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

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ADDITIONAL INDEX WORDS. photosynthesis, photosystem II light harvesting proteins, leaf development, fruit tree

ABSTRACT. A gene (*Lhcb2*Pp1*) encoding a type II chlorophyll *a/b*-binding protein (LHCB) was isolated from peach [*Prunus persica* (L.) Batsch]. The gene was sequenced and compared to a variety of other genes encoding LHCB polypeptides associated with photosystem II. Similarity at the nucleotide and amino acid level was highest between *Lhcb2*Pp1* and other type II genes and was lowest with type I and III genes from other species. Expression of *Lhcb2*Pp1* was followed by determining abundance of transcripts in developing leaves of field grown trees in the absence of flowering. Expression was monitored at three times during the first phase of the growing season and was shown to be highest in leaves which were at or near full expansion at each sampling time. These results are consistent with the function of this gene and indicate that it can serve as a marker of photosynthetic maturity under field conditions.

Assimilate supply and distribution play key roles in plant growth and differentiation. Although most researchers generally classify organs as functioning as a source (assimilate supply) or a sink (assimilate demand), these distinctions are often somewhat arbitrary, since all plant organs can act as sinks and sources in accord with their developmental state or in response to changing environmental conditions (for a review, see Ho, 1988). For example, leaves which are classified primarily as source organs can compete quite effectively for stored assimilates during the early stages of development before significant photosynthetic capability is achieved (Ho, 1984; Keller and Loescher, 1989). Conversely, fruit that are generally considered sink organs can contribute nearly 10% of the tree's total carbohydrate requirement during the early part of the growing season via photosynthesis (Pavel and DeJong, 1993).

Competition among aerial organs for assimilates is often complex and has a significant effect on final crop yields; indeed, managing fruit tree vegetative and reproductive growth is of considerable economic importance to today's typical high-density orchard culture (Williamson et al., 1992). Dorsey (1935) showed in early studies of peach growth and development that node initiation and flower bud formation were related to vegetative vigor. Although the potential for flower bud initiation was directly proportional to vegetative growth under normal conditions, excessive canopy growth actually suppressed flower bud development. In addition, Chalmers et al. (1981) suggested that vegetative growth of peach trees during stage III of fruit development could compete with fruit growth.

Assimilate supply ultimately depends on the light reactions of photosynthesis which provide energy for carbon fixation. Ultra-

structural and biochemical studies have identified a number of components comprising the pathway from light reception to starch synthesis. Constituents of the two photosystems involved in the light reactions, including the light-harvesting pigments and the proteins that aid in electron transfer, have been the most difficult to identify and characterize due in part to the complexity of these reaction centers and to their association with thylakoid membranes (for a review see Timko et al., 1985).

Recent advances in genetics and molecular biology have led to a better understanding of the characteristics and functions of the major constituents of the two photosystems. Among the most extensively characterized of these constituents are the chlorophyll *a/b*-binding proteins (LHCA and LHCB) associated with the light-harvesting complexes of photosystems I and II, respectively. The genes encoding these proteins constitute a complex family ranging in different species from 3 to 16 members (Buetow et al., 1988; Chitnis and Thornber, 1988; Demmin et al., 1989) and representing at least 10 distinct types of proteins (Green et al., 1991). Six different types of photosystem II LHCB polypeptides are recognized. Types I and II are distinguished by differences in transit peptide sequence and in the presence or absence of an intron within the coding sequence. Type I genes have no introns and are expressed at significant levels in the dark (Sullivan et al., 1989; Yamamoto et al., 1991).

