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Sequence of the fourth and fifth Photosystem II Type I chlorophyll *a/b*-binding protein genes of *Arabidopsis thaliana* and evidence for the presence of a full complement of the extended CAB gene family

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Abstract

A second locus (*Lhb1B*) encoding Photosystem II Type I chlorophyll *a/b*-binding (CAB) polypeptides was identified in *Arabidopsis thaliana*. This locus carries two genes in an inverted orientation. The predicted sequences of the polypeptides encoded by these two genes show substantial divergence in their amino termini relative to each other and to the proteins encoded by the three *Lhb1* CAB genes previously characterized [10], but little divergence within the predicted primary structure of the mature protein. DNA probes derived from seven additional types of tomato CAB genes, encoding chlorophyll *a/b*-binding polypeptides of several antenna systems of the photosynthetic apparatus, were tested against *A. thaliana*. Each of these hybridized in Southern blots to unique DNA fragment(s), demonstrating the existence of each of these different types of CAB genes in the genome of *A. thaliana*. The number of genes encoding each CAB type in *A. thaliana* was estimated to be similar to that of tomato.

Introduction

Nuclear-encoded chlorophyll *a/b*-binding (CAB) polypeptides are a distinct class of structurally and evolutionarily related pigment-binding proteins found in chloroplast thylakoid membranes. These proteins are localized in antenna complexes such as the light-harvesting complex I (LHCI) associated with Photosystem I (PSI), and LHCII, CP24, and CP29 antenna systems associated with PSII. Chlorophyll molecules bound to CAB proteins in these antenna complexes are the primary acceptors of light energy. The LHCI, LHCII, CP24 and CP29 antenna complexes have been identified in many plant species, including *Arabidopsis thaliana* [1]. To date, we have isolated and characterized tomato genes encoding four different types of CAB polypeptides of LHCI,

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X64459 (*Lhb1B1*) and X64460 (*Lhb1B2*).

three different types of LHCII CAB polypeptides, one type of CP29 CAB polypeptide, and one type of CP24 CAB polypeptide [4, 22]. In *A. thaliana*, three tandemly linked genes encoding PS II Type I proteins have been isolated [10], and recently the isolation of a PSI Type IV gene was reported [26]. We report here (1) the sequence of two additional PSII Type I genes from *A. thaliana*, and (2) that homologous sequences to most, if not all, types of CAB genes identified to date exist in *A. thaliana*.

Materials and methods

Plant material, probes, and genomic Southern blots

Three ecotypes of *A. thaliana* ('Columbia', 'Landsberg', and 'Niederzanz') were used in this study. DNA from each race, extracted as described [19], was digested (2 μg per sample) with restriction enzymes, electrophoresed in a 0.8% agarose gel and alkaline-blotted to Hybond membrane (Amersham) according to the manufacturer's recommendations. Random-primed ^{32}P-labelled probes were prepared from the coding regions of the tomato genes encoding the different types of CAB proteins, and also from *A. thaliana* sequences flanking *Lhb1* CAB genes (Table 1). Hybridization was carried out at 65 °C in a solution containing 5× SSPE, 5× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml fish sperm DNA [18]. With the single exception of the *Lhb1* and *Lhb2* genes, different types of tomato CAB genes are > 50% divergent from each other, and do not cross-hybridize under the conditions utilized (i.e. final wash at 65 °C, 1× SSC, 0.5% SDS). Blots probed with the tomato *Lhb2* gene were washed at higher stringency (65 °C, 0.2× SSC, 0.1% SDS).

Isolation and sequence analysis of recombinant bacteriophage

Recombinant bacteriophage were isolated from a lambda DASH library of *A. thaliana* (race Columbia) genomic DNA (courtesy of Dr Nigel Crawford). Library screening followed standard techniques [18]. Nucleotide sequences were determined for both strands, and sequence data were analyzed using the MacVector program and a MacIntosh II computer.

Linkage analysis

A total of 57 F_2 plants derived from a single F_1 individual of the Landsberg × Niederzanz cross were allowed to self. F_3 seeds from each of the individual F_2 plants were germinated and pooled for DNA extraction. The MAPMAKER com-

Table 1. Tomato and *A. thaliana* CAB gene probes utilized in this study. CAB gene nomenclature adopted from Jansson *et al.* [8].

