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Distinct Lhc mRNA stabilities in several vascular plant species

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Summary

The light harvesting complex protein genes (*Lhc*) were among the first isolated and characterized from vascular plants. Despite the accumulated knowledge about their steady-state mRNA levels in various organs/tissues and under certain light qualities and regimes, limited information is available regarding their transcription rates, and nothing is known about *Lhc* mRNA stabilities. Steady-state mRNA accumulation patterns under constant conditions suggested distinct *Lhc* mRNAs stabilities in various plant species. Decay studies with actinomycin D revealed half-life times of 3.8 hours in *Lycopersicon esculentum* (tomato), 2.5 hours in *Spinacea oleracea* (spinach), 24 hours in *Pinus sylvestris,* and 37 hours in *Ginkgo biloba*. The presence of ‹AUUUA›-destabilizing elements in the 3′ UTR, free energy calculations of secondary structures, and simultaneous expression of the tomato and *P. contorta Lhc* gene in transgenic tomato all support the notion that the intrinsic sequences of the transcript contribute significantly to the distinct *Lhc* mRNA stabilities present in different plant species.

Key words: *Ginkgo* – light harvesting complex protein genes (*Lhc*) – *Lycopersicon* – mRNA half-life times – mRNA stability – *Nicotiana* – *Pinus*

Introduction

The primary reaction in photosynthesis depends on the presence of chlorophyll *a/b* binding proteins in the antenna systems of photosystems I and II in the chloroplast membranes. Because of their importance, the genes encoding these proteins, designated *Lhc* (formerly *cab*), were isolated from various plant species and their structures, organizations and steady-state levels of mRNAs were determined. A complex set of factors has been unraveled that can influence time-, light-, tissue- and organ-dependent *Lhc* mRNA accumulation (Gilmartin et al. 1990, Piechulla 1999). However, transcript accumulation patterns only reflect steady-state levels and do not allow conclusions about the dynamic processes that may be involved, such as transcription or RNA turnover. Regulation at the level of transcription was manifested for the *Lhc* genes (e.g. Guiliano et al. 1988, Meyer 1993, Wehmeyer et al. 1990), yet still, very little is known about the turnover and stability of the *Lhc* transcripts in plants.

In recent years, research on RNA decay in plants has focused mainly on the identification and characterization of

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structural features of the mRNA molecule or on cis-acting elements. These studies show that general structural elements found at the ends of virtually all mRNAs, as well as specific sequence elements located within a transcript, might contribute to the overall stability. Sequence elements that could control inherent mRNA stability in plants are i) the DST (ATAGAT and GTA) and DST-like elements derived from the 3′ UTR of unstable SAUR (**s**mall **a**uxin-**u**p **R**NA), 15A, and SAURAC1 transcripts, respectively; ii) the A/U rich instability 3′ UTR element (AUUUA repeats); iii) the internal light responsive element from ferredoxin 1 gene present in the 5′ UTR and the coding region; and iv) the alpha-amylase3 hairpin/loop structures (reviewed in Gutierrez et al. 1999). Elucidation of mRNA decay pathways in plant cells is one critical aspect in understanding the molecular basis of RNA stability. To date, yeast is the only eukaryotic organism in which mRNA decay pathways have been extensively dissected (summarized in Gutierrez et al. 1999). Two main pathways are postulated, the deadenylation/decapping-dependent and the deadenylation-independent decay pathway. In the deadenylation-dependent pathway, the polyA tail is removed prior to the exonucleolytic cleavage of the transcript, while the other pathway does not depend on the primary elimination of the polyA tail. In addition to these possibilities, two other pathways are proposed for plants, i) soybean SRS4 type, where a stochastic endonuclease cuts up a transcript and the resulting fragments are the substrates for exonucleolytic cleavage; and ii) decay during post-transcriptional gene silencing (PTGS), involving possible RNase L-like or RNase III-like enzymes (reviewed in Gutierrez et al. 1999).

