Reverse Transcriptase, according to the manufacturer's instructions (Fisher Scientific, Pittsburgh, PA), employing a PTC-100 Thermal Cycler (MJ Research, Waltham, MA). The cDNAs were diluted to 100 μl with sterile water of which 2 μl was used per real-time PCR sample (Peng et al. 2007).

Real-time PCR conditions

Real-time PCR was carried out in a SmartCycler real-time thermal cycling system using real-time PCR Power Mix Kit (SYBR Green as fluorescent dye, ABI) (Peng et al. 2007). The reaction mixture (25 μl) contained 2 μl of first-strand cDNA, 0.5 μ*M* of each of the forward and reverse primers and appropriate amounts of other components as recommended by the manufacturer (ABI, Foster City, CA). SmartCycler was programmed as follows: 2 min at 95°C for predenature; 40 cycles of 15 s at 94°C, 15 s at 55°C and 20 s at 72°C for each gene. Data were collected during the extension step. Real-time PCR was carried out in triplicates. For control reactions, either no sample was added or RNA alone was added without reverse transcription to test if the RNA sample was contaminated with genomic DNA.

Absolute copy number of individual *RcELIP*s per nanogram total RNA was determined by the standard curves (copy number as a function of CT value) generated using a 10× mass dilution series of normal reverse transcriptase (RT)-PCR products. The copy number of *RcELIP*s was determined based on the size and the mass (ng or μg) of the RT-PCR products. Real-time RT-PCR of the 'test' cDNA samples (which were generated as explained above in another section) was conducted under identical conditions as the standard curve. Absolute copy number of individual

*RcELIP*s was determined by extrapolation of the CT value for 'test' cDNA sample on the standard curve and was expressed as copy number/ng total RNA (mean of three independent measurements) (Fig. 5). One housekeeping ubiquitin gene (*RhUBQ*), expressed at the same level throughout the year, was selected as reference gene to investigate the relative expression profiles of the *RcELIP*s during seasonal CA.

The oligonucleotide primers were designed with the PRIMER EXPRESS v. 2.0 software (Applied Biosystems-Perkin Elmer, Foster City, CA). The sequence of gene-specific primer sets was chosen to ensure that no primer shared more than 70% identity with any other *RcELIP* gene member (Jang et al. 2004). Considering the high sequence homology among *RcELIP* genes, the gene-specific primers were principally designed in the regions corresponding to 5′- or 3′-untranslated region (UTR). The PCR primer sets were also designed to produce the PCR products of about 120–150 bp. To test the suitability of these primer sets, the specificity and identity of the reverse transcription (RT)-PCR products were monitored by a melting curve analysis (65–99°C, 5°C s^{-1}) of the reaction products, which can distinguish the gene-specific PCR products from the non-specific PCR products (Jang et al. 2004). All primers to amplify *RcELIP*s and housekeeping genes were synthesized by Integrated DNA Technologies (Coralville, IA). Forward and reverse primer (indicated as F and R) pairs to amplify *RhUBQ* and *RcELIP*s genes, respectively, are as follows:

RhUBQ – F/R: 5′-AGA GGT GGT GTT GAA CGA TCG-3′ and 5′-TCT CGC ACT TAT TAC CGC ACA-3′; *RcELIP1* – F/R: 5′-CCG CAA CAG ATG TAC TCC TT-3′ and 5′-TCA CCG GGC TCA CCA GAA TG-3′; *RcELIP2* – F/R: 5′-CTC CGA ACA CCT TCC CTC CC-3′ and 5′-CTC CAG CCG AAC AGC AAC AG-3′; *RcELIP3* – F/R: 5′-ATT CGG CAC GAG GAT TTC TT3′ and 5′-TGA CAT GCC AAC CTT GAG AA-3′; *RcELIP4* – F/R: 5′-CGG TGC CCT CAC AAC CTT CG-3′ and 5′-CCA CTG ATG CCG ATA ATG AC 3′; *RcELIP5* – F/R: 5′-CTC ACC GAG TTT GTC AAG GG-3′ and 5′-TTG GGT CCA ACT GCA AGT GT-3′; *RcELIP6* – F/R: 5′-TAT CTC AAA CGC ATC AAT CAAC-3′ and 5′-AGC AAT CGG TAT GGC TGT AT-3′; *RcELIP7* – F/R: 5′-GAA CAT CGG GAT TCA TCA AACT-3′ and 5′-AAG ACA CTA TTC CAC GAC ATAC-3′.

