Isolation and Characterization of a Type I Gene Encoding a Light-harvesting Chlorophyll *a/b*-binding Protein of Photosystem II in Peach

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ABSTRACT. A nuclear gene, *Lhcb1*Pp1*, encoding a light-harvesting chlorophyll *a/b*-binding protein of photosystem II has been isolated from peach [*Prunus persica* (L.) Batsch. 'Stark Earliglo'] leaf genomic DNA, cloned, and sequenced. This gene encodes a precursor polypeptide of 267 amino acids with a transit peptide of 34 and a type I mature protein of 233 amino acids. The amino acid sequence of the mature polypeptide is 89% to 94% and 80% to 94% similar to those encoded by type I *Lhcb* genes of annual and other woody plants, respectively. In contrast, the amino acid sequence of the peach transit peptide is less conserved being 47% to 69% similar to those of annual plants and only 17% to 22% similar to those of other woody plants. The peach gene was used as a probe for *Lhcb* gene expression. *Lhcb* mRNA is detected in leaves of field-grown trees during June to October. *Lhcb* mRNA is detected at a high level in leaves of peach shoots grown in tissue culture in the light, but only at a trace level in leaves grown in the dark. Some *Lhcb* genes appear to be light-modulated in stems. *Lhcb1*Pp1* contains four potential polyadenylation sites. S1 nuclease analysis detected transcripts of the sizes expected from each of the four polyadenylation sites. All four are found in leaves of light-grown shoots and of field-grown trees throughout the growing season. In contrast, only three are detected in stems of light-grown shoots.

Environmental cues play critical roles in plant growth and development. The most studied of these cues is light. Among angiosperms, many genes have been identified whose expression is regulated by quantitative and qualitative changes in light (e.g., Tobin and Silverthorne, 1985). Certain fundamental mechanisms of the regulation of gene expression by light are being deduced from studies on annual plants (Gallie, 1993; Hankamer et al., 1997). Other processes, however, such as recurring cycles of photosynthesis and growth, are unique to perennial plants (Hutchinson et al., 1990; Hutchinson and Greenwood, 1990). Before the molecular basis underlying the control of recurring annual cycles by light, for example, can be productively investigated, basic information concerning light-regulated gene expression in perennial plants must be obtained. Little such information, however, is available.

Among the best characterized of the light-regulated genes in annual plants are those designated as light-harvesting chlorophyll *a/b*-binding protein genes, *Lhcb* (Jansson et al., 1992). These genes encode the apoproteins of the major light-harvesting complex (LHC-II) as well as three or four minor light-harvesting complexes associated with photosystem II (Green and Durnford, 1996; Hankamer et al., 1997). *Lhcb* genes exist as a multigene family in the nucleus. Those encoding the apoproteins of LHC-II are subdivided into three groups, types I, II, and III, depending on the

presence or absence of introns (Buetow et al., 1988) and the presence of signature amino acids in the deduced polypeptide sequences (Demmin et al., 1989). The products of these genes play a critically important function in photosynthesis by binding to and ensuring the proper orientation of chlorophylls and carotenoids in the light-harvesting complex thus facilitating rapid energy transfer between these pigments (Grossman et al., 1995; Kühlbrandt et al., 1994; Lee and Thornber, 1995). In contrast to annual plants, *Lhcb* genes have been investigated in only a few perennial plants (and mainly in gymnosperms). Many gymnosperms, unlike the usual annual angiosperm plant, strongly express *Lhcb* genes in the dark (Alosi et al., 1990; Canovas et al., 1993; Gustafsson et al., 1991; Kojima et al., 1992; Yamamoto et al., 1991). However, in another gymnosperm, *Ginkgo biloba* L., the organ-specific expression of certain *Lhcb* genes is light-dependent (Chinn et al., 1995). In the case of perennial angiosperms, information on *Lhcb* genes is especially lacking for the economically important fruit-bearing trees. So far, only a type I *Lhcb* gene in apple (*Malus ×domestica* Borkh.) (Chen et al., 1990) and recently a type II in peach (*Prunus persica* (L.) Batsch.) (Bassett et al., 1998) have been isolated and sequenced. The present study reports the first type I *Lhcb* gene isolated from peach. Further, this gene was used to characterize *Lhcb* expression in peach leaves during an annual growth cycle and in different organs of the peach following growth in the light and in the dark.

Materials and Methods

DNA AND RNA PREPARATION. A total genomic DNA library in lambda phage (EMBL3; Stratagene, La Jolla, Calif.) was prepared from peach leaves (*P. persica* 'Stark Earliglo') as described earlier (Chung et al., 1994).