The availability of transcripts in cells is crucial for translation efficiency and protein accomplishment, and is therefore responsible for the variation in amount of given proteins present at different developmental stages of the plant. In our previous investigations, we determined *Lhc* transcript levels in various plant species at different time points during the day, and a distinct difference could be observed. In 48 out of 49 *Lhc* genes investigated from 23 angiosperm plant species (reviewed in Piechulla 1999), a diurnal and/or circadian expression pattern could be observed. Within this group of plants, the *Lhc*b1*3 gene of *Arabidopsis thaliana* is the only known exception where no oscillations of the steady-state mRNA levels could be detected, and such mRNA patterns were proposed to be the result of post-transcriptional events (Millar and Kay 1991). In contrast to the general observations made with the angiosperm *Lhc* genes, constant expression levels were detected in four gymnosperm (Pinopsida) species, derived from the *Picea, Pinus, Abies,* and *Larix* genera (Oberschmidt et al. 1995). These results were the first hints that different modes of regulation exist for *Lhc* gene expression in gymnosperm and angiosperm plant species. To elucidate these differences in more detail, we investigated *Lhc* mRNA stabilities in various species.

Material and Methods

Plant growth and harvest of plant material

Seeds of *Pinus sylvestris* were obtained from the Staatliches Forstamt (Munster-Oerrel, Germany). *P. sylvestris* and *Nicotiana sylvestris* were germinated and grown for eight weeks in the greenhouse. *Ginkgo biloba* seeds obtained from Carter Seeds (Vista, California, USA) were germinated and grown for one year in the greenhouse. The young plants were transferred into a growth chamber for 5 to 11 days (light: 6 a.m. to 8 p.m., 20 °C, 120 µmol/m^2 sec) prior to incubation in continuous darkness for 15 days. The last day in light/dark conditions is day 0 in Figure 1. At the indicated days in darkness, leaves were harvested at 11 : 30 a.m. and stored at –70 °C until further use.

RNA extractions and hybridizations

RNA of *Pinus* and *Ginkgo* was extracted according to Chang et al. (1993). RNA of *Nicotiana* and *Lycopersicon* was isolated following the protocol of Piechulla et al. (1986). RNA was separated on formaldehyde agarose gels and standardized to rRNA. Gels were blotted onto nylon filters (Hybond N from Amersham, Braunschweig, or membrane positively charged from Roche, Mannheim, Germany) (Sambrook et al. 1989). The following gene probes were used for hybridisations (Figs. 1 and 2): the *Lhc*b1*2 (tomato) 0.55 kbp HincII-PvuII fragment (Piechulla et al. 1986), the oligonucleotide (5′-GCT CCG CCT TGG CCT CGG GGT CGT CGG CCA-3′) of the *Pinus sylvestris Lhc* gene (Jansson and Gustafsson 1990), the oligonucleotide (5′-TAG TCT CCC GGA AAT TCT CCC GTC AGA TAT-3′) of the *Ginkgo biloba Lhc*b1*1 gene (Chinn and Silverthorne 1993), and the 18S/28S rDNA (8.7 kb, pHA2, Jörgensen et al. 1981). Probes were radioactively labeled using the random priming method or the 5′ kinasing protocol (Sambrock at al. 1989). Digoxygenin-dUTP labeling was performed according to the manufacturer's protocol (Roche, Mannheim, Germany). Blots were hybridized at 60 °C with 5 × SSC or performed as described in Kellmann et al. (1996). Blot of Figure 4 was i) hybridized with the 5′ kinased *Lhc*-oligonucleotide (5′-AAG AGA TAA GCC AGG CCC G-3′) of *P. contorta;* ii) Digoxygenin-dUTP labeled *Lhc*b1*2 fragment of tomato; and iii) with Dig-dUTP labeled 305 bp pcr fragment of the 18S rDNA of tomato. To quantify mRNA hybridizations, absolute counts were determined using the Instant Imager (Canberra Packard, software program: electronic autoradiography, version 2.01 1995, Packard Instrument Co.). Relative mRNA levels (mRNA/rRNA) were calculated (Figs. 2 and 4) to allow the calculation of the half-life times (Table 1).