Results

Characterization of *RcELIPs*

In our previous study, 40 cDNAs for ELIPs were picked from the cDNA library of cold-acclimated *R. catawbiense* leaves; in contrast, none were picked from the NA EST library (Wei et al. 2005). In the present study, a series of 5′-RACE or 3′-RACE was used to obtain the full lengths of several of the partial-length cDNA clones that comprised the 40 ESTs (data not shown). After filtering the redundant and partial sequences of these 40 ESTs, seven unique transcripts containing typical *ELIP* domains could be identified as *RcELIP* genes (Table 1). The open reading frame (ORF) of *RcELIP*s encode

Table 1. Description of seven ELIP sequences found in the cDNA library prepared from cold-acclimated leaves of *R. catawbiense*. Accession numbers of RcELIPs nucleotide sequences are listed. The number of deduced amino acids residue, the protein pI and the molecular weight of each RcELIP protein are calculated.

Given name	EST no. and accession no.	Number of deduced amino acid	pI	Molecular weight (kDa)
RcELIP1	Z01-H11X (CV015023), Z02-E02X (CV015066)	181	9.03	19.2
RcELIP2	Z03-D05X (CV015148), Z02-E05X (CV015069); Z03-G06X (CV015182), Z03-F09X (CV015173)	181	9.00	19.2
RcELIP3	Z01-E12X (CV014989), Z01-F10X (CV014999), Z03-C02X (CV015134), Z03-E09X (CV015162), Z05-E04X (CV015329)	181	9.41	19.0
RcELIP4	Z02-B04X (CV015036); Z03-H07X (CV015193), Z03-G05X (CV015181)	164	9.69	17.3
RcELIP5	Z02-E12X (CV015076), Z02-F10X (CV015084), Z05-D02X (CV015317)	177	9.67	18.5
RcELIP6	Z02-G06X (CV015092), Z01-H06X (CV015018); Z04-D09X (CV015241), Z04-E03X (CV015246); Z05-E07X (CV015332); Z01-H10X (CV015022), Z04-H08X (CV015283), Z04-H10X (CV015285), Z05-B11X (CV015303), Z02-H07X (CV015105)	182	9.86	19.0
RcELIP7	Z04-E05X (CV015248), Z03-D03X (CV015146), Z04-H01X (CV015276)	194	9.8	20.4

deduced polypeptides of 164–194 amino acids, with a predicted molecular mass of 17.3–20.4 kDa and the pI of 9–9.8 (Table 1).

Alignment of the deduced RcELIP protein sequences revealed that each of the mature RcELIP contained a 46–49 amino acid long chloroplast transit peptide and three hydrophobic helic domains (Fig. 1). As another common characteristic, each RcELIP was also identified as a Chl *a/b*-binding protein by Conserved Domain BLAST (Search Service, v2.11 http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Phylogenetic analysis of RcELIPs

The distance method resulted in one tree using the alignment of full lengths of all the RcELIPs. *RcELIP*s are highly homologous with an average of 86.3% identity among these seven gene members; the divergence between each member was lower than 35%. The percent identity is 98% between RcELIP1 and RcELIP2, 97% between RcELIP3 and RcELIP4 and 91% between RcELIP5 and RcELIP6, while the lowest percent identity (73.5%) is between RcELIP1 and RcELIP7. Therefore, RcELIPs were categorized into subfamilies and were named as RcELIP1–RcELIP7. Bootstrap tests were performed to evaluate the stability of phylogeny; results of 1000 replicates are shown in Fig. 2.