Total RNA was prepared from fresh leaves collected early in the morning from first, second or third nodes of succulent shoots from 8-year-old peach trees growing in the field at the Pomology Research Center of the Univ. of Illinois (Urbana). Leaves were washed with dH₂O, frozen immediately in liquid N₂, and stored at -70 °C until used. Total RNA was isolated from 10 g frozen leaves

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by the method of Ausubel et al. (1990) which was modified as follows: leaves were ground to a fine powder in liquid N₂ using a mortar and pestle, mixed with 150 mL TLE buffer (200 mM Tris-HCl [pH 8.2], 100 mM LiCl, 5 mM ethylenediaminetetraacetic acid [EDTA, pH 8.0]) plus 50 mL TLE-equilibrated phenol, homogenized 1 min at full-speed in a Waring blender (model 700B), mixed thoroughly with 50 mL chloroform, made 1% in sodium dodecyl sulfate (SDS), incubated for 30 min at 50 °C, and centrifuged for 10 min at 10,000 g_n at 4 °C. The clear supernatant was precipitated for 2 h with 1.0 vol isopropanol at -20 °C and centrifuged for 30 min at 10,000 g_n. The pelleted RNA was resuspended with 60 mL TLE and 20 mL 8 M LiCl, precipitated overnight at 4 °C, collected by centrifugation at 15,600 g_n for 30 min, reprecipitated in 30 mL TLE and 10 mL 8 M LiCl at 4 °C for 2 h, collected by centrifugation at 15,600 g_n for 20 min, resuspended with 5 mL buffer (10 mM Tris-HCl and 1 mM EDTA, both at pH 8.0), divided into 500 µL aliquots, precipitated with two times the volume of cold (-20 °C) ethanol, and stored at -70 °C.

TISSUE CULTURE. Proliferating shoot cultures of *P. persica* 'Evergreen' were cultured for 2 weeks on a Murashige and Skoog (1962) medium containing 0.02 µM indole-3-butyric acid (IBA) and 10 µM 6-benzyladenine (BA) at 22 ± 1 °C under a regime of 16 h of light (cool-white fluorescent, 90 µmol·m⁻²·s⁻¹) followed by 8 h of darkness. Half of the cultures (six jars) were wrapped with aluminum foil for dark-growth while the other half were kept in the light. After 2 weeks, cultures were harvested, and leaves and stems were separated, frozen immediately in liquid N₂, and stored at -70 °C until used for total RNA isolation as described above.

ISOLATION, CLONING, AND SEQUENCING PEACH *Lhcb*. A probe (pAB96) consisting of an 833 bp fragment of pea (*Pisum sativum* L.) *Lhcb1** cDNA (Coruzzi et al., 1983) was radiolabeled overnight at 37 °C by random primer extension using a labeling kit (Ambion, Austin, Tex.) and 5 µL [α -³²P]-dCTP (10 mCi·mL⁻¹, 3000 Ci·mmol⁻¹; Amersham, Arlington Heights, Ill.). Peach genomic-library phage were grown on an agar plate, cross-linked to a Hybond-N membrane (Amersham) according to the manufacturer's protocol, and screened overnight at 42 °C with the probe in a solution containing 6× sodium chloride-sodium citrate buffer (SSC), 5× Denhardt's solution, 0.5% SDS, 0.5 µg·mL⁻¹ fragmented salmon sperm DNA and 50% formamide. Positive plaques were verified as containing peach *Lhcb* gene sequences by two further screenings. A 1.9 kb *Sst*I peach DNA fragment of a positive plaque was cloned in both orientations in pBluescript IKS⁺ (Stratagene, La Jolla, CA) and sequenced in both directions by the method of Sanger et al. (1977) using a Sequenase II kit (United States Biochemical, Cleveland, OH), T3 or T7 primer depending on the sequencing direction, and [α -³⁵S]-dATP (sp. act. > 1,000 Ci·mmol⁻¹, Amersham).

S1 NUCLEASE ANALYSIS. Uniformly-labeled probes were prepared from antisense strands of *Eco*R1-*Sst*I (550 kb) and *Pst*I-*Bam*H1 (648 bp) restriction fragments (Fig. 1) of the 1.9 kb *Sst*I-restricted peach DNA fragment using a transcription kit (Stratagene) with [α -³²P]-CTP (sp. act. 800 Ci·mmol⁻¹, Amersham). S1 nuclease mapping was modified from Perbal (1988) as follows: 10 mL (40 mg) peach total RNA were mixed with 30 mL hybridization solution (70% formamide, 0.9 M NaCl, 7.5 mM EDTA [pH 8.0], 60 mM Tris-HCl [pH 8.0], 0.05% SDS) and 2 ng (2 × 10⁶ cpm) of ³²P-labeled probe, and heated at 90 °C for 10 min. Hybridization was done at 49 °C for 15-18 h. S1 nuclease (Gibco-BRL, Life Technologies, Gaithersburg, Md.) digestion [300 units in 300 µL 0.3 M NaCl, 0.05 M sodium acetate, (pH 4.5), 9 mM ZnCl₂, 20 µg·mL⁻¹ calf thymus ssDNA (Sigma, St. Louis)] was done at 37 °C for 90 min, and then vortexed with 40 µL stop buffer (5.0 M ammonium