Most studies of the *Lhcb* gene family have focussed on herbaceous plants which can be conveniently manipulated genetically and grown under controlled laboratory or greenhouse conditions. Few studies of these genes have been attempted in woody plants. *Lhcb* genes of the type II subfamily have been isolated from *Pinus thunbergii* Parl. (Kojima et al., 1992; Yamamoto et al., 1988) and characterized in cotyledons of dark-grown seedlings. A recent study of rubisco activase expression in apple (*Malus x domestica*) leaves used an apple *Lhcb* gene probe to compare patterns of expression as a function of leaf development (Watillon et al., 1993). However, those studies examined expression in young (3 years old) 'McIntosh Wijcik' apple trees which possessed a single shoot apex. No studies of expression of *Lhcb* genes in other woody species, particularly as a function of leaf development in mature trees under field conditions, have been reported (however, see Chung et al., 1998).

Received for publication 19 Dec. 1997. Accepted for publication 2 Feb. 1998. We acknowledge the expert technical assistance of Michelle Shipley, Deanna Fischel and Michael Nickerson in identifying and characterizing the peach *Lhcb2*Pp1* gene. We also thank Dennis Buetow for allowing us to use the *Lhcb1*Pp1* gene sequence information in our comparisons and Richard Meagher for kindly providing the soybean 18S RNA probe. Use of a trade name or trade mark does not imply endorsement to the exclusion of other, similar products. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

PchcabII	MATSAIQQSAFAGQTALKQSNELIRKIGGLGGGRITMRRTVKSTPQSIWY	50
PetcabII	MATSAIQQSAFAGQTALKSQNELVRKIGSFGGGRATMRRTVKSAPQSIWY	50
TomcabII	MATCAIQQSAFVGQAVGKSNQEFIRKVGNFGEGRITMRRTVKSAPQSIWY	50
PeacabII	MATSAIQQSAFTGKTGLRQGNFIRKRGNFQGARFTMRRTVKSAPESIWY	50
PchcabII	GPDRPKYLGPFSEQTPSYLTGEFPGDYGWDTAGLSADPETFAKNGELEVI	100
PetcabII	GEDRPKYLGPFSEQTPSYLTGEFPGDYGWDTAGLSADPETFARNRELEVI	100
TomcabII	GEDRPKYLGPFSEQTPSYLTGEFPGDYGWDTAGLSADPETFARNRELEVI	100
PeacabII	GPDRPKYLGPFSEQIPSYLTGEFPGDYGWDTAGLSADPETFARNRELEVI	100
PchcabII	HSRWAMLGALGCVFPEILSKNGVKFGEAVWFKAGSQIFSEGGLDYLGNPN	150
PetcabII	HCRWAMLGALGCVFPEILSKNGVTFGEAVWFKAGSQIFSEGGLDYLGNPN	150
TomcabII	HCRWAMLGALGCVFPEILSKNGVKFGEAVWFKAGSQIFSEGGLDYLGNPN	150
PeacabII	HSRWAMLGALGCTFPELLEKNGVKFGEAVWFKAGSQIFAEGGLDYLGNPN	150
PchcabII	LVHAQSILAIWAVQVVLGMGFVEGYRVGGGPLGEGLDPLYPGGAFDPLGLA	200
PetcabII	LIHAQSILAIWAAQVVLGMGFVEGYRVGGGPLGEGLDKIYPGGAFDPLGLA	200
TomcabII	LVHAQSILAIWACQVVLGMGFVEGYRVGGGPLGEGLDKIYPGGAFDPLGLA	200
PeacabII	LIHAQSILAIWATQVVLGMGFVEGYRVGGGPLGEGLDPLYPGGAFDPLGLA	200
PchcabII	DDPEAFELKVKELKNGRLAMTSMFGFFVQAIVTGKGPVENLYDHVADPV	250
PetcabII	DDPEAFELKVKELKNGRLAMFSMFGFFVQAIVTGKGPIENLYDHVADPV	250
TomcabII	DDPEAFELKVKELKNGRLAMFSMFGFFVQAIVTGKGPIENLSDHINDPV	250
PeacabII	DDPDSFELKVKELKNGRLAMFSMFGFFVQAIVTGKGPIQNLVYDHVADPV	250
PchcabII	ANNAWAYATNFVPGK	265
PetcabII	ANNAWAFATNFVPGK	265
TomcabII	ANNAWAYATNFVPGK	265
PeacabII	ANNAWAFATNFVPGQ	265

and KS⁺ from Stratagene (La Jolla, Calif.). As DNA sequence information was generated, new oligonucleotides were synthesized on a Gene Assembler II (Pharmacia-LKB, Piscataway, N.J.). DNA sequences were analyzed using the PC/Gene program (Intelligenetics, Mountain View, Calif.).