Determination of RNA stability

Young tomato plants (4 weeks old, light 7 a.m. to 7 p.m.) and *Pinus sylvestris* seedlings (4 to 7 weeks old, light 6 a.m. to 8 p.m.) were cut above the soil at 8 : 30 a.m. and immediately incubated in scintillation vials containing buffer (15 mmol/L sucrose, 1 mmol/L KCl, 1 mmol/L Pipes, 1 mmol/L sodium citrate, pH 6.5) or buffer plus actinomycin D (200 µg/mL) (Klaff and Gruissem 1991). At the indicated time points, leaves were harvested, and the plant material was frozen in liquid nitrogen and stored at –70 °C until further use. Total RNA was extracted, and hybridizations were performed as described. Linear regression of the data points allowed the calculation of the half-life times.

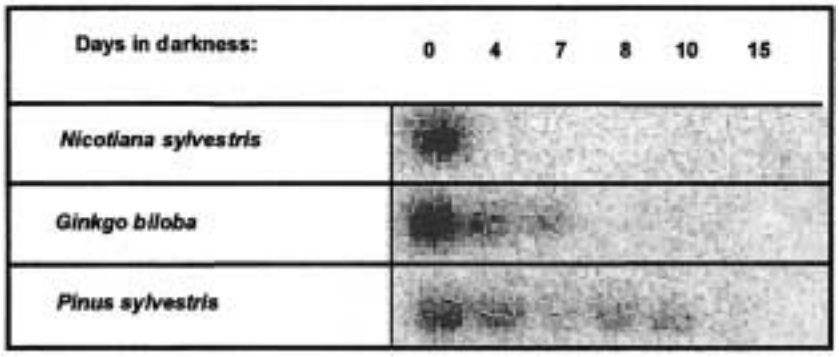

Figure 1. Decay of *Lhc* mRNA levels in continuous darkness. *Nicotiana sylvestris, Ginkgo biloba,* and *Pinus sylvestris* were grown in the greenhouse, and transferred for 5 to 11 days in light/dark cycles to a growth chamber prior to the incubation in continuous darkness. Total RNA was extracted from leaves harvested at 11 : 30 at day 0 (last day in light/darkness) and 4, 7, 8, 10, and 15 days in darkness, and hybridized with gene-specific probes.

