



HOKKAIDO UNIVERSITY

Title	LIL3, a light-harvesting-like protein, plays an essential role in chlorophyll and tocopherol biosynthesis
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Supplementary Information

Identification of chlorophyll derivatives with LC-MS Acetone-extracted photosynthetic pigments were separated on a C8 column and the absorption spectra between 400 nm and 700 nm were recorded by a photodiode array detector (See Fig. S4). In Fig. S4, representative chromatograms from WT and the mutant seedlings at 685 nm and 465 nm are shown indicating the absorption of Chl *a* and *b* derivatives, respectively. While WT samples resulted in two major peaks corresponding to Chl *a* and Chl *b*, the acetone extracts from *lil3:1*, *lil3:2* and *lil3:1/lil3:2* generated two to six extra peaks in addition to Chl *a* and Chl *b* (Fig. S4). Peaks 1, 2, and 3 showed identical absorption spectra to that of Chl *a* (peak 4). In contrast, peaks 5, 6 and 7 showed identical absorption spectra to that of Chl *b*. Note that peaks 2 and 8 comigrate at the same elution time on our HPLC system and they were distinguished by their absorption spectra and by mass spectrometry as described below. From these results, we speculated that peaks 1, 2 and 3 are derivatives of Chl *a*, and peaks 5, 6 and 7 are derivatives of Chl *b*.

Identification of Chl species by mass spectrometry. Chl derivatives were separated on an Agilent 1200 HPLC system according to the method of Zapata et al. (1) with the flow rate set to 1.0 ml/min. The eluate was split into two paths and 0.2 ml/min was sent to the time-of-flight mass spectrometer (JMS-T100LP, JEOL, Tokyo, Japan) that was equipped with an APCI (atmospheric pressure chemical ionization) source. The condition of the mass spectrometer was as follows: positive ionization mode and a scan range of m/z 100–2000. The needle voltage was set at 5000 V; the orifice 1 voltage was set at 40 V; the orifice 2 voltage was set at 5 V; the ring lens voltage was set at 10 V.

In our next step, we analyzed the mass spectra (MS) of these peaks to identify the pigments. Peak 1, 2, 3

and 4 gave major MS signals at 887.5, 889.5, 891.5, and 893.5. We observed that these peaks also shared an identical signal at 615, which most likely refers to chlorophyllide *a*. Peaks 5, 6, and 7 showed major MS peaks at 903.5, 905.5 and 907.5. These peaks share a common MS signal at 629, which most likely corresponds to chlorophyllide *b*. The MS signal of the peak 8 was below a detectable level in our analytical MS system. From these results, we concluded that peaks 1 - 8 represent Chl *a*-phytol (Chl *a*_{phy}), Chl *a*-tetrahydrogeranylgeraniol (Chl *a*_{THGG}), Chl *a*-dihydrogeranylgeraniol (Chl *a*_{DHGG}), Chl *a*-geranylgeraniol (Chl *a*_{GG}), Chl *b*-phytol (Chl *b*_{phytol}), Chl *b*-tetrahydrogeranylgeraniol (Chl *b*_{THGG}), Chl *b*-dihydrogeranylgeraniol (Chl *b*_{DHGG}), and Chl *a*-geranylgeraniol (Chl *b*_{GG}), respectively. (See Fig. 1A)

RT-PCR. Total RNA was extracted from the fourth whorl of four-week old plants using the RNeasy Plant RNA extraction kit (Qiagen). cDNA was synthesized with the PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan) and amplified with Sybr Premix Ex Taq (Takara Bio Inc.) according to the manufacturer's instruction using the following primer pair; *CHLP* forward primer, TGAGGTCTATCTCCTTTCTTCA and *CHLP* reverse primer, AGCCAAATGCTTTTGGACTA. The amplicon was quantified with a LightCycler real-time PCR (Roche Diagnostics K. K.). The relative levels of *CHLP* mRNA were normalized by the levels of *UBC21* (AT5G25760) which was amplified with the following primer set: *UBC21-F*, CTGCGACTCAGGGAATCTTCTAA and *UBC21-R*, TTGTGCCATTGAATTGAACCC.