5' RACE ANALYSIS. 5' RACE (rapid amplification of cDNA ends) was performed according to the manufacturer's instructions (Life Technologies) using the oligonucleotide *pch108SA* (5'-GGTACAACGACTAGG-TCAACGGTTG-3') to prime cDNA synthesis. The resulting cDNAs were ligated to an anchor primer (Life Technologies) and subjected to PCR using the universal amplification primer (UAP) supplied by the manufacturer and the gene specific oligonucleotide, *pch108SB* (5'-GGGTTTGGAAACAGGT-ACGGG-3'). The products were cloned into the TAIICloning vector (Invitrogen, Carlsbad, Calif.) and sequenced to obtain the full length of the coding region of the gene.

RNA ABUNDANCE ANALYSIS. RNA extracted from individual leaves was separated and hybridized as described in the previous sections. X-ray films exposed to RNA blots hybridized to *pch108* and pSR1.2B3 (soybean 18S rRNA, Eckenrode et al., 1985) were image analyzed using a Foto Eclipse system (Fotodyne Incorporated, Hartland, Wis.) and Collage software (Fotodyne). The ratio of the signal generated by each probe was determined for each sample and used as a measure of the relative amount of *pch108* RNA present.

Results

ISOLATION AND IDENTIFICATION OF A PEACH *Lhcb* GENE. Previous studies had identified a cDNA clone (*pch108*) from 'Suncrest' whose expression was highest in leaves and stage I developing peach fruit (Callahan et al., 1993). Sequence analysis of *pch108* revealed a 739 bp cDNA with a poly A tail. The sequence was compared to GenBank sequences and showed considerable homology to chlorophyll *a/b*-binding proteins from different plants.

Fig. 2. Comparison of the peach translated Lhcb polypeptide with other type II Lhcb proteins. Comparison of PchcabII (peach), PetcabII (petunia), TomcabII (tomato), and PeacabII (pea) was done with the CLUSTAL program of PC/Gene. Amino acids are represented by the one letter code. The number of amino acids is indicated on the right. Putative membrane-spanning regions are boxed.

D column. This was used as the template for DNA sequencing using a Sequenase 2 kit (USB, Cleveland, Ohio) following manufacturer's directions. Initial primers were commercial SK⁺

Table 1. Identity of the peach *Lhcb2*Pp1* gene with other photosystem II *Lhcb* genes.

Gene	Protein	Cab type	Identity with <i>Lhcb2*Pp1</i> gene ^z (%)	
			Amino acid	Nucleotide ^y
<i>Lhcb2*Ph1</i> ^x	PETCAB2	II	93	83
<i>Lhcb2*Le1</i>	TOMCAB2	II	90	81
<i>Lhcb2*Ps1</i>	PEACAB2	II	89	81
<i>Lhcb1*Ps4</i>	PEACAB1	I	78	71
<i>Lhcb1*Pp1</i>	PCHCAB1	I	79	71
<i>Lhcb1*Md1</i>	APPCAB1	I	64	64
<i>Lhcb3*Hv1</i>	BARCAB3	III	69	65

^zIdentities were determined using NALIGN and PALIGN programs of PC/Gene.

^yOnly the coding regions were compared.