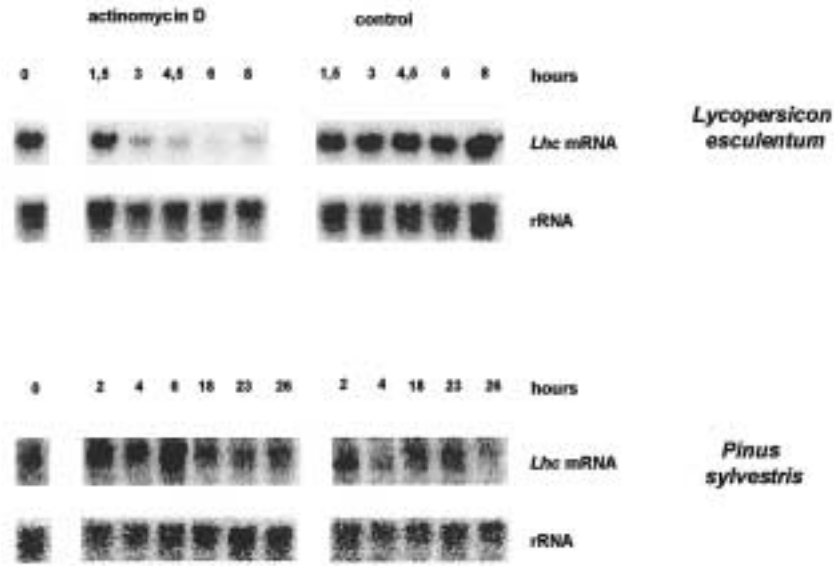

Figure 2. *Lhc* mRNA stability in *Lycopersicon esculentum* and *Pinus sylvestris*. Young tomato plants (4 weeks) and pine tree seedlings (4 to 7 weeks) were cut above the soil and immediately incubated with or without 200 µg/mL actinomycin D at 8 : 30 a.m. (= 0 hours). Leaves were harvested at indicated time points, total RNA was extracted, and Northern blots were hybridized. Relative mRNA/rRNA levels and half-life times of the *Lhc* mRNAs were calculated (Table 1).

mRNA-structure calculations

Predictions of thermodynamically optimal secondary structures of RNA were performed with LinAll (Schmitz and Steger 1992, Steger et al. 1984). The ionic strength of the calculations (1 mol/L NaCl) was extrapolated to cellular conditions by subtracting 15 °C from the calculated temperatures.

Vector constructions and plant transformation

The *Lhc* gene (including the 5′ UTR, the coding region, and the 3′ UTR, Barrett et al. 1993) from *P. contorta* was cloned into the SmaI/EcoRI restriction sites of the pBI121 vector, replacing the gus coding region and the nos terminator using standard cloning procedures (Sambrook et al. 1989). The gene was placed under the control of the CaMV 35S promoter. The plasmid was transformed into *Agrobacterium tumefaciens* C58C1 (pGV2260). After selection on rifampicin (150 mg/L), kanamycin (50 mg/L), and ampicillin (100 mg/L), leaf disks of tomato plants (*L. esculentum,* cv. Moneymaker) were inoculated with the respective *A. tumefaciens* strain (Horsch et al. 1985). Transgenic plants were selected on MS Medium (Murashige and Skoog 1962) containing 3 % sucrose, 1 mg/L zeatin, 35 mg/L kanamycin, and 250 mg/L betabactyl. Leaves from transgenic tomato plants (28 and 40 weeks after transformation, respectively) were cut above vermiculite and immediately incubated with or without 200 µg/mL actinomycin D.

Results and Discussion

In complete darkness, *Lhc* mRNAs were only detectable for three days in tomato leaves (Piechulla 1999 and references therein). Following this observation, we investigated the *Lhc* mRNA decay in three other plant species, *Nicotiana sylvestris, Ginkgo biloba,* and *Pinus sylvestris* (Fig. 1). Whereas no *Lhc* transcripts were detectable in tobacco after four days in complete darkness, *Lhc* mRNAs could be observed for 7 or 10 days in the two gymnosperm species. This result suggested that *Lhc* mRNA transcript decay rates vary greatly in different plant species.

Limited information is available regarding the influence of light on mRNA stabilities. Light regulation at the post-transcriptional level has only been characterized to some extent for the photosynthetic electron carrier ferredoxin I gene. The mRNA half-life time in transgenic tobacco seedlings is two-fold higher in light versus in darkness, indicating that light regulation occurs through a change in mRNA stability. A sequence element spanning the 5′ UTR and 20 codons of the coding region is responsible for the light responsiveness. Furthermore, the Fed mRNA accumulates to a higher extent on polyribosomes in light, which correlates with increased mRNA stability (summarized in Gutierrez et al. 1999).