Split-ubiquitin assay. The coding sequences of *LIL3:1* and *LIL3:2* were cloned into the pCub bait vector (Dualsystems Biotech AG, Schlieren, Switzerland). The coding sequence of *CHLP* was cloned into the pNub prey vector (Dualsystems Biotech AG). As negative controls of the prey and bait vectors, we used the potato

sucrose transporter gene *SUT1* that was cloned into pCub and pNub vectors (2). The bait vectors were transformed into the Lcc40uA yeast strain and positive colonies were selected on SD media lacking leucine (selection marker gene on Cub vector). The new generated strains were each transformed with the prey vectors and positive colonies containing both prey and bait vectors were selected a) on SD media lacking leucine and tryptophan (selection marker on Cub and Nub vector) and b) on SD media lacking leucine, tryptophan, histidine, uracil. Colonies harboring both the prey and bait vectors were able to grow on the former media. The *in vivo* interaction of the proteins encoded in the prey and bait vectors further facilitated growth of the colonies on the latter media.

BN-PAGE analysis Thylakoid membranes were isolated from 4-week-old WT rosette leaves by the method described by Block et al. (2). BN-PAGE was performed according to the methods described by Wittig et al. (3). Briefly, thylakoid membrane proteins (corresponding to 10 µg of chlorophyll) was resuspended in the following buffer: 50mM imidazole-HCl (pH 7.0), 20% glycerol, 5mM 6-aminocaproic acid, 1mM EDTA. Resuspended thylakoid membranes were solubilized with 1% (w/v) β-dodecyl maltoside on ice for 5 min and were electrophoretically separated on 4-13% acrylamide gradient gels. For immunoblot analysis, proteins were blotted on PVDF membranes with transfer buffer (25mM Tris, 192mM glycine, 20% methanol) by the wet transfer method. NativeMark unstained molecular weight markers (Invitrogen) was used for estimation of protein size.

Legends to supplementary materials

Fig. S1. LHC motifs in the LHC and LHC-like proteins of *Arabidopsis* (A) and prediction of transmembrane domains with LIL3:1 (B) and LIL3:2 sequences (C). LHC and LHC-like proteins were identified and aligned with the BLASTP program (4). At the top of the alignment, the generic LHC motif defined by Jansson (5) is shown for comparison. Genbank accession numbers are as follows:

LHCB1 (NP_564339.1), LHCB2 (NP_565786.1), PsbS (NP_175092.1), OHP1 (NP_195832.1), ELIP2 (NP_567438.1), ELIP1 (NP_188923.1), SEP2 (NP_565524.1), OHP2 (NP_564432.3), LIL3:2 (NP_199522.2), SEP1 (NP_567958.1), LIL3:1 (NP_567532.1), ferrochelatase 2 (FeC2, NP_180598.1).

Location of transmembrane domains within LIL3:1 and LIL3:2 sequences were predicted with the TMHMM program (6). The calculated probabilities for transmembrane helices, inside loops and outside loops are indicated on the Y axes. Amino acid positions are indicated on the X axes. Location of the LHC motifs described in the panel (A) are shown with bars in panels (B) and (C).

Fig. S2. Phylogenetic relationship of LIL3 proteins from green algae and land plants. LIL3 proteins were identified by a homology search with BLASTP (5) with a threshold detection limit of the E value = e^{-10} . The sequences were aligned with ClustalW (7) and manually trimmed. The multiple alignment of LIL3 sequences was used to deduce phylogenetic relationship with the Neighbor Joining method (8) by ClustalW. The bootstrap value calculated from 1,000 iterations was shown on each node.

LIL3 homologues are exclusively distributed in the organisms belonging to the green lineage of the photosynthetic eukaryotes. Green alga including *Chlamydomonas reinhardtii*, *Mesostigma*, *Micromonas*, and *Ostreococcus* appear to have one copy of *LIL3* homologues in their genomes. In contrast, mosses and higher

plants seem to have multiple *LIL3* homologues. Specifically, *Arabidopsis thaliana*, *Brassica napus*, rice (*Oryza*), maize (*Zea mays*) and *Physcomitrella* have two copies. A phylogenetic analysis indicates that most pairs of the angiosperm *LIL3* homologues arose very recently after the divergence of mono- and dicotyledonous plants, since a pair of homologues of one organism is more similar than the *LIL3* proteins between two different organisms. In contrast, a few species (*Ricinus*, *Populus* and *Vitis*) have relatively dissimilar sets of *LIL3* homologues. In these organisms, *LIL3* homologues were grouped into two different clades. All of the *LIL3* homologues of the green lineage share striking similarity in their C-terminal half of the amino acid sequences. This region includes the LHC motif, while the N-terminal half of the *LIL3* sequences are less similar and often intervened by gaps and insertions.