^x*Lhcb2*Ph1*: petunia (*Petunia hybrida* L.) *Cab37* [GenBank Ac. No. X04966, Stayton et al., 1986]; *Lhcb2*Le1*: tomato (*Lycopersicon esculentum* L.) *Cab4* [M17558, Pickersky et al., 1987]; *Lhcb2*Ps1*: pea *Cab-215* [X57082, Falconet et al., 1991]; *Lhcb1*Ps4*: pea *Cab-8* [X56538, Alexander et al., 1991]; *Lhcb1*Pp1*: peach [L36064, Chung et al., 1998] *Lhcb1*Md1*: apple *AB10* [X17697, Chen et al., 1990]; *Lhcb3*Hv1*: barley *Cab-3* [X63197, Brandt et al., 1992].

Fig. 3. Relatedness of derived LHCb polypeptides. Comparison of translated *Lhcb* genes encoding different types of chlorophyll *a/b*-binding proteins from a variety of plants was accomplished with the CLUSTAL program. TOMCAB2, *Lhcb2*Le1*; PETCAB2, *Lhcb2*Ph1*; PEACAB1, *Lhcb1*Ps4*; PEACAB2, *Lhcb2*Ps1*; PCHCAB1, *Lhcb1*Pp1*; PCHCAB2, *Lhcb2*Pp1*; APPCAB1, *Lhcb1*Md1*; BARCAB3, *Lhcb3*Hv1*.

It was determined from the sequence comparisons, as well as from the transcript size (800 bases, Callahan et al., 1993), that *pch108* was not full length and had been prematurely terminated at the 5'-most *EcoRI* site during the cloning process. To obtain full length sequence, 'Suncrest' RNA was subjected to 5' RACE to amplify the sequence 5' to the *pch108* cDNA. The combined sequence is presented in Fig. 1. The overlap between *pch108* and the 5' RACE-extended sequence (ca. 251 b) was identical thus verifying the reliability of the 5' RACE technique. Comparison of the composite sequence to other *Lhcb* genes further confirmed the identity of this gene, designated *Lhcb2*Pp1* in accordance with nomenclature proposed by Jansson et al. (1992).

Sequence comparisons with translated polypeptides of other type II chlorophyll *a/b*-binding protein genes of photosystem II (Fig. 2) indicated that *Lhcb2*1* encoded a type II LHCb protein. This was further supported by the identification of a 365 bp intron at a position conserved among other type II genes (Fig. 1, vertical arrow, data not shown). In addition a transit peptide similar to those proposed for other type II LHCb proteins can be postulated for *Lhcb2*1* (Fig. 1, arrowhead), and the putative membrane-spanning regions predicted in type II LHCb proteins are similar in sequence and location to those in *Lhcb2*1* (Fig. 2, boxes).

Comparison of *Lhcb2*1* with other types of photosystem II *Lhcb* genes from different species indicated homologies with type II genes of herbaceous species of 89% to 93% at the amino acid level (Table 1). While type I genes from peach and pea (*Pisum sativum* L.) shared 71% to 78% homology with *Lhcb2*1*, the type I gene from apple and the type III gene from barley (*Hordeum vulgare* L.), a monocot, were more distant ($\approx 65\%$) at both the nucleotide and amino acid levels (Table 1; Fig. 3).

LEAF EXPANSION DURING THE FIRST PART OF THE GROWING SEASON. To determine abundance of *Lhcb2*1* in leaves during the growing season, it was necessary to examine leaf development as a function of branch position and age. As a first approximation of leaf development, the increase in leaf blade length was determined

during the growing season of 1994, a season in which no flower buds survived the winter. Figure 4 shows the typical progression of expansion at 3 representative leaf positions along a peripheral branch during the first half of the growing season (from March to mid-June). Leaves at the base of the branch (first emergent four to five leaves) had ceased expansion at the time measurements were initiated. Leaves in the middle of the branch were still expanding at the beginning of these measurements, but ceased by the end of May. Those leaves at the tip of the branches when measurements were initiated were too small to measure without damaging the shoot tip; however, their expansion was essentially linear from mid-May to early June, averaging an increase in length of slightly more than 4 mm/d. A normal distribution of leaf lengths was seen when expansion was examined as a function of leaf position on the branch early in the season (Fig. 5A). As the growing season progressed, the peak of the curve became broader as illustrated in Fig. 5 B and C, with the number of leaves per branch >0.5 cm ranging from 16 to 22 at 70 d after vegetative budbreak (DAB). By early June, many ($\approx 65\%$) of the shoot tips had ceased initiating new leaves (data not shown).