In addition to the *Lhc* mRNA steady-state level observations under constant darkness, further support for our conclusion was obtained from decay studies in the presence and absence of actinomycin D, a potent inhibitor of RNA polymerases. Incubation of rootless *L. esculentum* and *P. sylvestris* plants, and leaves of *S. oleracea* and *G. biloba,* in actinomycin D solution revealed half-life times from 2.5 h to 37 h that were specific for each species (Fig. 2, Table 1). Furthermore, the *Lhc* mRNA half-life times in the gymnosperm species were 10-fold or more higher than those in the angiosperm species. Based on this result, it is conceivable that the different *Lhc* mRNAs turnover rates contribute to the different transcript accumulation patterns previously observed since low transcript stability would be a prerequisite for diurnal or circadian mRNA oscillations.

To find the reasons for the different half-lives of the *Lhc* mRNAs in gymnosperm and angiosperm species, we analyzed *Lhc* mRNA structures from *P. contorta* and *L. esculentum*. Structural calculations were performed at 40 °C to extrapolate cellular conditions to the ionic strength of 1 mol/L NaCl. The thermodynamically optimal structures are shown in Figure 3. Both mRNAs can form a variety of long-range interactions based on their sequences. ΔG_o for the *P. contorta Lhc* mRNA is $-969.2\ kJmol^{-1}$ at a length of 987 nucleotides, that of the *L. esculentum Lhc*b1*2 mRNA is $-812.7\ kJmol^{-1}$ at a length of 1108 nucleotides. The free energy per nucleotide based on the maximal number of base pairs that can be formed is 30 % higher in the *Pinus Lhc* mRNA than in tomato, indicating a higher overall structural stability. However, it must be taken into account that under cellular conditions, mRNA structures may be formed sequentially, not according to their lowest free energy.

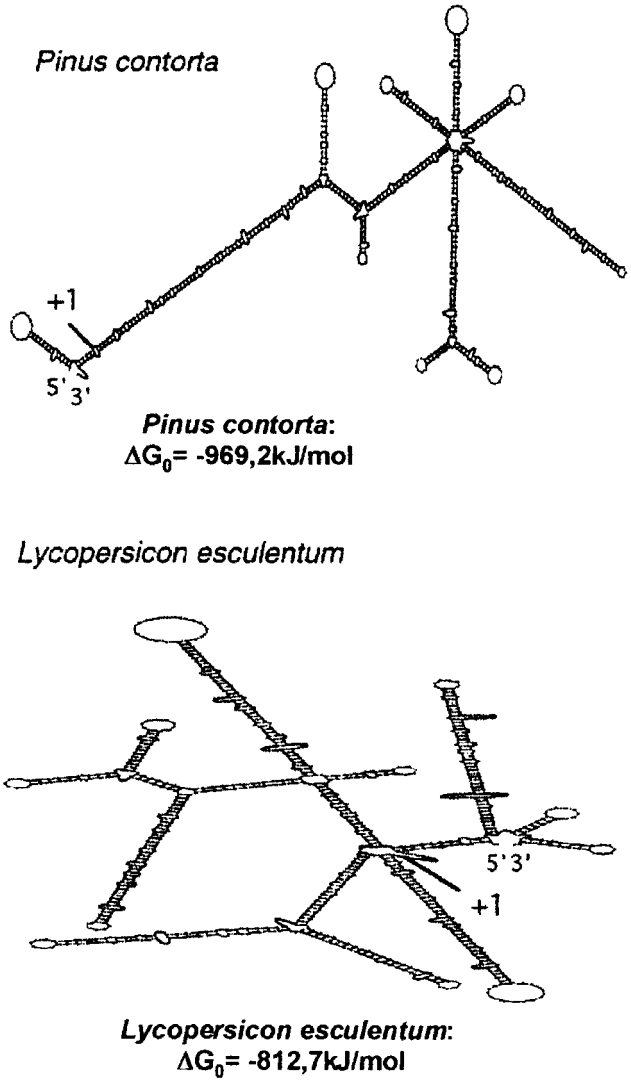

Figure 3. Predicted secondary structure of *L. esculentum* and *P. contorta Lhc* mRNA. The structures of the *Lhc* mRNA from *P. contorta* and the *Lhc*b1*2 mRNA from tomato were determined according to Schmitz and Steger (1992) at 40 °C, 1 mol/L NaCl. The translation start site is labeled with +1. ΔG_o for the *P. contorta Lhc* mRNA is −969.2 kJ/mol, while ΔG_o for the *L. esculentum Lhc* mRNA is −812.7 kJ/mol.