The full species names and the accession numbers are as follows: Brassica1 (*Brassica oleracea*, ABD64919.1); Brassica2 (*Brassica oleracea*, ABD65018.1); Populus1 (*Populus trichocarpa*, XP_002329601.1); Ricinus (*Ricinus communis*, XP_002524363.1); Populus2 (*Populus trichocarpa*, XP_002326480.1); Oryza1 (*Oryza sativa*, BAG92255.1); Oryza2 (*Oryza sativa*, BAD08011.1); Sorghum (*Sorghum bicolor*, XP_002451453.1); Zea1 (*Zea mays*, NP_001151867.1); Zea2 (*Zea mays*, ACG25699.1); Glycine (*Glycine max*, ACU19934.1); Picea (*Picea sitchensis*, ABK25387.1); Vitis1 (*Vitis vinifera*, XP_002275690.1); Physcomitrella1 (*Physcomitrella patens*, XP_001756081.1); Populus3 (*Populus trichocarpa*, XP_002337566.1); Vitis2 (*Vitis vinifera*, XP_002269204.1); Physcomitrella2 (*Physcomitrella patens*, XP_001782647.1); Chlamydomonas (*Chlamydomonas reinhardtii*, XP_001699421.1); Ricinus (*Ricinus communis*, XP_002511036.1); Arachis (*Arachis hypogaea*, ACF74338.1); Micromonas (*Micromonas* sp. RCC299, XP_002501670.1); Mesostigma (*Mesostigma viride*, ABD58894.1); Ostreococcus (*Ostreococcus tauri*, CAL52848.1); Populus4 (*Populus trichocarpa*, XP_002321782.1).

Fig. S3. Comparison of the *Arabidopsis lil3* mutants. The WT, *lil3:1*, *lil3:2* and *lil3:1/lil3:2* mutants were grown for four weeks under continuous illumination at a light intensity of approximately 80 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ as described in Materials and Methods. Scale bar = 1 cm.

Fig. S4. Accumulation of Chl and its derivatives that are characterized by the conjugation of incompletely reduced side chains. Chl derivatives were separated by HPLC as described in Materials and Methods. Elution was monitored by absorbance from 400 nm to 700 nm. The four panels on the left side show the elution profiles of Chl derivatives at 685 nm to mainly emphasize the different forms of Chl *a*, as Chl *b* derivatives have a much lower absorption coefficient at 685 nm than Chl *a*. The four panels on the right side show the elution profiles at 465 nm to emphasize the derivatives of Chl *b*, visualizing the Chl *a* derivatives to a lesser extent. Peak 1, Chl *a*-GG; Peak 2, Chl *a*-DHGG; Peak 3, Chl *a*-THGG; Peak 4, Chl *a*-phytol; Peak 5, Chl *b*-GG; Peak 6, Chl *b*-DHGG; Peak 7, Chl *b*-THGG; Peak 8, Chl *b*-phytol,

Fig. S5. Immunoblotting analysis of the geranylgeranyl reductase levels in the *lil3* mutants with a larger volume of protein extracts and a higher concentration of the primary antibody compared to Fig. 3. A larger volume of protein extracts which is equivalent to 2 mg leaf material from WT, *lil3:1*, *lil3:2* and *lil3:1/lil3:2* were separated on SDS-PAGE and transferred onto a PVDF membrane. The geranylgeranyl reductase protein was detected with anti-geranylgeranyl reductase antiserum (x 4,000 dilution) using an ECL-plus chemiluminescent system (GE Healthcare).

Fig. S6. Analysis of α -tocopherol in the *lil3* mutants. HPLC chromatograms show the separation of α -tocopherol (5.2 min) in WT, *lil3:1*, *lil3:2* and *lil3:1/lil3:2*. α -tocopherol was excited at 290 nm and fluorescence emission was monitored at 320 nm. The Y-axis is roughly proportional to the amount of leaf material used for the extraction of α -tocopherol. Note that α -tocotrienol could not be detected which should appear at around 4.1 min if present.