EXPRESSION OF *Lhcb2*1* RNA AS A FUNCTION OF LEAF AGE AND POSITION. Leaf samples were taken from ≈ 15 different trees. Immediately after sampling, the length of each leaf was recorded

Fig. 4. Leaf expansion from April to June. The expansion of representative leaves as a function of the increase in leaf blade length was determined from late April to mid-June. The data were graphed as a function of d from vegetative bud break (5 Apr. 1994); months represented during the collection times are labelled below the x-axis. Leaf 3: filled squares; leaf 8: open circles; leaf 13: filled triangles. Bars represent confidence limits at the 95% confidence level. N ranged from 3 to 20 depending on the position of the leaf and the time of the season.

and the leaves were pooled by relative position i.e. the branch base, middle and tip for measurements at 38 and 52 DAB. Based on the confidence intervals calculated for leaf length vs leaf position on the standard trees, the approximate positions of the leaf samples were estimated using the leaf length measurements. RNA blots were run on these samples using *pchl108* as probe. Figure 5 A and B show that the abundance of *Lhcb2*1* RNA is greatest in mature leaves presumably functioning at or near full photosynthetic capacity. Leaves sampled in mid-June (70 DAB) were collected, measured and pooled according to individual positions. Again, confidence intervals allowed the confirmation of individual leaf position based on length measurements taken after sampling. RNAs were prepared and analyzed as before, except that the RNA blots were washed and reprobed with a soybean gene encoding an 18S RNA (Eckenrode et al., 1985) to normalize differences in loading. Examination of the relative abundance of *Lhcb2*1* RNA in individual leaves shown in Fig. 5C demonstrates that RNA abundance is highest in leaves which are close to full expansion (leaves 14 to 17 in Fig. 5C), based on comparison of measurements taken at d 63 and 70 DAB.

Discussion

*Lhcb2*1* represents the first description of a photosystem II type II chlorophyll *a/b*-binding protein gene from a fruit tree. That it encodes a bona fide type II polypeptide is supported by its sequence identity with other dicot type II genes and its evolutionary distance from type I and III genes. In addition, it shares other structural features common to type II genes, namely the location of a single intron at a conserved position within the gene and a low copy number in the genome (Callahan et al., 1993, Pichersky et al., 1987). Other features of *Lhcb2*1* common to LHCb proteins include a putative transit peptide with a predicted cleavage site at a position conserved among these genes and three predicted membrane-spanning regions believed to represent insertion points into the thylakoid membrane.

The relatedness of *Lhcb2*1* with other type II genes supports the hypothesis that different members of the *Lhcb* gene family maintain their identity through concerted evolution. As a result, the peach type II gene shares more identity with the pea type II gene despite the taxonomic distances between these plant groups than with the peach type I gene. The apple *Lhcb1*1* gene appears to be an exception in that it is significantly different in identity to *Lhcb2*1* compared to the other two type I genes examined. It has been previously noted that the apple type I gene has more nucleotide substitutions in the 1st or 2nd codon positions, which lead to greater divergence during evolution (Chen et al., 1990).

