Degradation prohibited?

Are prohibitins protecting newly synthesized copies of the D1 protein from degradation prior to their incorporation into reassembling PSII complexes in *Synechocystis* sp. PCC 6803?

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1. Introduction

Although light is essential for oxygenic, photoautotrophic organisms as the driving force for photosynthesis, excess light can cause significant damage on the cellular level. A major target of light-induced, irreversible damage is the photosystem II (PSII) complex in the thylakoid membrane, and in particular the D1 reaction centre subunit (see figure 1). In order to maintain PSII activity the cell needs to remove and degrade damaged D1 protein from the reaction centre of PSII and replace it with a newly synthesized copy of D1. The molecular details of the PSII repair cycle are unclear; however recently the FtsH protease (slr0228) was assigned a key role in the degradation of the D1 protein in *Synechocystis* sp. PCC 6803 (Silva et al., 2003).

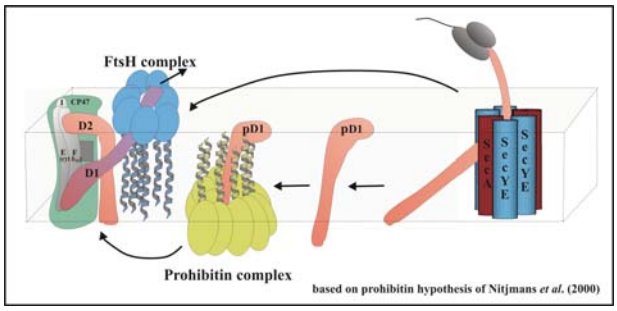

figure 1 (from Silva et al., 2003): Simplified model for D1 turnover. D1 degradation is catalysed by a large FtsH complex. The replacement D1 polypeptide may be inserted into PSII co-translationally via the Sec translocon or post-translationally via a large prohibitin complex. This model is based on the prohibitin hypothesis described in yeast by Nitjmans *et al.*(2000).

In *S. cerevisiae* it has been found that unassembled proteins of the mitochondrial inner membrane are removed and degraded by proteases homologous to the FtsH protease in *Synechocystis* sp. PCC 6803 (Steglich et al., 1999), whereas the newly synthesized copies of the protein are protected from degradation by heterooligomeric prohibitin (PHB) complexes (Nijtmans et al., 2000). Protein complexes containing the FtsH protease and prohibitin homologues (HflK/C) have been found in *E. coli* (Saikawa et al., 2004).

We have identified five putative prohibitin proteins (PHB1-5) in *Synechocystis* sp. PCC 6803 that share the characteristic SPFH domain (**s**tomatins, **p**rohibitins, **f**lotillins and **H**flK/C) (Tavernarakis et al., 1999). It therefore seems possible that these proteins may interact with FtsH (slr0228). So far the role and the function of prohibitins in *Synechocystis* sp. PCC 6803 remain unclear and the aim of this study is to test a possible involvement in photoinhibition and the PSII repair cycle.

2. Directed mutagenesis

Mutants are important tools in biological studies and are used to test whether a certain gene product is involved in a particular process. After deleting a specific gene any changes in the phenotype compared to the WT situation suggest a link between the gene and the process. In this study various prohibtin deletion mutants have been generated (see table 1).

table 1: Available prohibitin mutants.

#	segregated mutants	knocked out genes
1	ΔPHB1-KAN	slr1106
2	ΔPHB2-CAM	slr1768
3	ΔPHB1-KAN + ΔPHB2-CAM	slr1106 + slr1768
4	ΔPHB3-SPEC	slr1128
5	ΔPHB3-CAM	slr1128
6	ΔPHB1-KAN + ΔPHB2-CAM + ΔPHB3-SPEC	slr1106 + slr1768 + slr1128
7	ΔPHB4-SPEC	sll0815
8	ΔPHB3-CAM + ΔPHB4-SPEC	slr1128 + sll0815
9	ΔPHB5-CAM	sll1021

The generation of deletion mutants in *Synechocystis* sp. PCC 6803 is achieved by disruption of the target gene with an antibiotic resistance cassette. The desired gene is amplified via PCR from WT DNA and cloned into a vector. Then an antibiotic resistance cassette is inserted into the cloned gene and the resulting construct (see figure 2A) is transformed into *Synechocystis* sp. PCC 6803 cells. Homologous recombination leads to the disruption of the WT gene in the transformed cells. The transformants are grown on selective plates with rising levels of antibiotics in order to enforce segregation, as *Synechocystis* sp. PCC 6803 possesses multiple copies of its genome. The progress of segregation is monitored by PCR (see figure 2B). Once the cells are completely segregated, they can be transformed with other constructs to obtain multiple mutants and their phenotype can be studied.