Table 1. Determination of *Lhc* mRNA half-life times in different plant species.

plant species	
Lycopersicon esculentum	3.8 h
Spinacea oleracea	2.5 h
Pinus sylvestris	24 h
Ginkgo biloba	37 h

To determine whether it is the nucleotide sequence of the transcript or the differences in cellular RNase activity that account for the different decay patterns of *Lhc* mRNAs in the examined plant species, we transferred the *P. contorta Lhc* gene (63 nt of 5′ UTR, 823 nt of the coding region, and 309 nt of the 3′ UTR) into the *L. esculentum* (cv. Moneymaker) genome via *Agrobacterium tumefaciens*. Experiments with and without actinomycin D allowed the simultaneous determina-

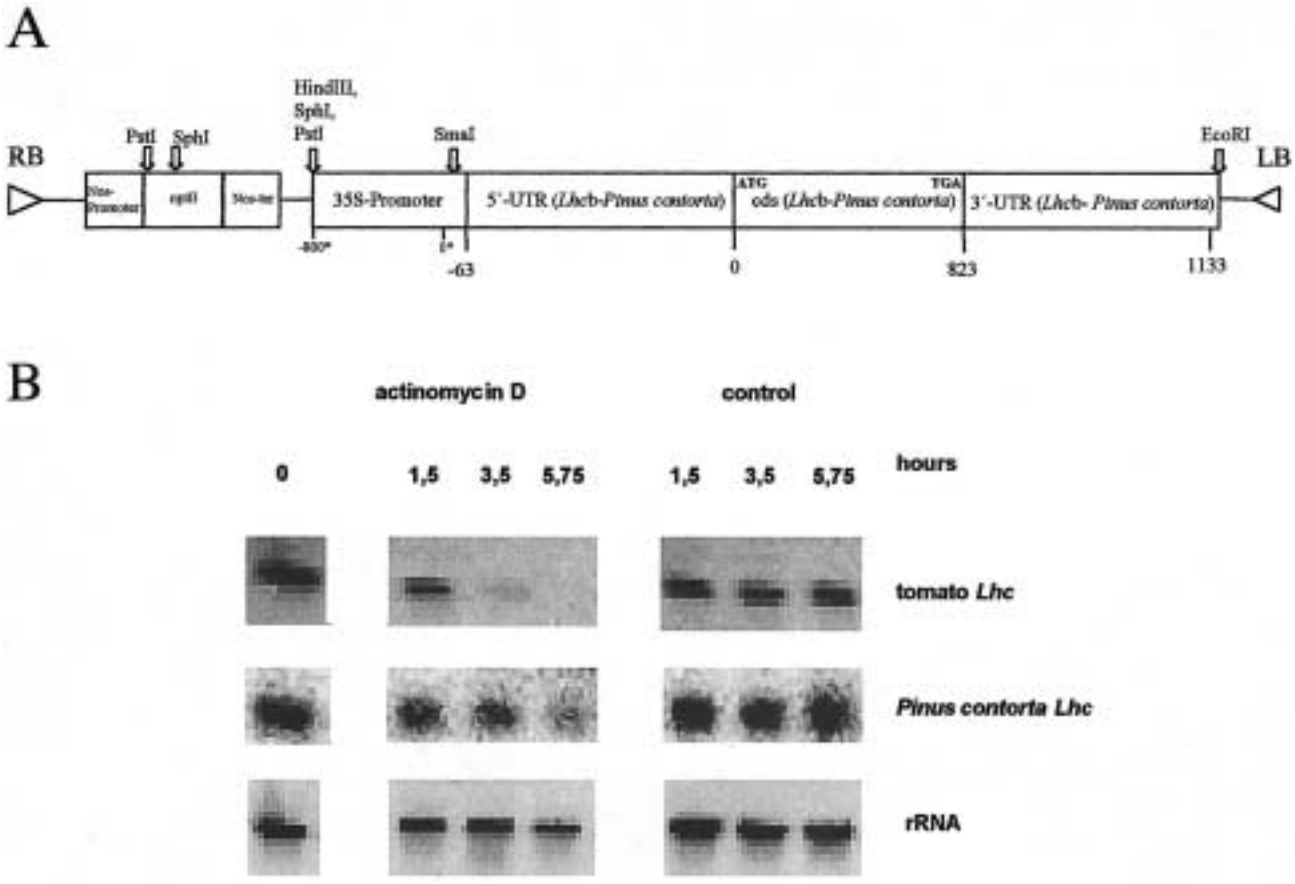

Figure 4. Determination of *Lhc* mRNA transcript stabilities of the tomato and *Pinus Lhc* gene in transgenic tomato plants. **A:** The *P. contorta Lhc* gene construct in the modified pBI121 vector, cds: coding sequence, UTR: untranslated region, 0*: transcription start site of the 35S promoter, 0: translation start site of the P.c.-*Lhc* gene, RB: right border, LB: left border. **B:** Leaves from transgenic tomato plants were cut and immediately incubated with or without 200 µg/mL actinomycin D. Leaves were harvested at indicated time points (two independent experiments), total RNA was extracted, and 3–5 Northern blots of each sample set were hybridized. Relative mRNA/rRNA levels (at least 6 values for each time point) and half-life times of *Lhc* mRNAs were calculated (Table 1).