In peach trees, as in many dicots, the pattern of leaf development proceeds acropetally, that is from base to tip, with the oldest

Fig. 5. Leaf expansion and *Lhcb2*1* RNA abundance as a function of leaf position along the branch. Leaves at the indicated positions were sampled at three times during the growing season (A, B, C), and leaf RNA was extracted as described in material and methods. Blotted RNAs were probed with *pchl108* to visualize the transcript. Relative transcript abundance from the RNA blots is depicted above the bars in A and B; the relative position of the leaves sampled in A and B is indicated by brackets below the RNA blots. In A, leaves sampled at 38 DAB; B, leaves sampled at 52 DAB. In C, leaves sampled at 70 DAB. RNA blots of 70 DAB samples were probed separately with an 18S RNA probe and the levels were used to normalize abundance of the *Lhcb2*1* transcripts to correct for loading artifacts. Leaf lengths are indicated by open bars while the relative abundance of *Lhcb2*1* is indicated by filled bars. Open arrowheads mark the position of the most photosynthetically active leaf predicted at the times samples were taken. Bars at individual leaf positions represent confidence intervals at the 95% level. N varied, ranging from 3 to 20, depending on whether the leaves were senescing, mature or recently initiated.

leaves being those at the base of the branch. Since individual peach leaves are considerably longer than their width, increases in leaf length can be used as a first approximation of leaf expansion. Results of leaf length measurements during the first portion of the growing season indicated that older leaves, e.g., the leaf at position 3 (leaf 3), had essentially ceased expansion before the first measuring date (25 Apr.). Leaves in the mid-portion of the branch continued to expand until the end of May, whereas the youngest leaves [represented by the leaf at position 13 (leaf 13)] expanded linearly from the middle of May up to the middle of June. Based on data obtained from leaf 13, an estimated average expansion rate of $4.4 \text{ mm} \cdot \text{d}^{-1}$ is obtained. It is difficult to compare the expansion rate obtained here with other studies, as vegetative growth in fruit trees is usually measured as an increase in leaf number per unit time (Haun and Coston, 1983), leaf dry mass or area (Miller and Walsh, 1988), or shoot length and/or mass (Blanco et al., 1995). Miller and Walsh (1988) observed that 'Loring' trees reached a maximum leaf area at the end of stage II of fruit development which occurred on 23 July in the season studied. This is in general agreement with our observations that, although 65% of the tagged branches were no longer initiating new leaves at 70 DAB (14 June 1994), the remainder continued to produce leaves which expanded through July (data not shown).

Leaves of dicot plants begin the transition from sink to source status just after the young leaf has begun to unfold (for a review see Turgeon, 1989); based on this morphological phenomenon one can estimate the most photosynthetically active leaf on a given branch at a specified time by counting four nodes basipetally from the tip-most completely unfolded leaf (Horton and Edwards, 1976). By this criterion the most photosynthetically active leaf during the early phase of the growing season progressed from leaf 8 at 38 DAB to leaf 15 at 70 DAB. Expression of genes associated with photosynthesis can also be used as an indicator of photosynthetic maturity. As expected, *Lhcb2*1* RNA abundance was highest in leaves which were at or near full expansion at the sampling times indicated (Fig. 5). Older leaves had lower concentrations of *Lhcb2*1* RNA which could reflect dilution of the RNAs due to an increase in leaf mass and/or leaf senescence occurring at the time samples were taken. This reduction in concentration is most clearly seen in samples taken at 70 DAB when individual leaves were collected. The lower abundance of *Lhcb2*1* RNA seen in leaf 16 (Fig. 5C) is probably an artifact reflecting the difficulty in staging these leaf positions, as the confidence limits were larger in measurements from position 15 and higher; indeed, leaves at positions beyond 18 could not be staged with confidence and so were not sampled in this experiment. Very young leaves also had lower levels of *Lhcb2*1* RNA than fully expanded leaves, reflecting the fact that like other photosynthetic genes, the *Lhcb* family is developmentally regulated.

We have isolated and characterized a gene from peach encoding one of the family members of chlorophyll *a/b*-binding proteins associated with LHCII. The expression of this gene was correlated with leaf development during the early phase of the growing season in a year where no fruit was set, thus allowing us to examine expression in the absence of competition from reproductive growth. Further work will be needed to provide insight into the regulation of *Lhcb2*1* under a variety of environmental conditions and during different phases of tree vegetative and reproductive growth.

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