Gene	Synonym	Probe	Fragment used (kb)	Ref.
Lha1	PS I Type I	*cab6A*[a]	0.28 (*Nco* I + *Eco* RI) + 0.45 *Nco* I	[6]
Lha2	PS I Type II	*cab7*[a]	0.9 *Hin*d III + *Eco* RI	[14]
Lha3	PS I Type III	*cab8*[a]	0.55 *Hin*d III	[15]
Lha4	PS I Type IV	*cab11*[a]	0.7 *Pst* I + *Hun*d III	[21]
Lhb1	PS I Type I	*cab3C*[a]	1.3 *Xba* I + *Hin*d III	[12]
Lhb1A		p1655[b]	0.8 *Eco* RI + *Xba* I	This paper
Lhb1B		p3′CD[b]	3.1 *Xba* I + *Sal* I	This paper
Lhb2	PS II Type II	*cab5*	(p3–48) 0.5 *Pst* I	[13]
Lhb5	CP29 Type I[a]	*cab9*[a]	0.8 *Sst* I + *Bal* I	[17]
Lhb6	CP24	*cab10B*[a]	0.7 *Pst* I + *Hin*d III	[20]

[a] Tomato gene.
[b] Locus-specific probe from *A. thaliana* isolated for this study.

puter algorithm was used for linkage analyses [9].

Results and discussion

Identification and characterization of two genes residing at a second Lhb1 *locus*

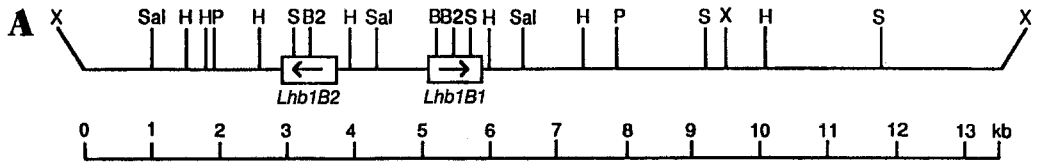
Leutwiler *et al.* [10] characterized three *Lhb1* genes residing on an 11 kb fragment of *A. thaliana* genomic DNA. Following guidelines for a standard CAB gene nomenclature system [8, see Table 1 for a cross-reference between CAB gene nomenclatures], we have re-designated the genes characterized by Leutwiler *et al.* [10] as *Lhb1A1* (= AB140 [10]), *Lhb1A2* (= AB165) and *Lhb1A3* (= AB180). Here we report the isolation and characterization of two additional, tandemly linked *A. thaliana Lhb1* genes. Because our evidence indicates these two genes reside at a separate locus from the three previously characterized *Lhb1* genes (see below), we have designated them as *Lhb1B1* and *Lhb1B2*.

Four genomic *Lhb1B* clones were recovered by plaque hybridization with a probe derived from the coding region of *A. thaliana Lhb1A1* [10]. A 6.0 kb *Pst* I fragment common to all four was found to contain two tandemly linked genes (in inverse orientation) separated by 1.3 kb of non-coding DNA (Fig. 1A). No other CAB genes were found within 10 kb spanning either side of the *Lhb1* genes carried by these four clones (Fig. 1 and data not shown). Sequence comparison of the two *Lhb1B* CAB genes presented here, *Lhb1B1* and *Lhb1B2*, and the three *Lhb1A* CAB genes previously characterized [10] indicates an overall similarity $>85\%$ at the nucleotide sequence; sequence divergence between *Lhb1B1* and *Lhb1B2* lies largely within the regions encoding the transit peptide and amino terminus of the mature protein (Figs. 1B, 1C). In contrast, nucleotide sequence differences between *Lhb1B* and *Lhb1A* genes are more evenly spread throughout the coding regions. The deduced amino acid sequences of all five mature *Lhb1* polypeptides are nearly identical to one another ($>95\%$), but there are a few locus-specific differences (Fig. 1C).