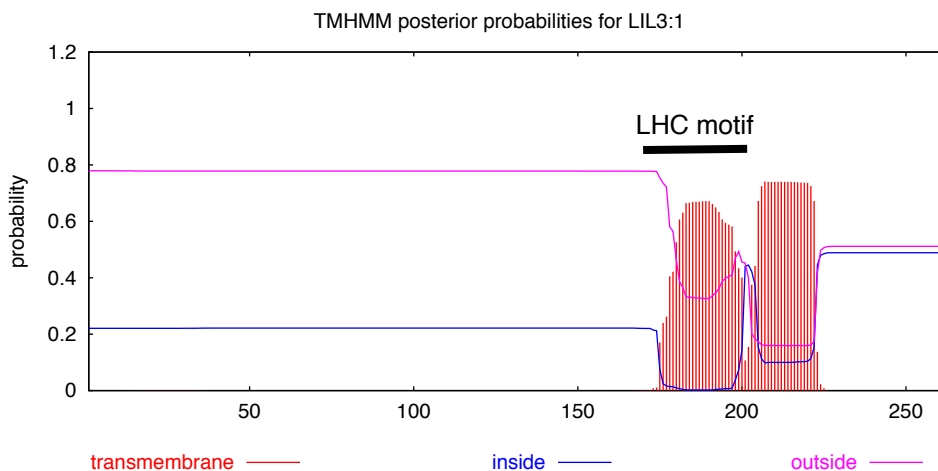
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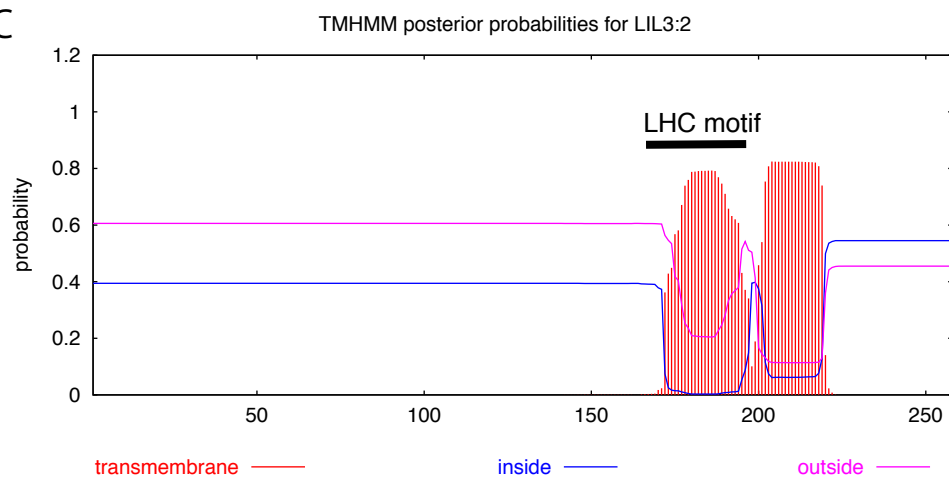
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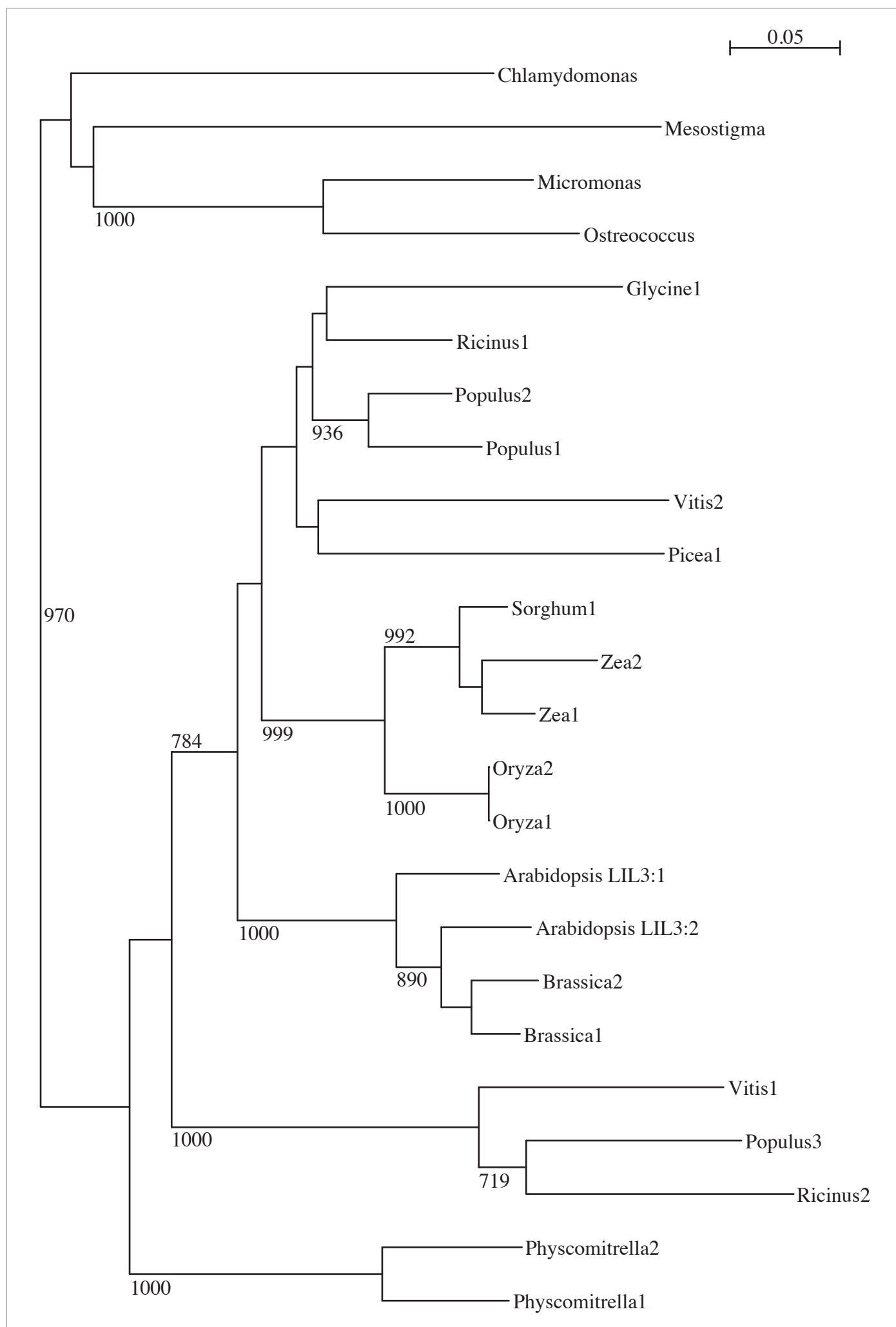
LHC motif	1	ELINGRLAMIGFLGFLVPELIT	22
LHCB1 helix1	99	EVIIHSRWAMIGALGCVFPELLARNGVKFGE	128
LHCB1 helix3	215	ELKNGRLAMFSMFQFFVQAIVTGKGPINL	244
LHCB2 helix3	213	ELKNGRLAMFSMFQFFVQAIVTGKGPINL	242
LIL3:1	174	ELLNGRAMIGFFMAYFVDSLTVGVLVDQM	203
LIL3:2	171	ELLNGRAMIGFFMAYFVDSLTVGVLVDQM	200
FeC2	477	ETWNGRAMLAVLALLVLEVTIGKGFLLQW	506
SEP1	85	DIWLGRGAMVGFVAIVTVEISTGKGLLENF	114
SEP2	105	ETISGRLAMIVFAVTVTETIVTGNLFFKKL	134
OHP1	69	EIWNLRACMIGLIGTFIVELILNKGILELI	89
OHP2	130	EISNGRWAMIGFAVGMLTEYATGSDLVDQV	159
ELIP1	98	ERINGRLAMVGFVAALAVELSKGENVLAQI	127
ELIP2	96	ERINGRLAMVGFVAAIAMELSKGENVFAQI	125
PsbS helix1	90	ELFVGRVAMIGFAASLLGEALTGKGI LAQL	119
PsbS helix3	194	ELFVGR LAQLGIAFSLIGETITGKGA LAQL	203

B



C





WT

lil3:1



lil3:2

lil3:1/lil3:2