Deduced amino acid sequences of RcELIPs and ELIP-like proteins from other plant species were analyzed and compared to examine the structural relationship. An unrooted neighbor-joining cladogram is depicted in Fig. 3.

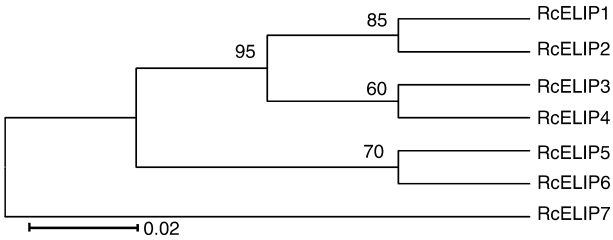

Fig. 2. Bootstrap majority-rule consensus tree of RcELIPs generated with the distance method. Numbers above the lines represent bootstrap percentages (based on 1000 replicates). A bootstrap value of 100% indicates branches that were supported in all replicates of resampling of data. Branches with a bootstrap value of less than 50% are collapsed. The scale bar indicates the number changes per unit length.

Relationships shown in this gene tree generally reflect standard groupings of monocots, eudicots, ferns and green alga. Obviously, RcELIPs fall within eudicot cluster rather than with other species; within this group, however, they were closer to flowering plants than to conifers, indicating evolutionary divergence between angiosperm and gymnosperms.

Relative expression profiles of the *RcELIPs* during seasonal CA

All seven *RcELIP*s were distinctly upregulated (by several fold compared with the NA levels) during the seasonal development of CA in *R. catawbiense* leaves (Fig. 4).

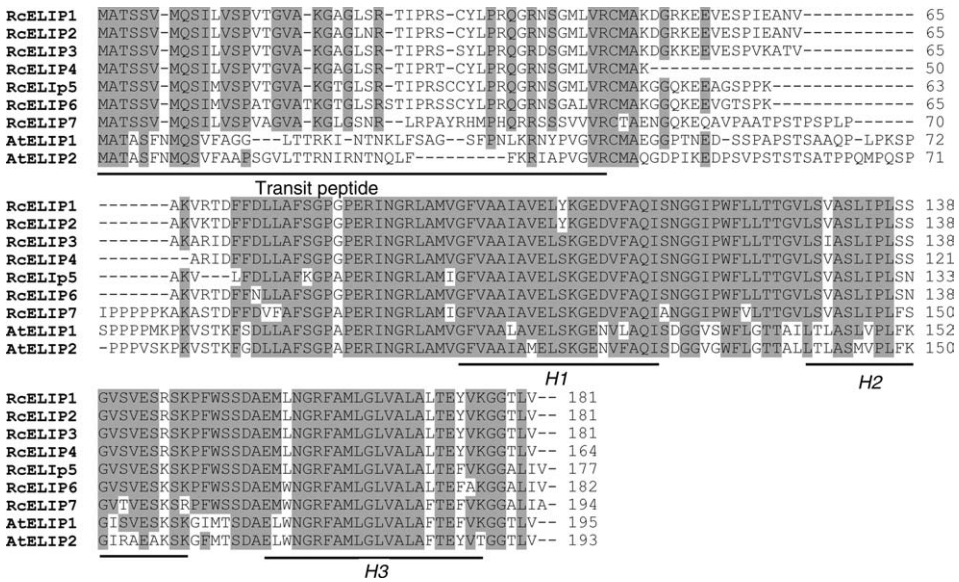

Fig. 1. Alignment of the deduced amino acid sequences of ELIPs from *R. catawbiense* (RcELIP 1–7) and *Arabidopsis* (AtELIP1 and 2). A predicted transit peptide and three hydrophobic helices (H1, H2 and H3) are indicated. A conserved Chl *a/b*-binding domain (from approximately 75–170 amino acid) was contained in each RcELIP.