Evidence for two unlinked Lhb1 *CAB loci*

The Southern blot patterns we obtained with the tomato *Lhb1* gene probe displayed multiple fragments with all restriction enzymes used (Figs 2–4). They were also more complex than patterns previously observed [10]. Previously, restriction maps of the cloned *Lhb1A* genes and Southern blots of *A. thaliana* genomic DNA (probed with one of the *A. thaliana* cloned genes) showed that the three *Lhb1A* genes reside on 1.65 kb (*Lhb1A2*), 1.8 kb (*Lhb1A3*), and 6.0 kb (*Lhb1A1*) *Eco* RI fragments, and 1.4 kb (*Lhb1A1*), 3.8 kb (*Lhb1A2*) and 5.5 kb (*Lhb1A3*) *Hin*d III fragments [10]. We observed these same fragments on our Southern blots as well (Fig. 2, marked with filled squares). A previously observed but uncloned 2.0 kb *Hin*d III fragment [10] is indicated in Fig. 2 as an open square. It carries the *Lhb1B1* gene (Fig. 1A). A second previously uncloned *Hin*d III fragment of 1.4 kb, carrying the *Lhb1B2* gene, was originally missed [10] because it overlaps with the 1.4 kb *Hin*d III fragment of the *Lhb1A* gene (Figs. 1A, 2). However, our Southern blots displayed additional fragments (Fig. 2) as compared with the Southern blots in [10]. It seems likely that differences between our results and the previous results are due both to our utilization of lower-stringency hybridization conditions and to our recovery of high-molecular-weight DNA. The latter supposition may explain why the previous study did not detect the additional strongly hybridizing, high-molecular-weight (>25 kb) *Eco* RI fragment detected in our blots (Fig. 2) (E. Meyerowitz, personal communication). In addition, an *Eco* RI fragment >25 kb would not be present in a lambda phage-based genomic library of *Eco* RI-digested *A. thaliana* DNA [10], which explains why the *Lhb1B* genes were not recovered in the previous study.

Identification of two unlinked *Lhb1* loci was aided by the isolation of cloned DNA adjacent to both *Lhb1* gene clusters. Locus-specific probes derived from a non-coding region of *Lhb1B* identified *Xba* I fragments of ca. 15 kb from 'Niederzanz' (and 'Columbia') and ca. 17 kb from 'Landsberg' as *Lhb1B* (Fig. 2). The *Lhb1B* locus-