acetate, 12.5 M EDTA [pH 8.0], 25 µg·mL⁻¹ yeast tRNA [Sigma]) for 5 s, extracted with an equal volume of phenol:chloroform (1:1) and centrifuged 5 min at 15,600 g_n. The aqueous solution was mixed with an equal vol of cold isopropanol, precipitated at -20 °C for 2 h, and centrifuged 30 min at 15,600 g_n. Pelleted digestion products were washed with 100% ethanol, centrifuged 5 min at 15,600 g_n, air dried, resuspended with 10 mL loading buffer (80% formamide, 20 mM EDTA [pH 7.5], 0.1% bromophenol blue, 0.1% xylene cyanol), and electrophoresed in a 6% polyacrylamide gel containing 8 M urea for 3 h at 300 V. The gel was placed on a Whatman 3 MM filter paper, covered with Saran wrap and exposed to Hyper X-ray film (Amersham) at room temperature for 2 to 18 h.

Results

SEQUENCE ANALYSIS OF *Lhcb1*Pp1*. A 1.9 kb *Sst*I-restricted peach leaf DNA fragment was isolated and sequenced (Fig. 1). The sequence includes the putative polypeptide coding region, the putative transit peptide, and several hundred bases of flanking DNA as well as the deduced amino acid sequence of the putative polypeptide. Based on a comparison with types I, II, and III *Lhcb* genes obtained from a GenBank search, the peach gene, *Lhcb1*Pp1* (designated according to Jansson et al., 1992), encodes a precursor polypeptide of 267 amino acids composed of a transit peptide of 34 amino acids and a type I mature polypeptide of 233 amino acids. As is typical of a type I gene, no intron is present.

The *Lhcb1*Pp1* precursor polypeptide and those encoded by representative type I *Lhcb* genes of annual plants are 83% to 91% similar at the amino acid level (Table 1). Mature proteins are 89 to 94% similar. The amino acid similarity between the *Lhcb1*Pp1* precursor polypeptide and those encoded by woody plant type I *Lhcb* genes is 70% to 86% (Table 1). Mature proteins are 80% to 94% similar. Further, the *Lhcb1*Pp1* mature polypeptide contains 15 of the 16 signature amino acids (Fig. 1) characteristic of type I *Lhcb* apoproteins in angiosperms (Demmin et al., 1989). However, amino acid 207 (isoleucine) in the peach apoprotein is a type II signature residue in angiosperms. In contrast to the mature protein, the *Lhcb1*Pp1* transit peptide is considerably less similar to those of either annual or other woody plants (Table 1). The similarity to annual plants ranges between 47% and 69% at the amino acid level while the similarity to woody plants is even less, ranging between 15% and 22%.

EXPRESSION OF *Lhcbs*. *Lhcb* gene expression was examined by S1 nuclease analysis using ³²P-labeled antisense probes transcribed from 5' and 3' regions of *Lhcb1*Pp1* (Fig. 1). When total peach 'Stark Earliglo' leaf RNA was hybridized with the 5' probe and digested with S1 nuclease, two fragments of 286 and 240 nucleotides in length (designated as fragments 1 and 2, Fig. 2) were protected. Hybridization with the 3' probe produced six protected fragments (designated as fragments 3 to 8, Fig. 2) ranging in size from 365 to 200 nucleotides. Of these, the two smaller bands of 220 and 200 nucleotides (fragments 7 and 8, Fig. 2) were heavily labeled with the radioactive probe. The same 5' and 3' protected fragments were found in two additional cultivars, 'Stark Elberta Queen' (data not shown) and 'Evergreen' (Fig. 4).

Amino acid sequences of coding regions of many different *Lhcbs* are highly similar to one another (Jansson, 1994; Thorner et al., 1994). Therefore, since both 5' and 3' probes used in the present experiments include coding as well as flanking sequences, they will hybridize to all related *Lhcb* family members. Even so, any *Lhcb1*Pp1* transcripts will be among those protected by these probes. Of the two protected regions that hybridized with the 5' probe, the larger 286-base protected fragment likely includes

Fig. 1. Nucleotide sequence of the RNA-like strand of *Lhcb1*PpI* and its flanking regions and the deduced amino acid sequence of its encoded precursor polypeptide. The presumed transcription initiation site is numbered as 1. 2–4. The encoded precursor polypeptide starts at +67 and an arrow marks the apparent cleavage site for the transit peptide. Boxed boldface sequences are a TATA-like sequence at -41, CCAAT at -102 and four putative polyadenylation signals in the 3' flanking region. Numbers 2, 7, 8 and corresponding bold letters indicate the ends of *Lhcb* transcripts as suggested by the results in Fig. 2–4. Numbers 3 through 6 and corresponding bold letters indicate potential polyadenylation sites as suggested by results in Fig. 2–4. Restriction enzymes, *SsrI*, *EcoRI*, *BamHI* and *PstI*, used to make probes for SI nuclease analyses are shown at their respective restriction sites. Amino acid signature sequences that characterize type I genes are marked with an asterisk. A solid dot marks a residue that is a type II signature in annual angiosperms but is present in the type I peach gene.