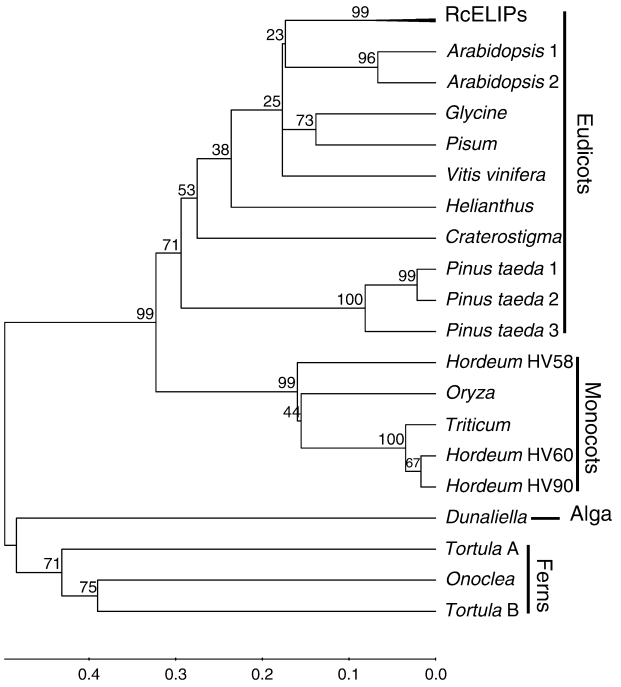

Fig. 3. Unrooted neighbor-joining tree derived from a data set of deduced amino acid sequences for *R. catawbiense* ELIPS and other 19 ELIPs represented by 13 different taxa. Numbers above the lines represent bootstrap percentages (based on 1000 replicates). A bootstrap value of 100% indicates branches that were supported in all replicates of resampling of data. See Material and methods for species names and accession numbers. The scale bar indicates the number changes per unit length.

While small or no change in the expression was observed from August to September, a significantly increased expression for most *RcELIP*s was observed from September onwards peaking in December or February, when their expression was approximately 5–8-fold of that in NA (August-collected) tissues. In our previous study, one of the *ELIP*s, designated as *RcELIP3* in the current study, was observed to be upregulated in CA leaves of *R. catawbiense* by Northern blotting (Wei et al. 2005).

Quantitative expression, as copy number, of *RcELIPs* in cold-acclimated (December-collected) leaves

For an accurate amplification of each specific target gene among the highly homologous *RcELIP* family, primer sets for each of the seven *RcELIP*s were carefully designed based on the sequences corresponding to 5′- or 3′-UTR region, as described in Materials and methods. The PCR products were verified by melting curve analysis, and by DNA sequencing, if necessary. The real-time RT-PCR data were analyzed to calculate the absolute transcript levels as described in Materials and methods. Fig. 5 shows gene expression, as the copy number per nanogram of total RNA, for all *RcELIP*s from the leaves collected in December; these samples represented fully cold-acclimated tissues with the LT_{50} of approximately −50°C. Real-time RT-PCR analysis was repeated three times (in duplicates), and the histograms represent the mean values and SES (bar) of three independent runs.

The seasonal expression levels varied considerably among the seven *RcELIP*s, based on which they were classified into three groups. *RcELIP6* had the highest expression with more than 3000 copies per nanogram of total RNA. The low-expression group includes *RcELIP1* and *RcELIP7* with no more than 750 copies, while *RcELIP2*, *RcELIP3*, *RcELIP4* and *RcELIP5* belong to the moderate-expression group with 1000–2000 copies.