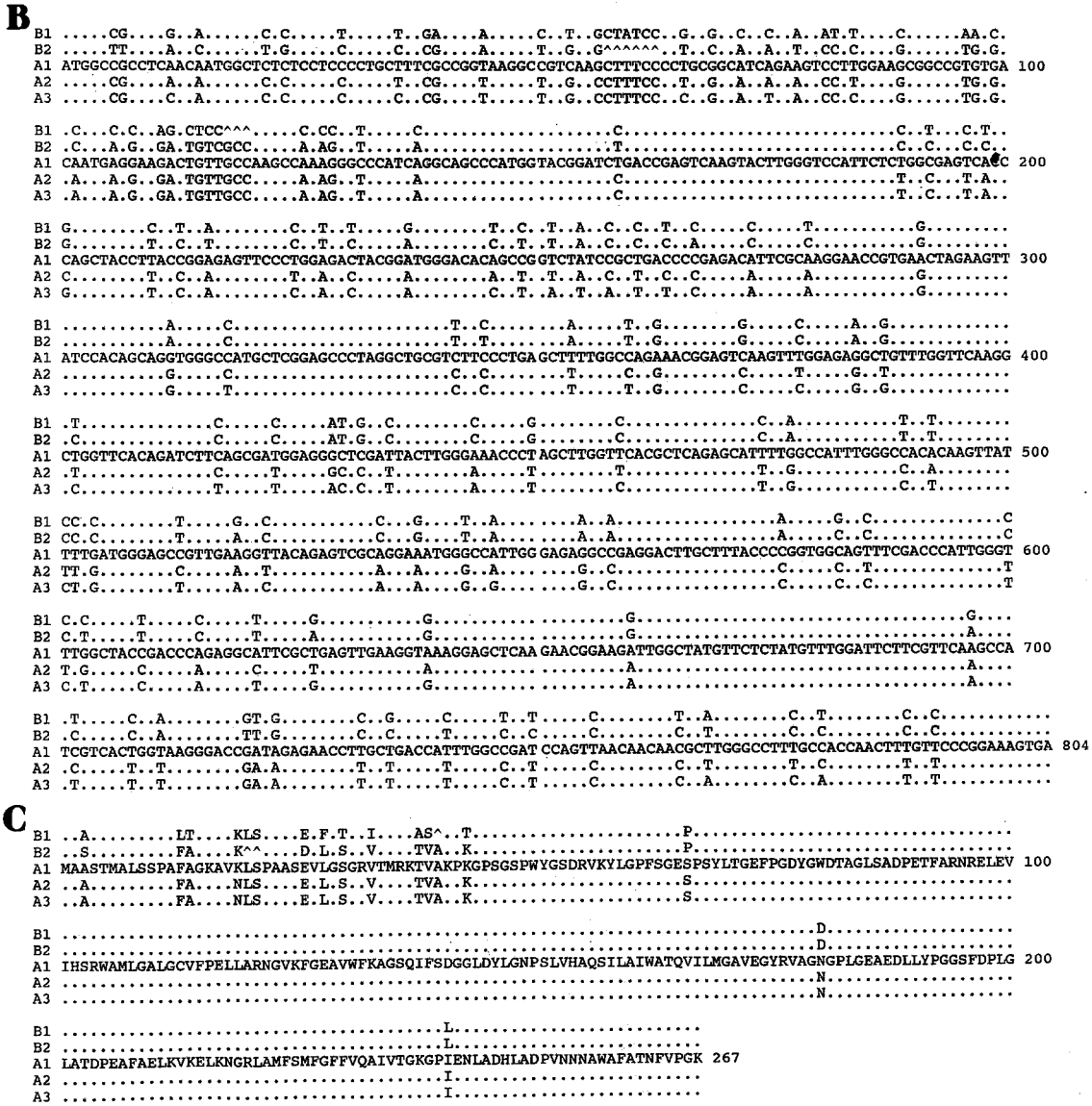


Fig. 1. A. Restriction map of *Lhb1B* CAB genes (B, *Bam* HI; B2, *Bgl* II; H, *Hind* III; P, *Pst* I; S, *Sac* I; Sal, *Sal* I; X, *Xba* I). B. Nucleotide sequence comparison of *A. thaliana Lhb1* CAB genes aligned and numbered relative to *Lhb1A1* [data from 10]. Note that both *Lhb1B* CAB genes have suffered deletions (indicated by ^) within the first 150 bp compared with *Lhb1A*. C. Predicted polypeptide sequence of *Lhb1* CAB genes aligned relative to *Lhb1A1* [data from 10].

specific probes also hybridized strongly with a single *Eco* RI fragment >25 kb (Fig. 2). Similarly, a gene-specific probe derived from a non-coding region of *Lhb1A* (the promoter of *Lhb1A2*) identified the polymorphic *Xba* I fragments >20 kb as the *Lhb1A* locus and did not hybrid-