Table 1. Percent similarity of the deduced amino acid sequence between the *Lhcb1*Pp1* polypeptide and those encoded by type I *Lhcb* genes in annual and woody plants.^z

Plant	<i>Lhcb1*Pp1</i>			
	<i>Lhcb</i> gene	Precursor polypeptide	Mature polypeptide	Transit peptide
Annual plants				
<i>Arabidopsis</i>	<i>Lhcb1*At1</i>	87.3	91.0	61.8
Pea	<i>Lhcb1*Ps2</i>	88.1	94.0	51.4
Soybean	<i>Lhcb1*Gm3</i>	88.8	91.8	68.6
Tobacco	<i>Lhcb1*Nt3</i>	91.0	94.4	67.6
Wheat	<i>Lhcb1*T1</i>	83.5	88.8	47.1
Woody plants				
Apple	<i>Lhcb1*Md1</i>	69.9	80.4	19.5
Scots pine	<i>Lhcb1*Ps1</i>	81.8	89.8	22.2
Scots pine	<i>Lhcb1*Ps2</i>	80.1	91.5	17.4
Lodgepole pine	<i>Lhcb1*Pc1</i>	79.2	89.9	17.4
Japanese black pine	<i>Lhcb1*Pt1</i>	78.2	90.6	15.2
Maidenhair	<i>Lhcb1*Gb1</i>	86.1	94.0	21.4

^zComparisons are made with *Arabidopsis* (Leutwiler et al., 1986), pea (Cashmore, 1984), soybean (*Glycine max* L.) (Walling et al., 1988), tobacco (*Nicotiana tabacum* L.) (D.S. Jin and L. Bogorad, GenBank accession no. NTCAB40, 1991), wheat (*Triticum* spp.) (Lamppa et al., 1985), apple (Chen et al., 1990), scots pine (*Lhcb1*Ps1* and *Ps2*, Jansson and Gustafsson, 1990), lodgepole pine (Barrett et al., 1994), Japanese black pine (Yamamoto et al., 1993), and maidenhair (Chinn and Silverthorne, 1993). Comparisons were computed at the National Center for Biotechnology Information using the BLAST network service.

transcripts from *Lhcb1*Pp1*. This size fragment places the transcription initiation site about 66 bases upstream of the translation start codon of *Lhcb1*Pp1* (Fig. 1). Further, this potential initiation site is preceded by putative TATA and CCAAT sequences at about 44 and 102 bases further upstream, respectively. The smaller 240-base protected fragment (2, Fig. 2) is equivalent to the 5' coding region plus 21 bases of the 5' untranslated region (UTR) of *Lhcb1*Pp1* and probably represents hybridizations of the 5' probe to related *Lhcb* mRNAs. Certain *Lhcb* genes in *Arabidopsis* (Leutwiler et al., 1986) have conserved 5' UTRs.

Examination of the 3' flanking region in *Lhcb1*Pp1* showed four potential polyadenylation signals at 34, 77, 108, and 151 bases downstream from the stop codon (Fig. 1) based on their sequence similarities to multiple potential polyadenylation signals in the 3' flanking region of other plant genes (Chinn et al. 1995; Dean et al., 1986; Gallie, 1993; Li and Hunt, 1997; Rothnie, 1996). Notably here, the 3' probe from *Lhcb1*Pp1* protected four fragments ranging from 265 to 365 nucleotides in length (fragments 3 through 6, Fig. 2). These fragments matched the expected protected portions of mRNAs transcribed from *Lhcb1*Pp1* if all four of its potential polyadenylation signals were used. The 3' probe also protected two smaller fragments. One of 200 nucleotides (fragment 8, Fig. 2) was equivalent mainly to the 3' coding region *Lhcb1*Pp1* and the other of 240 nucleotides (fragment 7, Fig. 2) was equivalent to the 3' coding region plus 26 bases of the 3' UTR. These smaller protected fragments likely represented hybridization of the 3' probe to related *Lhcb* mRNAs.

Lhcb gene expression during an annual growing season in peach leaves ('Stark Earliglo') was examined using the same 5' and 3' probes as above and S1 nuclease analysis. Results showed that all *Lhcb*s identified above (Fig. 2) were detected in leaves during the growing season at least into the month of September (Fig. 3 A and B). However, at the end of the growing season in October, not all the *Lhcb* mRNAs were detected. This was especially demonstrated by the results obtained with the 3' probe where the four larger fragments (3 through 6, Fig. 3B) were not detected in October. Similar experiments with 'Stark Elberta Queen' leaves produced same results (data not shown).