Discussion

In the present work, we identified and characterized seven *ELIP* genes from a 423 EST library generated from cold-acclimated *R. catawbiense* leaves; we determined their relative expression profiles during a seasonal change

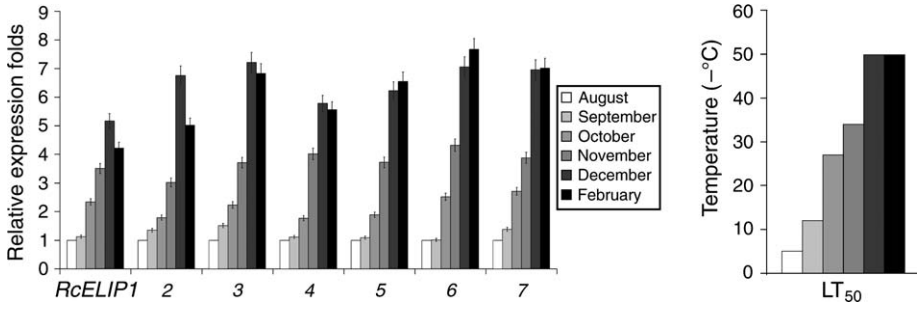

Fig. 4. Seasonal expression profiles of *RcELIP*s. Total RNA from leaf tissues of field-grown *R. catawbiense* collected (at approximately monthly intervals) in August, September, October, November, December and February was used for cDNA synthesis. The expression level of each *RcELIP* was plotted relative to their expression in August (taken as 1). LT_{50} for August, September, October, November, December and February samples are −5°C, −12°C, −25°C, −34°C, at least −50°C and at least −50°C, respectively.

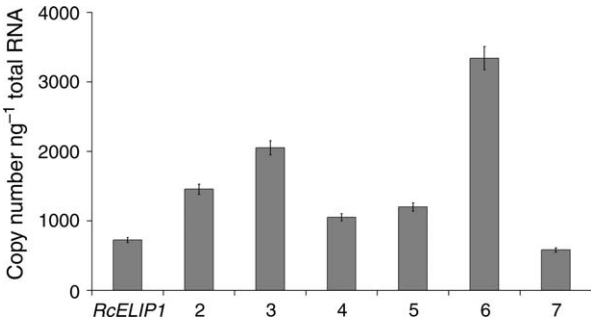

Fig. 5. Absolute quantitative expression (copy number) of seven *RcELIP*s in cold-acclimated (LT_{50} = approximately −50°C) *R. catawbiense* leaves. Total RNA was isolated from the December-collected leaf samples. The transcript level of each *RcELIP* gene is given as molecular copy numbers in 1 ng of total RNA. The data are mean values and SE (bar) of three independent runs (in duplicates).

(August to February) in the LT_{50} as well as the absolute expression (as the copy number) of individual *RcELIP*s in winter-collected, fully cold-acclimated tissues.

The highly conserved amino acid sequences, similar molecular weights and the narrow range of theoretical pI suggest an allied function for *RcELIP*s (Table 1). *RcELIP*s were shown to share many common characteristics with their homologs across other plant species, such as having one transit peptide, three hydrophobic helices and a Chl *a/b*-binding protein motif (Fig. 1). However, a relatively larger gene family size (at least seven members) and a relatively smaller than mostly reported protein size are some of the unique features of RcELIPs. ELIP families were found to have no more than three members for the species surveyed in this study including monocots, eudicots, ferns and green alga. Based on the deduced amino acid composition and molecular weight, RcELIP4 is the smallest member, while RcELIP7 is the largest; molecular weight of other members is approximately 19 kDa, which is somewhat smaller than that for most other species (data not shown). With regards to ELIPs, a distinct evolutionary divergence seems to exist between *Rhododendron* and other species used for comparison in this study (Fig. 3). A high amino acid sequence similarity (91–98%) between the RcELIPs pairs 1 and 2, 3 and 4 and 5 and 6 raises a curious possibility that perhaps these might be allelic pairs rather than separate genes, especially given the heterozygous nature of the rhododendron genotype. However, this cannot be confirmed because of the lack of requisite data.