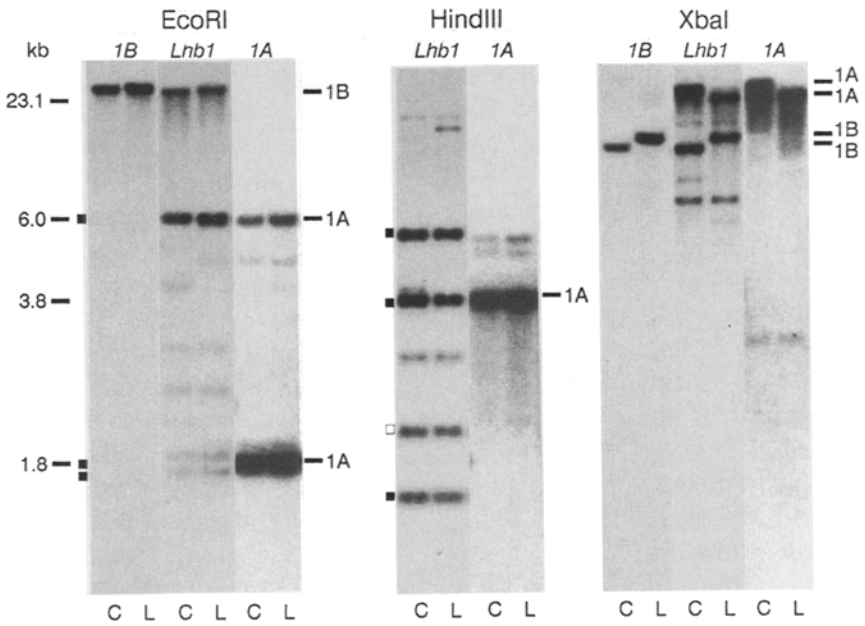


Fig. 2. Comparisons of Southern blots of 'Columbia' (C) and 'Landsberg' (L) DNA digested with three restriction enzymes and probed with tomato *Lhb1* gene probe (lane *Lhb1*), *A. thaliana Lhb1A* locus-specific probe (lanes 1A) and *A. thaliana Lhb1B* locus-specific probe (lanes 1B). Alphanumerics at the right of each panel indicate the locus designation of each polymorphic fragment identified in the segregation analysis (i.e., 1A = *Lhb1A*; 1B = *Lhb1B*). Boxes indicate the position of fragments observed in the Southern blots of Leutwiler *et al.* [10] (see text for details).

ize with the smaller *XbaI* fragments or >25 kb *Eco* RI fragment (Fig. 2). Independent segregation of these polymorphisms in Landsberg × Niederzanz F_3 families indicated that the two loci, *Lhb1A* and *Lhb1B*, are unlinked (Fig. 3).

These data indicate that *A. thaliana* has at least five *Lhb1* genes. Tomato has eight such genes [16], and all other species examined, with perhaps one exception, possess five or more *Lhb1* genes (for examples see [2, 3]). The exception was from cucumber in which a Southern blot of *Hin*d III-digested DNA showing six bands was interpreted to indicate that the cucumber genome possesses only two *Lhb1* genes [5]. However, this estimate was based on an untested assumption that each cucumber *Lhb1* gene contained an internal *Hin*d III site which the probe spanned (although this should still give an estimate of three genes, rather than two). Also not taken into consideration was the possibility that genes were tandemly linked, a common arrangement for *Lhb1* genes [2, 10, 12], and thus several genes may

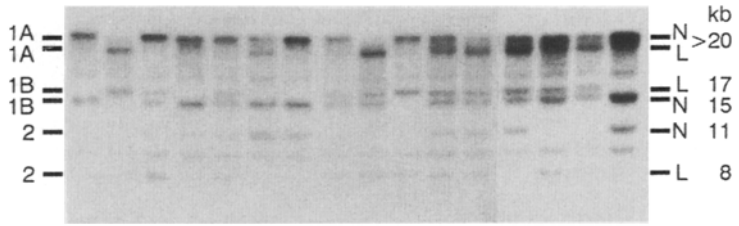


Fig. 3. F_3 segregation at three loci. The DNA of pooled F_3 plants from individual F_2 plants was digested with *Xba* I, and the probe was a tomato *Lhb1* CAB gene. Fragments labelled 1A and 1B are alleles of the *Lhb1A* and *Lhb1B* loci, respectively. Fragments labelled 2 are alleles of *Lhb2* (see text). N = 'Niederzanz' allele; L = 'Landsberg' allele.

wholly or partially reside on a single fragment. Hence, re-evaluation of the cucumber data suggests that the number of *Lhb1* genes in cucumber is likely to be greater than two, and possibly as many as five.

Evidence for an Lhb2 *locus in* A. thaliana

Genes encoding Photosystem II Type II CAB proteins (designated as *Lhb2* genes) are 70% identical to *Lhb1* genes [13]. Thus, some of the fragments we observed in our blots probed with the *Lhb1* probe are likely to carry *Lhb2* genes. Consistent with this hypothesis, Southern blots probed with the tomato *Lhb2* gene displayed a set of hybridizing fragments which largely overlapped the set of fragments hybridizing to the *Lhb1* probe. However, the two probe types hybridized with different efficiency to individual fragments, as seen in side-by-side comparisons of autoradiographs probed with the *Lhb1* and *Lhb2* probes and examined for relative differences in intensities of all fragments within the same DNA sample (compare Figs. 4a, b).