Lhcb gene expression also was examined in leaves and stems of 'Evergreen' grown in tissue culture in the dark and in the light. S1 nuclease analysis with the 5' probe again protected the same two fragments as above (Figs. 2 and 3A). Transcripts corresponding to these two fragments (1 and 2, Fig. 4A) were readily detectable in both organs in light-grown plants. In dark-grown leaves, the smaller fragment (2, Fig. 4A) was detected, while the larger fragment (1, Fig. 4A) was observed only at a trace level. The 3' probe again protected the same six fragments in light-grown leaves (3 through 8, Fig. 4B) as above (Figs. 2 and 3B). In contrast, in dark-grown leaves, only the two smaller fragments (7 and 8, Fig. 4B) were detected. In light-grown stems (Fig. 4 A and B), all but the 365 nucleotide fragment (3, Fig. 4B) were detected. In dark-

Fig. 2. S1 nuclease analysis of peach *Lhcb* transcripts. Total leaf RNA was hybridized with a 5' or 3' antisense probe made from *Lhcb1*Pp1* (Fig. 1), digested with S1 nuclease, electrophoresed and autoradiographed. The portion of the gel containing protected fragments (arrows 1–8) is shown. Yeast tRNA was used as a negative control.

Fig. 3. S1 nuclease analysis of *Lhcb* gene expression during an annual growth cycle. (A) A 5' antisense RNA probe (see Fig. 1) was hybridized to 1, 5, and 25 μ g of total leaf RNA collected from youngest produced leaves during the growing season. Hybrids were subjected to S1 nuclease analysis as Fig. 2. The portion of the gel containing protected fragments (arrows 1 and 2) is shown. Yeast tRNA was used as a negative control. (B) As in A except that a 3' antisense RNA probe (Fig. 1) was used. The portion of the gel containing protected fragments (arrows 3-8) is shown.

grown stems, the 5' probe revealed only a trace amount of the smaller fragment (2, Fig. 4A); while, the larger fragment (1, Fig. 4A) was not detected. With the 3' probe, only the two smallest fragments (7, 8, Fig. 4B) were detected in dark-grown stems.

Discussion

For the first time, a genomic clone that encodes a type I chlorophyll *a/b*-binding protein has been isolated and characterized from a peach-leaf genomic library. The gene, *Lhcb1*Pp1*, is expressed in three cultivars of peach, and encodes a precursor polypeptide composed of a transit peptide of 34 amino acids and a mature protein of 233 amino acids. The deduced amino acid sequence of the *Lhcb1*Pp1* mature protein is similar to those encoded by other type I *Lhcb* genes in both annual and woody plants. Considering the critical function in photosynthesis of *Lhcb*-encoded apoproteins, it is not surprising that their deduced amino acid sequences are highly conserved in the phylogenetically different annual and woody plants, and even in more evolutionarily distinct organisms (Thornber et al., 1994). The amino acid conservation of the *Lhcb1*Pp1* product extends to signature amino acids that characterize type I *Lhcb*s (Demmin et al., 1989). The mature polypeptide encoded by the peach gene has 15 of the 16 signature residues found in type I *Lhcb*s of annual angiosperms. The remaining residue in the peach differs, however, in that it is a type II signature in annual plants. In contrast, the only other woody angiosperm type I *Lhcb* so far reported, the apple (Chen et al., 1990), has a type I signature residue (leucine) at this position. Of

interest, however, the type II residue found in the peach is also present in type I *Lhcb*s of several gymnosperms, including scots pine (*Pinus sylvestris* L.) (Jansson and Gustafsson, 1990), maiden-hair (*Ginkgo biloba* L.) (Chinn and Silverthorne, 1993), lodgepole pine (*Pinus contorta* Loud.) (Barrett et al. 1994), Japanese black pine (*Pinus thunbergii* Parl.) (Yamamoto et al., 1993), and longleaf pine (*Pinus palustris* Mill.) (Peer et al., 1996).

When compared to type I *Lhcb* products of annual plants, the amino acid sequence of the *Lhcb1*Pp1* transit peptide is less conserved than that of its mature protein (Table 1). Interestingly, even lower similarity is found when the peach transit peptide is compared to those encoded by type I *Lhcb* genes of other woody plants. A detailed review by DeBoer and Weisbeek (1991) concludes that transit peptides of similar proteins in different plants tend to have comparable lengths. However, the *Lhcb1* transit peptides of the perennial woody plants listed in Table 1 are generally longer (with 37 to 44 residues) than those found in annual plants (with 32 to 36 residues). Among woody plants, only the peach transit peptide, with 34 residues, is of similar size to those found in annual plants. The question of why the transit peptide is so highly variable in amino acid sequence and length in perennial woody plants deserves further investigation. Understanding of the changes that have occurred in transit peptides of woody plants may provide some novel insights into the evolutionary relationships of higher plants.

Fig. 4. S1 nuclease analysis of *Lhcb* gene expression in organs of light- and dark-grown peach shoots in tissue culture. (A) The 5' antisense RNA probe (see Fig. 1) was hybridized to 1, 5, and 25 μ g of total RNA from light- and dark-grown peach ('Evergreen') leaves and stems. Hybrids were subjected to S1 nuclease analysis as Fig. 2. The portion of the gel containing protected fragments (arrows 1 and 2) is shown. Yeast tRNA was used as a negative control. (B) As in A except that a 3' antisense RNA probe (Fig. 1) was used. The portion of the gel containing protected fragments (arrows 3-8) is shown.