Expression analysis showed all *RcELIP*s to be active genes (Figs 4 and 5). During the seasonal increase in LT_{50} from summer to winter, the expression of all *RcELIP*s was significantly upregulated. This is the first report to our knowledge on the seasonal upregulation of *ELIP* genes and its association with CA in an evergreen woody perennial. Considering the potential protective function of ELIPs during photoinhibition under high light and/or against photooxidative damage, the inducible expression of ELIPs during CA particularly in overwintering green tissues, such as rhododendron leaves, is a reasonable expectation. ELIPs have also been found, however, to be among the most highly induced genes in CA *Arabidopsis* leaves (Fowler and Thomashow 2002) and blueberry buds (Dhanaraj et al. 2004).

Our observations that *ELIP* genes are upregulated in winter-collected samples are in line with the recent findings by Zarter et al. (2006) at the protein level. Using immunoblots, these authors reported a distinct accumulation of ELIP proteins and other PsbS relatives in the winter-collected leaves of bearberry (*Arctostaphylos uva-ursi*), also a broad-leaved evergreen; essentially no ELIP protein was detected in the summer-collected leaves in their study. Moreover, ELIP accumulation was greater in sun-exposed and high-altitude ecotypes than in the shade-adapted and lower-altitude ones. Similar to our previous report on rhododendron (Wei et al. 2005), Zarter et al. (2006) too noted a downregulation of photosynthesis in winter-collected leaves of bearberry, particularly in high-altitude ecotype, and suggested that ELIPs and other PsbS relatives may protect photochemically inactive leaves of bearberry against the winter sun.

The photoprotective role of ELIPs has been suggested on the basis of the following evidence: (1) enhanced expression related to conditions manifesting photoinhibition and/or photooxidative stress, (2) localization in the stroma-exposed thylakoids, the site of the repair of damaged photosystems, (3) a putative binding capacity for chlorophylls and carotenoids and (4) the observation that *chaos* (a mutant of chloroplast signal recognition particle system required in the rapid targeting of ELIPs to thylakoid membranes) is more photosensitive to high light and chilling than its isogenic wild-type counterpart and that its photosensitivity is rescued by constitutive ELIPs expression (Hutin et al. 2003). However, using an inverse genetic approach (*elip1/elip 2* double mutant of *Arabidopsis*), Rossini et al. (2006) have questioned the protective role of ELIPs against photoinhibition and photooxidation but hypothesized that ELIPs accumulation might still be beneficial to plants under light stress via stabilization of, and/or interaction with, the xanthophyll cycle pigments (zeaxanthin), a suggestion put forth earlier by others as well (Ensminger et al. 2004, Montane and Kloppstech 2000, Zarter et al. 2006).

Zeaxanthin accumulation has been highly correlated with an increased proportion of the absorbed light energy being dissipated as heat (Bjorkman and Demmig-Adams 1994), which helps plants to avoid photooxidation. Plants

that routinely experience high-light stress are known to have larger xanthophyll cycle pools (Königer et al. 1995, Thayer and Bjorkman 1990) and several evergreen species, including rhododendrons, indeed increase these pools during winter months (Adams and Demmig-Adams 1994, 1995, Harris et al. 2006, Zarter et al. 2006). Possibly, as chloroplast-localized proteins, RcELIPs could maintain a hydrophobic environment within the stroma that promotes zeaxanthin stabilization in response to high light. It is postulated that the RcELIPs represent an adaptive response to stress-induced photodamage within rhododendron chloroplast. Further research to confirm the subcellular localization of these polypeptides and investigate their ability to protect and/or repair the photochemistry in overwintering rhododendrons is underway. Expression data as per the copy number (Fig. 5) indicated that among the seven *ELIP* members, *RcELIP6* was the most abundantly expressed in winter samples, perhaps suggesting its greater functional significance than other *RcELIP*s.

The upregulated expression of *RcELIP*s during the seasonal change is perhaps a long-term adaptation to extreme stress conditions. Clearly, further studies will be necessary to prove or disprove whether RcELIPs have any functional relevance to the persistent pool of zeaxanthin. As a first step, however, our study of *RcELIP* genes and family characteristics and their seasonal expression profiles in *R. catawbiense* provides an informational foundation for investigating the physiological functions of ELIPs in woody evergreens' winter tolerance.

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