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Lil3 dimerization and chlorophyll binding in *Arabidopsis thaliana*

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ABSTRACT

The two-helix light harvesting like (Lil) protein Lil3 belongs to the family of chlorophyll binding light harvesting proteins of photosynthetic membranes. A function in tetrapyrrol synthesis and stabilization of geranylgeraniol reductase has been shown. Lil proteins contain the chlorophyll a/b-binding motif; however, binding of chlorophyll has not been demonstrated. We find that Lil3.2 from *Arabidopsis thaliana* forms heterodimers with Lil3.1 and binds chlorophyll. Lil3.2 heterodimerization (25 ± 7.8 nM) is favored relative to homodimerization (431 ± 59 nM). Interaction of Lil3.2 with chlorophyll a (231 ± 49 nM) suggests that heterodimerization precedes binding of chlorophyll in *Arabidopsis thaliana*.

1. Introduction

The light harvesting like protein 3 (Lil3) belongs to the two-helix stress-enhanced proteins (SEPs) in higher plants. The protein carries a light harvesting complex (LHC) motif that classifies the LHC-like protein family. The family is divided according to the number of transmembrane helices: three-helix early light-induced proteins (ELIP), two-helix SEP and one-helix proteins (OHP) [1,7,13]. The LHC motif was originally described as an overall hydrophobic amino acid sequence composed of 22 amino acids with two charged amino acids: glutamic acid (E), arginine (R) and three conserved glycine (G) residue with the consensus sequence ELINGRLAMLGFLGFLVPELIT [20]. Two 16-mers peptides synthetically designed in the N-terminus of this motif were shown to bind Chlorophyll (Chl) [5]. A motif was defined and shown to be required for Chl binding: E-X-X-H/N-X-R or R-X-N/H-X-X-E [5,12,19,20] in which residues E and H/N were found responsible for coordination of the central Mg^{2+} ion in Chl. The anion carbonyl group in E- and the guanidinium group in R-residues were also shown to play an important role for salt ion paring in E139-R142, E65-R185 and E180-R70 [22].

The function of Lil3 in protecting the plant against stress has been discussed the last decade [4,31,33,37]. Lil3 has been shown to associate with Chl and tocopherol synthesis in *Arabidopsis thaliana* [37]. Recently, the transmembrane amino acids of the LHC-motif in Lil3 were reported to structurally anchor geranylgeranyl reductase (GGR) to the membrane, and to be responsible for the oligomerization of GGR [35]. It has been shown that a recombinant GGR protein functionally did not require Lil3 for the reduction of geranylgeranyl pyrophosphate (GGPP) to phytyl pyrophosphate (PYPP) in plant [35]. GGR catalyzes the NADPH dependent three-step reduction of the pyrophosphate (PP) form of GG or its esterified form in Chl_{GG}. The products of this enzymatic reaction is PYPP or Chl_{PY} [36].

Cyanobacterial high-light inducible proteins (Hlips) are the ancestors of LHC antennae and other members of the LHC super family. A recent study showed that HliD purified from Synechocystis bound Chl a and β-carotene and exhibited an energy dissipative function [33]. The above-mentioned study suggested that the quenching mechanism works *via* a direct energy transfer from a Chl a Q_y state to the β-carotene S_1 state in the LHC superfamily. In barley (*Hordeum vulgare* L.), Lil3 was identified as the first protein to bind Chl during deetiolation [31]. In etioplast membranes, Lil3 was characterized to bind chlorophyllide and Chl in supercomplexes during Chl-synthesis [25]. In this study, the dissociation

Abbreviations: LHC, light harvesting complex; Chl, Chlorophyll

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constants for interaction of Lil3 proteins with chlorophyll a were determined with microscale thermophoresis. We find that the dissociation constant for heterodimerization of proteins Lil3.1 and Lil3.2 from *A. thaliana* is lower than for binding of chlorophyll a by Lil3.2.

2. Materials and methods

2.1. Split ubiquitin

The split ubiquitin assay was carried out according to the DUALmembrane starter kit (Dualsystems Biotech Inc. Schlieren, Switzerland). Genes were amplified from cDNA using *Pwo* polymerase (Roche, Basel, Switzerland), cloned into pPCR-Script (Stratagene, California, USA). The coding sequence of *Lil3:1* (At4g17600), *Lil3:2* (At5g47110), were cloned into the bait (pBT-C) and prey (pPR-N and pPR-C) vectors (Dualsystems Biotech AG, Schlieren, Switzerland). Yeast NMY51 cells were co-transformed with the resulting plasmids according to the manufacturer's instructions (Dualsystems Biotech AG, Schlieren, Switzerland). In the split ubiquitin assay with Lil3:1 as bait, the selective media (SD-WLAH) was supplied with 5 mM *of 3-aminotriazole* (*3-AT*). Coexpression analysis experiments were repeated four times, two times in each direction.

2.2. Protein expression

The Lil3 genes, (Lil3.1 – AT4G17600 and Lil3.2 – AT5G47110), of *A. thaliana* were PCR amplified from pUNI51 plasmids containing the Lil3 sequences obtained from TAIR. The amplified sequences were cloned into the pET151d expression vector (Invitrogen, Carlsbad, CA, USA). The Lil3_pET151d plasmids were transformed to BL21 *Escherichia coli* chemically competent cells for expression of recombinant protein. Cultures were induced at OD_{600} = 400 nm with a final concentration of 1 mM IPTG and incubated at 16 °C on prior to harvest (6000×*g*, 15 min, 4 °C).