Lhcb gene expression during an annual growing season in a woody angiosperm has not been documented before. Therefore, this expression has been examined in the peach using S1 nuclease analysis with uniformly labeled antisense 5' and 3' RNA probes transcribed from *Lhcb1*Pp1*. Results show that *Lhcb* genes are expressed throughout the growing season, but that different *Lhcb*s detected with present probes are altered late in the season. With the 5' probe, both leaf *Lhcb* transcripts detected may decrease at the end of the growing season in October (Fig. 3A). Further, the 3' probe has not detected the four largest *Lhcb* transcripts (fragments 3 through 6, Fig. 3B) in October. Further studies, however, are needed to determine whether this change in detectable peach *Lhcb* mRNAs correlates with onset of leaf senescence and/or with other programmed cell-death responses (Gan and Amasino, 1997).

Certain *Lhcb* genes are expressed in the dark in some angiosperms, such as barley (*Hordeum vulgare* L.) (Apel, 1979; Baumgartner et al., 1989), pea (*Pisum sativum* L.) and mung bean (*Vigna radiata* L.) (Thompson et al., 1983), maize (*Zea mays* L.) (Sullivan et al., 1989), and *Arabidopsis thaliana* L. (Brusslan and Tobin, 1992). However, *Lhcb* mRNAs in most annual plants are light-dependent and/or accumulate rapidly in response to light (Tobin and Silverthorne, 1985; Buetow et al., 1988). As shown here, *Lhcb* gene expression in the peach appears to be modulated by light. The number of different *Lhcb* mRNAs detected in dark-grown peach leaves and stems is reduced compared to those in either organ in light-grown plants (Fig. 4 A and B).

Multiple potential polyadenylation signals characterize many plant genes (Li and Hunt, 1997). Transcripts terminating at alternative polyadenylation sites have been reported, for example, for individual genes, including *Lhcb* genes, in annual plants (Dean et al., 1986). Multiple transcripts apparently arising from differential polyadenylation of certain *Lhcb* genes also have been identified in the gymnosperm, *G. biloba* (Chinn et al., 1995). The present peach gene contains four potential polyadenylation signals. S1 nuclease analysis uncovered transcripts expected if all four signals are used during expression of this gene. Further, these varied-sized transcripts are differentially expressed during the growing-season (Fig. 3) and in different organs in the peach (Fig. 4). However, a definitive conclusion cannot be made yet concerning alternative polyadenylation during expression of *Lhcb1*Pp1*. This is so because conserved 50-base long regions have been documented in the 3' UTRs immediately adjacent to the coding regions of two distinct *Lhcb* genes in *Arabidopsis* (Leutwiler et al., 1986). Therefore, to answer the question of alternative polyadenylation with *Lhcb1*Pp1*, it will be necessary to classify the *Lhcb* genes in the peach and to assess nucleotide-sequence similarities among them especially in 3' regions flanking their coding regions. The preponderance of multiple polyadenylation sites in plant genes suggests that alternative site usage may be an important regulatory mechanism for plant gene expression (Li and Hunt, 1997). So far, however, alternative polyadenylation has not been definitively shown to be involved in the regulated expression of any plant gene. Further study with peach *Lhcb* genes and especially on the expression of *Lhcb1*Pp1* may help resolve this question.

Finally, there has been little study on the nature of the recurring influence of light and other environmental factors on the expression of genes during annual cycles of photosynthesis and growth in perennial woody plants. In the case of Eastern larch (*Larix* spp.), a gymnosperm, a relationship has been established between the level of *Lhcb* gene expression and the age of the tree (Hutchison et al., 1990; Hutchison and Greenwood, 1991). As the tree matures, *Lhcb* gene expression in the foliage decreases. In contrast, the level of expression of another light-regulated gene, *rbcS*, is not altered

as the tree matures and ages. However, it is not known how this age-related influence operates nor how it distinguishes among genes. The present study sets the stage for subsequent investigations on the form of control by recurring annual environmental cues on the expression of a light-regulated gene in a perennial angiosperm.

