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The role of FtsH proteins in photosystem biosynthesis and turnover in *Arabidopsis* and *Synechocystis*

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Introduction

Homologues of the *Escherichia coli* FtsH protein are widespread in prokaryotes and eukaryotes. These membrane-bound ATP-dependent metalloproteases attract great interest because of their diverse roles, sometimes acting as proteases and sometimes as molecular chaperones. FtsH mediates a multitude of functions in eubacteria, including phospholipid and lipopolysaccharide biosynthesis, protein assembly and export, and degradation of heat-shock factor σ^{32} (for review, see Schumann, 1999): deletion of the *E. coli* FtsH is lethal. Homologues of FtsH also appear to be important in photosynthesis: the model photosynthetic organisms *Arabidopsis thaliana* and *Synechocystis* sp. PCC 6803 both encode several FtsH-like proteins. Previous work (Mann *et al.*, 2000) showed that deletion of one of the four FtsH proteins found in *Synechocystis*, encoded by open reading frame (ORF) slr0228, is responsible for a marked reduction in the photosystem I (PSI) content of the organism. In addition, Silva *et al.* (2001) have found that the same FtsH is involved in the turnover of damaged photosystem II (PSII) D1 protein following photoinhibition. Likewise, a mutant 'Var2' FtsH in *A. thaliana* (one of at least nine such proteins encoded by the plant; see Adam *et al.*, 2001, for summary) is responsible not only for severe variegation (Takechi *et al.*, 2000; Chen *et al.*, 2000) but for the D1 repair cycle, just as seen in the *Synechocystis* mutant (Bailey *et al.*, 2001). Measurement of PSII activity reveals greater photoinhibition in Var2 than wild-type (WT) leaves during moderate- or high-light treatment. Western blots confirm that in Var2 the D1 polypeptide is not removed and replaced following such photo-oxidative damage, whereas in WT there is rapid D1 turnover. We are continuing work to characterise photosynthetic FtsH mutants, and the role of the proteins in degradation and biosynthesis of thylakoid membrane protein complexes. In particular, the accumulation of pigments and photosystems in FtsH mutants is being investigated to assess the impact of the proteases on PSI biogenesis. Because of the role of FtsH in lipid synthesis in *E. coli* we have also been examining the lipid content of the mutant strains' thylakoid membranes.

Materials and methods

Synechocystis was grown in BG11 medium (Castenholz) supplemented with 10 mM sodium bicarbonate at 30°C under white light of 8 μmol/m^2/s. Spectinomycin or kanamycin, where required, were added at 50 μg/ml. VAR2-2 *A. thaliana* (stock no. N272; Nottingham Arabidopsis Stock Centre, Nottingham, UK) and the parent line Col0 (stock no. N1092) were grown at 20 ± 2°C at a light intensity of 30 μmol/m^2/s (18 h light, 6 h dark). Chlorophyll in cyanobacterial samples was calculated from absorbance of methanol extracts: for *Arabidopsis*, extracts in 80% buffered acetone of either leaves powdered in liquid nitrogen or thylakoid membranes were used, after pelleting leaf debris (Porra *et al.*, 1989).

Routine DNA manipulation was carried out according to Sambrook *et al.* (1989). For glucose-tolerant (GT) *Synechocystis* mutant construction, PCR-amplified slr0228 was ligated into the

cloning vector pBluescript SK+/– (Stratagene, La Jolla, California, USA) and then disrupted with the pUC4K kanamycin resistance cassette (Pharmacia, Uppsala, Sweden). *Synechocystis* cells were then transformed with the mutated gene (Chauvat *et al.*, 1989). The spectinomycin-resistant slr0228::Ω mutant in the motile non-GT strain was constructed as described previously (Mann *et al.*, 2000).

For regreening assays, pelleted aliquots of *Synechocystis* cultures were inoculated into BG11 medium that contained no phosphate (plus 5 mM glucose for the GT strains) and incubated as detailed above. When chlorophyll levels had diminished to <0.5 μg/ml, pelleted cells were reinoculated into standard BG11 medium. Samples of cells containing 5 μg/ml chlorophyll were prepared at intervals for absorption spectra, recorded over the range 400–750 nm (SLM Aminco DW2000 spectrophotometer; SLM Instruments, Urbana, Illinois, USA). The content of chlorophyll and phycocyanin was calculated from spectra using the formulae of Myers *et al*. (1980). Growth and pigment content were monitored until cells were fully regreened.