2.3. Cell lysis and protein purification

Cells were lysed under non-denaturing conditions using 20× volume lysis buffer (50 mM K_2HPO_4, 400 mM NaCl, 100 mM KCl, 10% Glycerol, 0.5% Triton X, 10 mM Imidazole, 0.8 mg/mL lysosome and complete protease inhibitor cocktail tablet (Roche)) and incubated on ice 1 h prior to sonication 6× 10 s at 30 amplitudes. The filtrated crude protein extract was purified on a 1 mL His-Trap HP column (GE Healthcare Life Sciences) according to the manufacturers instructions with a non-denaturing buffer (25 mM HEPES pH 7.5, 300 mM NaCl and 10 mM-500 mM Imidazole), 1 mL fractions were collected. Fractions were separated on a 12% SDS–PAGE [23], stained by Coomassie Brilliant Blue (CBB) and transferred to nitrocellulose (NC) membranes [38,39] for subsequent immunological identification of recombinant Lil3_his (monoclonal anti-polyHistidine Antibody produced in mouse, Sigma Aldrich) (not shown). Lil3_his fractions were separated on a HiTrap desalting column (GE healthcare, Buckinghamshire, United Kingdom) with a desalting buffer (25 mM HEPES pH 7.5, 30 mM NaCl) to desalt and ensure refolding of protein. Protein concentrations were determined by the BCA method and purified Lil3 was verified by mass spectrometry (Waters Corporation, Milford, MA, USA).

2.4. Surface plasmon resonance

The SPR measurements were carried out on a Biacore® T-100/T-200 instrument (GE-Healthcare) using CM5 chips. Purified Lil3.1 and Lil3.2 were either immobilized (ligand) on a CM5 chip or in the mobile phase (analyte, 0–3000 nM) and run using single channel analysis. The CM5 chips used were preconditioned with 3× 10 s injections of running buffer (1× HBS-EP: 10 mM HEPES pH 7.4, 0.15 M NaCl, 3.4 mM EDTA and 0.005% surfactant P20), 2× 10 s injections of 100 mM HCl, 2× 10 s injections of 50 mM NaOH and finally 2× 10 s injection of sodium monododecyl sulfate with the flow rate set to 100 μl/min. Reactive succinimide esters were created by injecting a 1:1 mixture of *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (Life technologies, Oslo, Norway) and *N*-hydroxysuccinimide (Life Technologies, Oslo Norway) for 420 s with the flow rate set to 5 μl/min. Ligands where then covalently immobilized (1 min., 10 μl/min) and unreacted esters blocked by ethanolamine-HCl (240 s, 5 μl/min). The ligands were covalently linked to the CM5 chip surface at densities of 200 RU for multiple channel runs and 500 RU for single channel runs. Flow channel one (FC1) was treated as FC2-FC4 except no ligand was coupled to the sensor chip channel. Analyte flow rate was set to 100 μl/min to avoid mass transport. Regeneration of chip surfaces was obtained by injecting flowing buffer (30 μl/min, 10–30 min).

2.5. Pigment isolation

Etioplasts were isolated from 4.5 days old *H. vulgare* seedlings as described in [6,21,26]. Acetone was added at 80% (v/v) final concentration in the dark and extracts incubated 20 min prior to transfer and over night incubation at −80 °C. Aggregated proteins were removed by centrifugation (20 800×*g*, 10 min, 0 °C). The concentration of Chl a standard (Sigma, St. Louis, USA) was determined in 100% aceton [29] and a respective volume added to the supernatant prior to evaporation of acetone in a vacuum centrifuge.

2.6. Thin layer chromatography

For pigment characterization, pigment/lipids frozen on dry ice were thawed into an organic phase composed of 100% (v/v) acetone on ice. Samples were loaded on reversed phase (C18) high-pressure thin-layer chromatography (HPTLC) plates (Merck, Darmstadt, DE) and plates were developed in a solvent composed of 58.8% (v/v) acetone, 39.2% (v/v) methanol, and 2% (v/v) water. HPTLC plates were scanned for fluorescence emission (excitation 633 nm/670 BP30 emission filter) in a Typhoon scanner (GE Healthcare, Buckingham, GB).

2.7. Fluorescence spectroscopy assays

Pigment/lipid extracts were characterized by emission (740 nm) and excitation (440 nm) spectra measured at 77 K in a Horiba Yvonne Florolog-3 Spectrophotometer (Fluorolog®, HORIBA, France). Fluorescence emission (600–800 nm) at room temperature was measured upon excitation of HPTLC spots at 440 nm using a *y*-scale optical light cable.

2.8. Microscale thermophoresis, MST

MST experiments were performed on a Monolith NT.115 system using 20% LED and 20% IR laser power and consumables (NanoTemper Technologies, München, Germany). The intrinsic fluorescence of the externally added Chl a in the pigment/lipid mix was monitored and applied at a final concentration of 4 μM Chl a diluted in MST buffer with 1 mg/mL BSA and 0.025% Tween 20. A twofold dilution series starting at 39 μM was prepared for the unlabelled Lil3.2 in 25 mM HEPES pH 7.5 and 30 mM NaCl. Samples were filled into Premium coated capillaries for measurement. The negative control was conducted by substituting Lil3.2 with (Glu1)-Fibrinopeptide B human (Sigma Aldrich, St. Louis, USA).