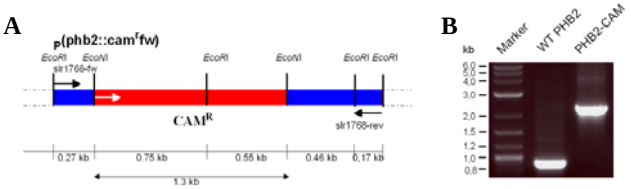

figure 2: (A) Schematic drawing of the construct that has been used to disrupt the phb2 gene (slr1768) with a chloramphenicol cassette. (B) PCR with WT DNA and DNA from a segregated mutant using specific primers for PHB2 yielding the WT (~0.8 kb) and the disrupted (~2.1 kb) fragment.

3. Expression and purification of prohibitins

Our working model for the prohibitins in *Synechocystis* sp. PCC 6803 (see figure 1) proposes a multimeric prohibitin complex that shields a newly synthesized copy of the D1 protein from degradation by the FtsH protease. As it is our aim to identify and characterize this prohibitin complex and its interacting partners, we will generate polyclonal antibodies against the prohibitins. These antibodies will be used in western blotting analyses following 1D- and 2D-gelelectrophoresis and for immunoprecipitation experiments. Previously a polyclonal antibody against PHB1 has been produced in the lab and only four antibodies remain to be generated.

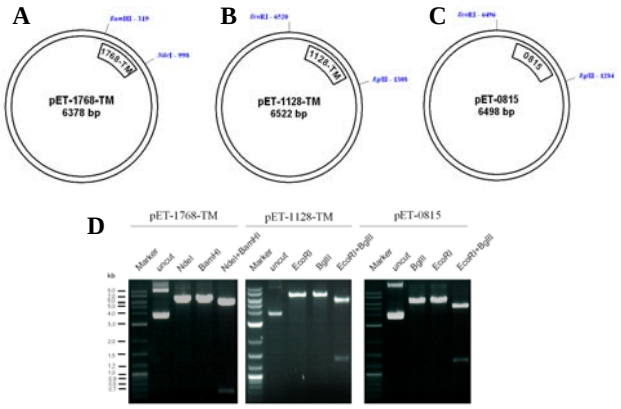

figure 3: (A-C) Schematic drawings of the expression constructs for PHB2-TM (A), PHB3-TM (B) and PHB4 (C). (D) Diagnostic digests of the expression constructs.

We generated full length prohibitin PCR products, as well as truncated fragments, from which the transmembrane (TM) domains have been deleted, and cloned them into the expression vector pET16b, that adds a His-tag to the expression products (see figure 3A-C). The obtained constructs were transformed into various bacterial expression strains and expression was induced with IPTG (see figure 4A). PHB5 has so far not been expressed in any strain using either the full lenght or the truncated DNA expression construct. The other proteins (PHB2-TM, PHB3-TM and PHB4) were purified with a His-tag column (see figure 4B) and send to SEQLAB (Göttingen, Germany) for immunization.

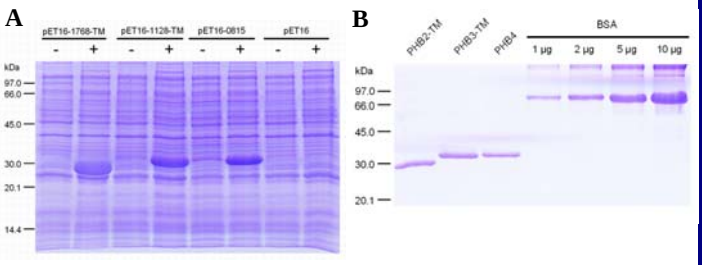

figure 4: (A) Whole cell extracts of induced and uninduced bacteria carrying the expression constructs after a 3h incubation period. (B) His-tag column purified prohibitin proteins and various amounts of BSA.

4. Conclusions and future work

Several single and multiple prohibitin deletions mutants have been generated.

Three prohibitin proteins (PHB2-TM, PHB3-TM and PHB4) have been expressed, purified and send to SEQLAB for rabbit immunization and generation of polyclonal antibodies.

In the future the phenotypes of the generated mutants will be studied. This will involve oxygen evolution measurements and pulse-chase experiments. Furthermore the mutants will be grown under various stress conditions and analysed by 2D gelelectrophoresis, Western blotting and possibly mass spectrometry.

5. References

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