For fluorescence emission spectra of *Synechocystis*, whole-cell samples in BG11 were frozen in liquid nitrogen; *Arabidopsis* samples were thylakoid membranes or frozen powdered leaves resuspended in buffer (0.33 M sorbitol, 5 mM $MgCl_2$, 5 mM EDTA, 10 mM HEPES pH 7.6), stored in liquid nitrogen. 77 K-temperature fluorescence emission spectra were recorded (LS50 luminescence spectrometer with low-temperature housing; Perkin Elmer, Gaithersburg, Maryland, USA) using excitation at 435 nm for chlorophyll *a*, and 600 nm for phycobilisomes (PBS) in *Synechocystis* only. Spectra were measured over 600–750 nm to reveal fluorescence emitted by PSII and PSI. The same equipment was used in its room-temperature set-up to monitor state transitions in the cyanobacteria. Low-light-grown WT and slr0228$^-$ cells (1–2 μmol/m^2/s for >24 h) were dark-adapted and then illuminated to produce state 2 (PSI favoured by light-harvesting complexes; LHC), then state 1 (PSII favoured). Finally, the cells were allowed to return to state 2. Fluorescence emission at 680 nm was measured using 620 nm excitation light.

The lipid content of *Synechocystis* WT/slr0228$^-$ and of *Arabidopsis* Col0/Var2 thylakoid membranes was analysed as detailed by Kruse *et al.* (2000). Briefly, thylakoid lipids were extracted using methanol, chloroform then acetone washes of filtered membranes, and the dried pellets containing pigments and lipids were redissolved in chloroform:methanol. Thin-layer chromatography was used to identify carotenoid, phospholipid and galactolipid content of the membranes, staining as appropriate.

Results

We have constructed site-directed mutants in the slr0228 ORF that encodes one of four FtsH proteins in *Synechocystis*, in motile and GT WT strains. The mutants display the characteristic slr0228$^-$ phenotype (Mann *et al*., 2000) of increased PSII:PSI fluorescence ratio (Fig. 1a) in 77K fluorescence emission spectroscopy.

In both *Arabidopsis* and *Synechocystis*, reduced levels of chlorophyll are found in mutant compared with WT. However, antenna function appears quite normal in VAR2 *Arabidopsis* (Fig. 1a), where the chlorophyll *a/b* ratio (Col0, 3.02 ± 0.22; Var2, 3.16 ± 0.21) and 77K fluorescence emission spectra indicate little difference between WT and the FtsH mutant. In high light, however, spectra show marked differences in adaptation and recovery between Col0 and Var2 plants (data not shown), which might be expected given the faulty D1 repair cycle. In the mutant cyanobacterium, Mann *et al.* (2000) have already reported that there is a 60% reduction in functional PSI centres/cell, and this is reflected in the 77K fluorescence spectrum (Fig. 1a): the PSII:PSI ratio is raised relative to WT. The dramatically altered fluorescence characteristics of the slr0228$^-$ mutant

might indicate state transitions are defective. The mutant is able to perform state transitions as well as the WT strain, however (data not shown).

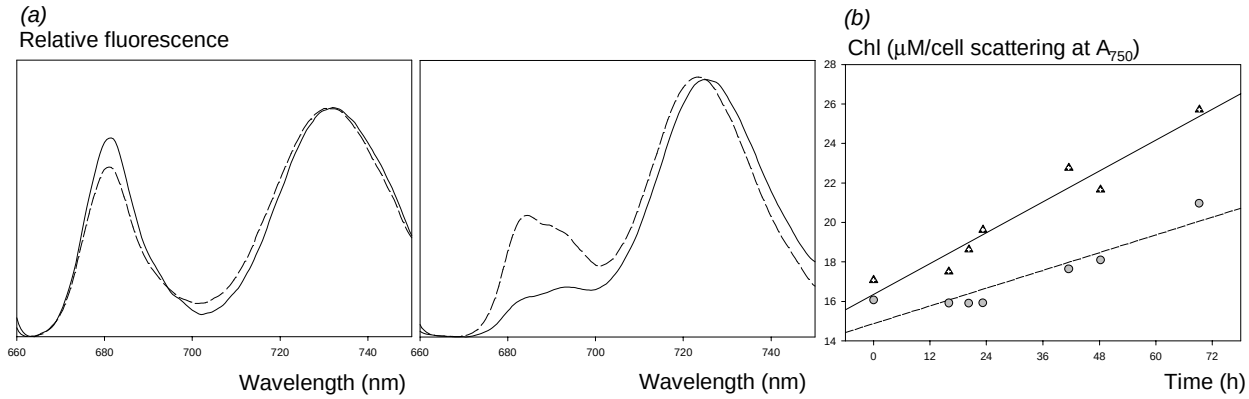

Figure 1. (a) 77K fluorescence emission spectra at 435 nm excitation of, left panel, *Arabidopsis thaliana* wild type (WT; Col0) and mutant (Var2; dotted line), and, right panel, WT and slr0228^{-} (dotted line) *Synechocystis* sp. PCC 6803. Spectra are normalised to photosystem I (right peak). **(b)** Chlorophyll (µM)/cell scattering increasing over time in WT (black triangles) and slr0228^{-} (grey circles) *Synechocystis* during regreening.