All fragments which showed preferential hybridization to the *Lhb2* probe and which were polymorphic between accessions were tested for segregation. With each of the four restriction enzymes tested, these fragments mapped to the same genetic locus, designated *Lhb2*, and this locus segregated independently from *Lhb1A* and *Lhb1B* (Fig. 3 and data not shown). For example, *Xba* I fragments of 8 and 11 kb hybridized more intensely with the *Lhb2* probe than with the *Lhb1* probe (compare Figs. 4a, 4b) and they segregated independently from the *Lhb1* fragments (Fig. 3).

As in the case of *Lhb1A* and *Lhb1B* loci, frag-

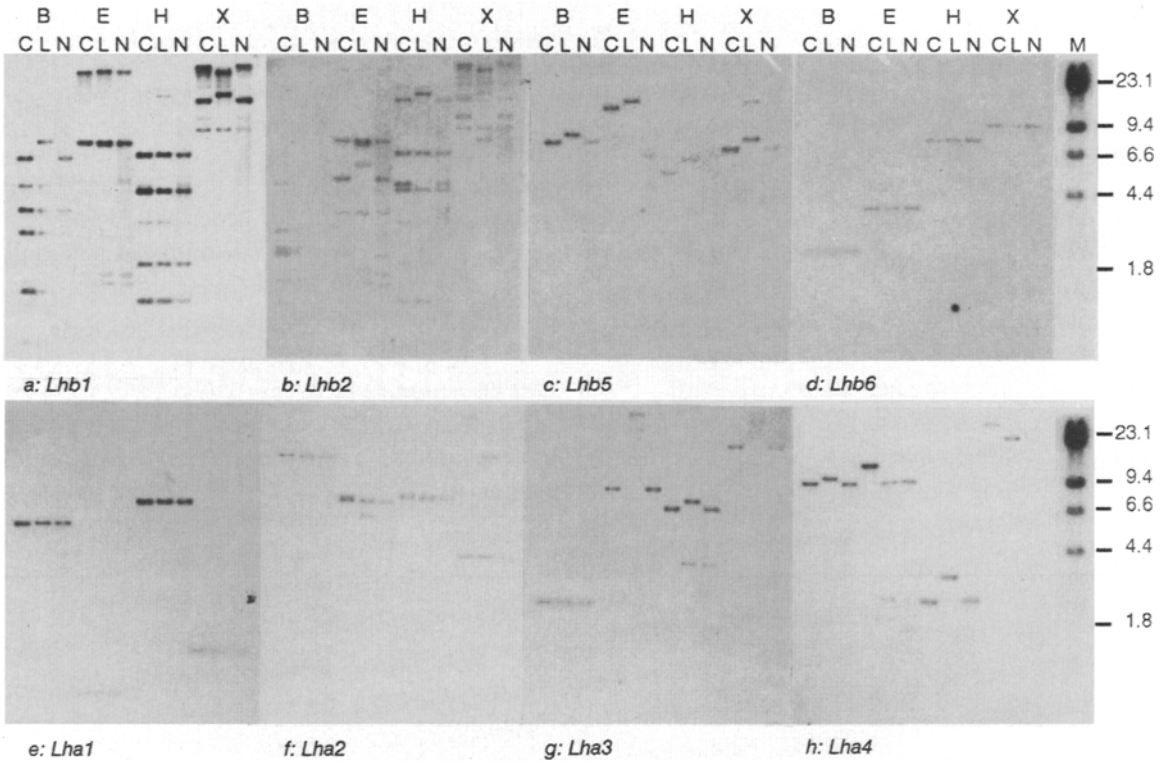


Fig. 4. Southern blots of *A. thaliana* DNA from three ecotypes (C = 'Columbia'; L = 'Landsberg'; N = 'Niederzanz';) probed with eight different tomato CAB genes types. In each panel, the three lanes on the left are DNA digested with *Bgl* II (B), next three lanes are DNA cut with *Eco* RI (E), the next three lanes with *Hin*d III (H), and the last three lanes on the right with *Xba* I (X). (See Table 1 for the source and size of the probes).

ment(s) identifying the *Lhb2* locus were also large enough to contain multiple copies of this gene, and further study is required to determine the exact number of the *Lhb2* genes in the *A. thaliana* genome. Since not all hybridizing fragments in all blots with the *Lhb1* and *Lhb2* probes could be mapped, estimates presented for the number of loci and the number of genes of these two CAB types (Table 2) are minimal estimates. In addition, other faint bands, which could not be mapped for lack of polymorphism, were present in these two blots, and may indicate the presence of a third PSII CAB gene type (*Lhb3*) recently identified in tomato [22]. Probing *A. thaliana* DNA with the tomato *Lhb3* gene gave a weak hybridization signal which was difficult to interpret (data not shown), but the existence of the *Lhb3* gene product in *A. thaliana* has recently been reported [11].