tion of the half-life times of the mRNAs transcribed from the *Pinus* transgene and the tomato endogenous gene (Fig. 4). The transgene *Pinus Lhc* mRNA turned out to be approximately 10-fold more stable than the endogenous *Lhc* mRNA (5.4 h of the *P. contorta Lhc* transcript, 2.9 h and 7.9 h in two independent transgenic lines), 0.5 h of the *L. esculentum Lhc* transcript (0.4 h and 0.5 h in two independent transgenic lines). This result suggests that indeed nucleotide and sequence differences of the transcripts lead to the altered mRNA stabilities.

For sequence comparisons, the *P. contorta* and the *L. esculentum Lhc*b coding sequences, as well as the 3′ and 5′ untranslated regions, were aligned (Fig. 5). Overall, the nucleotide sequences of both transcripts are 64 % identical; the 5′ UTRs are 40 %, the coding regions are 72 %, and the 3′ UTRs are 47 % identical. The 3′ untranslated regions of the tomato *Lhc*b1*2 mRNA contain two ‹AUUUA› destabilizing elements while none was detected in the short 3′ UTR of the *P. contorta* mRNA. These ‹AUUUA› sequence motifs have been shown to confer instability on mRNAs in plants and animals (Chen and Shyu 1995, Ohme-Takagi et al. 1993, reviewed in Gutierrez et al. 1999). Furthermore, sequence alignments of all published 3′ UTRs of *Lhc* genes from different plant species revealed that 82 % of them contain such destabilizing elements, and it is very likely that the presence or absence of this motif contributes to the different *Lhc* mRNA half-lives in the different plant species.