Literature Cited

- Alosi, M.C., D.B. Neale, and C.S. Kinlaw. 1990. Expression of *cab* genes in Douglas-fir is not strongly regulated by light. *Plant Physiol.* 93:829–832.
- Apel, K. 1979. Phytochrome-induced appearance of mRNA activity for the apoprotein of the light-harvesting chlorophyll *a/b*-protein of barley (*Hordeum vulgare*). *Eur. J. Biochem.* 97:183–188.
- Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl. 1990. Current protocols in molecular biology. vol. 1. p. 4.3.1–4.3.4. Wiley, New York.
- Barrett, J.W., R.N. Beech, B.P. Dancik, and C. Strobeck. 1994. A genomic clone of a type I *cab* gene encoding a light harvesting chlorophyll *a/b* binding protein of photosystem II identified from lodgepole pine. *Genome* 27:166–172.
- Bassett, C., A. Callahan, and L. Dunn. 1998. Characterization of a type II chlorophyll *a/b* binding protein gene (*Lhcb2*Pp1*) in peach: I. Isolation, identification and abundance in developing leaves of mature 'Loring' trees in the absence of flowering. *J. Amer. Soc. Hort. Sci.* 123:486–492.
- Baumgartner, B.J., J.C. Rapp, and J.E. Mullett. 1989. Plastid transcription activity and DNA copy number increase early in barley chloroplast development. *Plant Physiol.* 89:1011–1018.
- Brusslan, J.A. and E.M. Tobin. 1992. Light-independent developmental regulation of *cab* gene expression in *Arabidopsis thaliana* seedlings. *Proc. Natl. Sci. USA* 89:7791–7795.
- Buetow, D.E., H. Chen, G. Erdos, and L.S.H. Yi. 1988. Regulation and expression of the multigene family coding light-harvesting chlorophyll *a/b* binding proteins of photosystem II. *Photosyn. Res.* 18:61–97.
- Canovas, F., B. McLarney, and J. Silverthorne. 1993. Light-independent synthesis of LHCIIb polypeptides and assembly of the major pigmented complexes during the initial states of *Pinus palustris* seedling development. *Photosyn. Res.* 38:89–97.
- Cashmore, A.T. 1984. Structure and expression of a pea nuclear gene encoding a chlorophyll *a/b* binding polypeptide. *Proc. Natl. Acad. Sci. USA* 81:2960–2964.
- Chen, H., S.S. Korban, and D.E. Buetow. 1990. Nucleotide sequence of an apple nuclear gene encoding a light-harvesting chlorophyll *a/b* binding polypeptide of photosystem II. *Nucl. Acids Res.* 18:679.
- Chinn, E. and J. Silverthorne. 1993. Light-dependent chloroplast development and expression of a light-harvesting chlorophyll *a/b* binding protein gene in the gymnosperm *Ginkgo biloba*. *Plant Physiol.* 103:727–732.
- Chinn, E., J. Silverthorne, and A. Hohtola. 1995. Light-regulated and organ-specific expression of Types 1, 2 and 3 light-harvesting complex b mRNAs in *Ginkgo biloba*. *Plant Physiol.* 107:593–602.
- Chung, K.H., S.S. Korban, and D.E. Buetow. 1994. Construction of a genomic library using high-molecular-weight DNA prepared from peach leaves. *Plant Mol. Biol. Rpt.* 12:304–309.
- Coruzzi, G., R. Broglie, A. Cashmore, and N-H. Chua. 1983. Nucleotide sequences of two pea cDNA clones encoding the small subunit of ribulose-1,5-bisphosphate carboxylase and the major chlorophyll *a/b*-binding thylakoid polypeptide. *J. Biol. Chem.* 258:1399–1402.
- Dean, C., S. Tamaki, P. Dunsmuir, M. Favreau, C. Katayama, H. Dooner, and J. Bedbrook. 1986. mRNA transcripts of several plant genes are polyadenylated at multiple sites in vivo. *Nucl. Acids Res.* 14:2229–2240.
- DeBoer, A.D. and P.J. Weisbeek. 1991. Chloroplast protein topogenesis: Import, sorting and assembly. *Biochim. Biophys. Acta* 1071:221–253.
- Demmin, D.S., E.J. Stockinger, Y.C. Chang, and L. Walling. 1989. Phylogenetic relationships between the chlorophyll *a/b* binding protein (CAB) multigene family: An intra- and interspecies study. *J. Mol. Evol.* 29:266–279.