Evidence for other members of the extended CAB gene family in A. thaliana

We prepared eight duplicate Southern blots of DNA from three different *A. thaliana* ecotypes each digested with four different restriction enzymes. In addition to *Lhb1* and *Lhb2* probes, each of the other six blots was probed with one of six other tomato CAB gene types (see Table 1). Results indicate that each probe hybridized to one or a few fragments (Fig. 4). The fragments to which these probes hybridized were unique and were not recognized by other tomato CAB gene probes.

Table 2. Comparison between the number of genes from each different CAB type in tomato with the estimate for the minimum number of corresponding genes in *A. thaliana*.

	A. thaliana		Tomato	
	Number of loci	Number of genes	Number of loci	Number of genes
Lha1	1	1	1	2
Lha2	≥1	≥1	1	1
Lha3	1	1	1	1
Lha4	1	1	2	2
Lhb1	≥2	≥5	3	8
Lhb2	≥1	≥1	2	2
Lhb5	1	≥1	1	1
Lhb6	1	1	1	2

The tomato genes encoding *Lha1* and *Lhb6* each recognized a single fragment with all enzymes tested (Fig. 4e and 4d, respectively). The minimum fragment size observed with the *Lha1* probe was ca. 0.75 kb, and the minimum size fragment identified by the *Lhb6* probe was ca. 2.5 kb. With the tomato probes for *Lha3*, *Lha4*, and *Lhb5* single fragments were commonly seen (Fig. 4g, h, c). The *Lha3* probe showed a minimum fragment size of ca. 3.0 kb with the enzyme *Bgl* II. Likewise, the smallest fragment seen with the *Lha4* probe was ca. 2.5 kb. Fragment sizes of <3 kb were considered to be large enough to code for only a single gene (i.e. coding region, introns and promoter region). The minimum fragment size observed with the tomato *Lhb5* probe was ca. 6.0 kb, which is sufficient to contain a tandem duplication in this region. Therefore, further experiments need to be done to determine if *A. thaliana* has one or two genes for the *Lhb5* protein. With the *Lha2* probe, two or three fragments were seen in most enzyme digests (Fig. 4f). It seems possible that *A. thaliana* has more than one gene encoding the *Lha2* protein; tomato has a single gene of this kind [16], while petunia has several [23].

We conclude from the Southern blot data that the *A. thaliana* genome contains genes homologous to all eight different types of tomato CAB genes used in this study. These data also demonstrate that, although the different CAB genes are structurally and evolutionarily related, substantial divergence has occurred between CAB gene types such that probes derived from different CAB genes within the same organism do not usually cross-hybridize. Thus an estimate of the minimum number of CAB genes within a species cannot be obtained from Southern blots unless an entire set of CAB genes are used as probes. Our data also demonstrate that CAB genes of a specific type are more similar to genes encoding the same type in other species than are CAB gene types within species, and that the number of CAB

genes in *A. thaliana* is roughly equivalent to the numbers seen in tomato (Table 2).

Chlorophyll *a/b*-binding polypeptides were first discovered in the light-harvesting complex of PSII [24, 25]. Since then, the discovery of structurally and evolutionarily related CAB polypeptides in other antenna systems (LHCI, CP29, CP24) has resulted in the characterization of an 'extended' gene family whose members encode these proteins [4, 16]. The data presented here demonstrate the existence in the *A. thaliana* genome of all the different types of CAB genes presently known, consistent with the observation that all the different types of antenna complexes are present in the thylakoid membranes of *A. thaliana* chloroplasts [1]. These results also suggest that other plant species will be found to possess all or most of these genes, as has been partly demonstrated in the gymnosperm Scots pine [7].

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