It has become clear that our knowledge of the mechanisms for temporal, spatial, and environmental regulation of gene expression is particularly limited in gymnosperms, despite the fact that a large number of laboratories are aiming to define the structure and organization of genes in pine trees. It is also clear that the mechanisms of gene regulation are distinct in gymnosperms and angiosperms, and it would

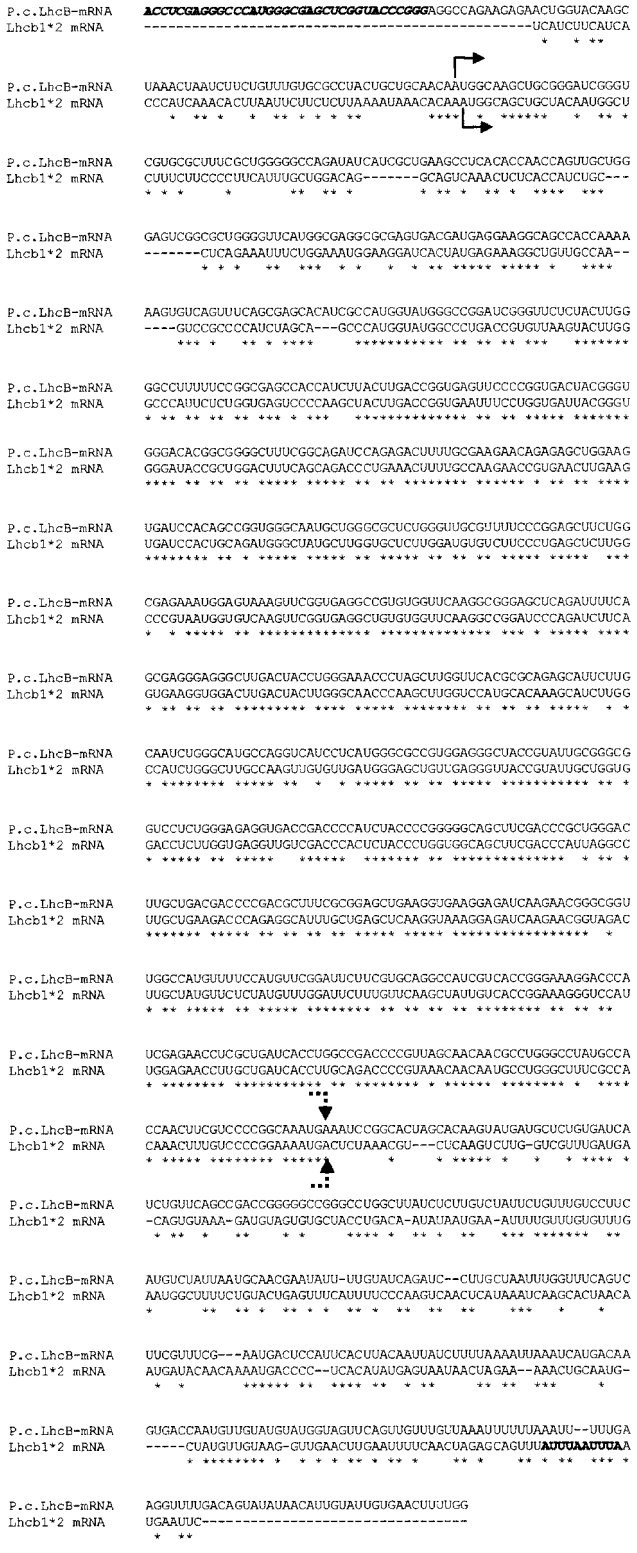

Figure 5. Sequence comparison of the tomato and *Pinus contorta Lhc*b1*2 genes. The coding and 3′ and 5′UTR sequences of the *Lhc* mRNA from *P. contorta* (P.c.*Lhc*B) and *L. esculentum* (*Lhc*b1*2) were compared and found to exhibit 64 % overall nucleotide sequence identity. The 5′UTR, 3′UTR and the coding regions share 40 %, 47 %, and 72 % sequence identity, respectively. The translational start site (AUG) (filled arrow) and stop site (UGA) (broken arrow) are indicated. The transcription start site of the 35S promoter is shown in bold and italics. In the 3′UTR, the ‹AUUUA›-destabilizing elements are shown in bold face.

therefore be very fruitful to investigate gene regulation processes in gymnosperms in more detail in the future.

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