- Gallie, D.R. 1993. Posttranscriptional regulation of gene expression in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:77–105.
- Gan, S. and R.M. Amasino. 1997. Making sense of senescence: Molecular genetic regulation and manipulation of leaf senescence. 1997. *Plant Physiol.* 113:313–319.
- Green, B.R. and D.G. Durnford. 1996. The chlorophyll–carotenoid proteins of oxygenic photosynthesis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 47:685–714.
- Grossman, A.R., D. Bhaya, K.E. Apt, and D.M. Kehoe. 1995. Light-harvesting complexes in oxygenic-photosynthesis: Diversity, control and evolution. *Annu. Rev. Genet.* 29:231–288.
- Gustafsson, P., S. Jansson, J. Lidholm, and A.-K. Lundberg. 1991. Structure and regulation of photosynthesis genes in *Pinus sylvestris* (Scots pine) and *Pinus contorta* (lodgepole pine). *For. Ecol. Manage.* 43:287–300.
- Hankamer, B., J. Barber, and E.J. Bockema. 1997. Structure and membrane organization of photosystem II in green plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:641–671.
- Hutchison, K.W. and M.S. Greenwood. 1991. Molecular approaches to gene expression during conifer development and maturation. *For. Ecol. Manage.* 43:273–286.
- Hutchison, K.W., C.D. Sherman, J. Weber, S.S. Smith, P.B. Singer, and M.S. Greenwood. 1990. Maturation in larch. II. Effects of age on photosynthesis and gene expression in developing foliage. *Plant Physiol.* 94:1308–1315.
- Jansson, S. 1994. The light-harvesting chlorophyll *a/b*-binding proteins. *Biochim. Biophys. Acta* 1184:1–19.
- Jansson, S. and P. Gustafsson. 1990. Type I and Type II genes for the chlorophyll *a/b*-binding protein in the gymnosperm *Pinus sylvestris* (Scots pine): cDNA cloning and sequence analysis. *Plant Mol. Biol.* 14:287–296.
- Jansson, S., E. Pichersky, R. Bassi, B.R. Green, M. Ikeuchi, A. Melis, D.J. Simpson, M. Spangfort, L.A. Staehelin, and J.P. Thornber. 1992. A nomenclature for the genes encoding the chlorophyll *a/b*-binding proteins of higher plants. *Plant Mol. Biol. Rpt.* 10:242–253.
- Kojima, K., N. Yamamoto, and S. Sasaki. 1992. Structure of the pine (*Pinus thunbergii*) chlorophyll *a/b*-binding protein gene expressed in the absence of light. *Plant Mol. Biol.* 19:405–410.
- Kühlbrandt, W., D.N. Wang, and Y. Fujiyoshi. 1994. Atomic model of plant light-harvesting complex by electron crystallography. *Nature* 367:614–621.
- Lamppa, G., G. Morelli, and N.-H. Chua. 1985. Structure and developmental regulation of a wheat gene encoding the major chlorophyll *a/b*-binding polypeptide. *Mol. Cell Biol.* 5:1370–1378.
- Lee, A. and J.P. Thornber. 1995. Analysis of the pigment stoichiometry of pigment-protein complexes from barley (*Hordeum vulgare*): The xanthophyll cycle and intermediates occur mainly in the light-harvesting complexes of photosystem I and photosystem II. *Plant Physiol.* 107:565–574.
- Leutwiler, L.S., E.M. Meyerowitz, and E.M. Tobin. 1986. Structure and expression of three light harvesting chlorophyll *a/b* binding protein genes in *Arabidopsis thaliana*. *Nucl. Acids Res.* 14:4051–4064.
- Li, Q. and A.G. Hunt. 1997. The polyadenylation of RNA in plants. *Plant Physiol.* 115:321–325.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.
- Peer, W., J. Silverthorne, and J.L. Peters. 1996. Developmental and light-regulated expression of individual members of the light-harvesting complex b gene family in *Pinus palustris*. *Plant Physiol.* 111:627–634.
- Perbal, B. 1988. A Practical guide to molecular cloning. 2nd ed. p. 542–549. Wiley-Interscience, New York.
- Rothnie, H.M. 1996. Plant mRNA 3' end formation. *Plant Mol. Biol.* 32:43–61.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* 74:5463–5467.
- Sullivan, T.D., A.H. Christensen, and P.H. Quail. 1989. Isolation and characterization of a maize chlorophyll *a/b* binding protein gene that produces high levels of mRNA in dark. *Mol. Gen. Genet.* 215:431–440.
- Thompson, W.F., M. Everett, N.O. Polans, R.A. Jorgensen, and J.D. Palmer. 1983. Phytochrome control of RNA levels in developing pea and mung bean leaves. *Planta* 158:487–500.
- Thornber, J.P., R.J. Cogdell, P. Chitnis, D.T. Morishige, S. Gomez, G.F. Peter, S. Anandan, S. Preiss, B.A. Welty, A. Lee, T.S. Takeuchi, and C. Kerfeld. 1994. Antenna pigment-protein complexes of higher plants and purple bacteria. *Adv. Mol. Cell Biol.* 10:55–118.
- Tobin, E.M. and J. Silverthorn. 1985. Light regulation of gene expression in higher plants. *Annu. Rev. Plant Physiol.* 36:569–593.
- Walling, L.L., Y.C. Chang, D.S. Demmin, and F.M. Holzer. 1988. Isolation, characterization and evolutionary relatedness of three members from the soybean multigene family encoding chlorophyll *a/b* binding proteins. *Nucl. Acids Res.* 16:10477–10492.
- Yamamoto, N., K. Kojima, and M. Matsuoka. 1993. The presence of two types of gene that encode the chlorophyll *a/b*-binding protein (LHCPII) and their light-independent expression in pine (*Pinus thunbergii*). *Plant Cell Physiol.* 34:457–463.
- Yamamoto, N., Y. Mukai, M. Matsuoka, Y. Kano-Murakami, Y. Tanaka, Y. Ohashi, Y. Ozeki, and K. Odani. 1991. Light-independent expression of *cab* and *rbcS* genes in dark-grown pine seedlings. *Plant Physiol.* 95:379–383.