The rate of regreening of chlorophyll-deficient *Synechocystis* cultures has been followed using motile and GT strains. In cells recovering after reinoculation into phosphate-containing medium, the rate of regreening of the slr0228^{-} mutants was always slower than that of WT (Fig. 1b). The mean regreening rate of slr0228^{-} cells is 0.66 that of WT (P<0.05), calculated from rates measured in both the motile and GT strains. Interestingly, the rate of increase of phycocyanin per cell is also slower in mutant than in WT cells. Preliminary data, using flash spectroscopy to quantify functional PSI during regreening, indicate that PSI recovery is similarly affected in the mutant. Meanwhile, growth rates are the same in WT and mutant under standard culture conditions and during the regreening assays.

According to our preliminary investigations, the phospho- and galactolipids of both *Synechocystis* and *Arabidopsis* FtsH mutants are identical to those of their respective WT. Var2 has more than double the lipid:chlorophyll ratio of the Col0 plant membranes, however, as might be expected because of the profound variegation of its leaves. Work is continuing to analyse in more detail the exact fatty acid composition of the thylakoid membranes.

Discussion

We have been characterising the effect of FtsH proteins in *A. thaliana* and in the photosynthetic cyanobacterium *Synechocystis*. Much interest has centred on the role of FtsH in photosynthesis since Lindahl *et al*. (1996), using an antibody to the *E. coli* FtsH, identified the protein in spinach thylakoids. They also reported that an FtsH was encoded by an *Arabidopsis* cDNA, before the plant's genome was sequenced. Subsequent work has showed that at least one FtsH in both the plant and cyanobacterium is responsible for repair of photodamaged PSII D1 protein. Here we present preliminary work supporting the case for a role of the same protein in formation of functional PSI in the cyanobacterium, also.

In regreening assays, the slr0228^{-} mutants consistently recover their chlorophyll more slowly than do WT strains. In the case of the GT strain, cells are able to grow heterotrophically in the presence of glucose, and dispense with chlorophyll quickly when grown in cultures without phosphate. Chlorophyll affects the translation, stability and assembly of chlorophyll-binding proteins, most of which are components of PSI and PSII (Yu *et al*., 1999, and references within). As most chlorophyll in the cyanobacterium *Synechocystis* is associated with PSI, it could be inferred that in the slowly-

regreening mutant cells, PSI is also being regained at a lower rate than in WT. This is being investigated using flash spectroscopy to quantify functional PSI, and preliminary data do indeed indicate that PSI is reaccumulated more slowly in slr0228^{-} *Synechocystis*. A slower recovery of PSI is notable because FtsHs sometimes act as chaperones as well as proteases, and it has been postulated that this may be the case with photosynthetic FtsHs as well. As mentioned above, the slr0228^{-} mutant contains fewer functional PSI centres/cell than does the WT. Slower biogenesis of the reaction centre because of the loss of a chaperone function might account for this. Interestingly, when we have generated mutants with the disrupted slr0228 ORF in PSII-deficient *Synechocystis*, the mutated gene will not segregate to all copies of the genome. If the slr0228-encoded protein were to reduce PSI levels in a PSII-less cell, this mutation may indeed be very deleterious. The reason for the reduced rate of increase of phycocyanin is not clear. Phycocyanin is associated solely with light-harvesting, and is not an integral component of the reaction centres like chlorophyll. Yu and colleagues (1999), however, also noted a difference in PBS characteristics when they regreened chlorophyll-depleted cultures of their protochlorophyllide oxidoreductase-minus *Synechocystis* strain. The role of FtsH in PSI assembly and pigment accumulation in both the cyanobacterium and *A. thaliana* merits further study.

As well as monitoring pigment and reaction centre accumulation, the lipid content of thylakoid membranes of the Var2 plant and slr0228^{-} bacterium has been examined. Since FtsH may be involved in lipid biosynthesis in *E. coli*, it is important to rule out changes in the lipid composition of thylakoid membranes that might influence photosystem behaviour. No dramatic differences are obvious, but the detailed fatty acid composition of the membranes is still under investigation.

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