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Towards targeted *psbW* mutants in *Physcomitrella patens*

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Introduction

The PSII complex comprises over 25 different polypeptides, which are encoded for by either the nuclear or chloroplast genome (Nugent, 1996). The complex and its subunits have been extensively studied in unicellular organisms such as the prokaryotic *Synechocystis* and the eukaryotic *Chlamydomonas*. Both organisms are model organisms for the study of the PSII complex due to firstly the ease with which the either the chloroplast (*Chlamydomonas*) or the entire genome (*Synechocystis*) can be targeted and secondly the ability to dispense with photosynthesis and grow autotrophically, enabling the viability of otherwise lethal photosynthetic mutants. There are, however, limits to using these lower organisms for our understanding of the plant PSII complex. In particular, the nuclear sub units of eukaryotic PSII have been less well studied than their chloroplast counter parts, mainly due to the absence of efficient manipulation of the nuclear genome.

Homologous recombination, which occurs at high frequencies in *Synechocystis* and the chloroplast genome of *Chlamydomonas*, allows gene targeting. It occurs in the nuclear genome of higher organisms only at very low frequencies ranging from 1 in 1,000,000 to 1 in 100 and occurs at frequencies greater than 1 in 10 only in lower eukaryotes such as yeast (Schaefer and Zrÿd, 1997). The popular model plant organism *Arabidopsis* has a homologous recombination frequency of 1 in1000 making it impractical for gene targeting experiments. Only one plant to date has been reported to have efficient gene targeting frequencies.

The moss *Physcomitrella patens* has been used for many years as a model for plant development (Cove *et al*., 1997). The life cycle comprises of a haploid vegetative state and a diploid sporophyte state that is used for sexual reproduction only. The haploid nature of vegetative *P. patens* enables relatively easy genetic and molecular analysis. It can also be grown, either on agar plates or in liquid culture, photoautotrophically or using glucose or sucrose as a carbon source making the viability of photosynthetic mutants a possibility.

Recent advances in the understanding of moss genetics together with the development of existing techniques has enabled *P. patens* to be used as a model system in many areas. In 1991, *P. patens* protoplasts were successfully transformed with plasmids carrying a selectable marker from a plant-active gene promoter (Schaefer *et al*., 1991) and in 1994, nuclear gene targeting was demonstrated (Schaefer *et al*, 1994). There are now many reports of similar gene targeting, with some of these showing that such targeting is as efficient in *P. patens* as it is in *Saccharmyces cervisiae* (Schaefer, 2001). At least three of these have resulted gene disruption that has led to null phenotypes (gene knockout), allowing gene and protein function to de determined (for review, see Schaefer, 2001).

We aim to exploit this novel technology to study the nuclear genes of photosynthesis. This paper outlines initial work on the isolation and cloning of the *psbW* gene from a *P. patens* genomic library with a view to creating gene disruption, histidine tagged and site directed mutants.

Materials and Methods

Four *psbW* ESTs were obtained from the *Physcomitrella* EST Programme (PEP) based at the University of Leeds, UK. A *P. patens* genomic library in λ fix II was also obtained from C. Knight at the University of Leeds, UK. All the ESTs were sequenced and the longest one (Figure 1) used for library screening and southern analysis. All library screening, and molecular biology procedures were carried out as described in Maniatis *et al*. (1982).

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  1  ggcaccagat ccccccccaac agccatggct gccatcgcct ctgcatcctg cgctaccgct
 61  gctacccgcc ttgccaccac atcgcttgcc tcttctagct ctggcttcgc agccactctg
121  aagcctgtcg ctggtctccc cgctctgcgc atgcccaagg ttgtttgcgc ggctgagagg
181  tcggagtcca aggcggagcg tgtcaatggc gtcgctcagc tcgctgcagc tgtgacatcc
241  gcggctaccc ttgcttactc tcaccccgtg ctggctctcg tggacgagag gctaagcact
301  gaaggaacag gtttgggtct cggtatcagc aacaccaagt tgacatggat cttggtcggc
361  gtgactgccc ttatctggac attgtactta cttactcctc gacgttgcca gagggtgacc
421  gacgactccg tttggacctg tagatccagg aaacaggaat tttgtgtacc attttgaggt
481  gtagtgttaa ttgttccaat tagccggagt ggactttcaa tgg
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Figure 1. DNA Sequence of the *Physcomitrella patens psbW* EST used for screening the genomic library (genbank accession numberAF312742).

Results

Using the four ESTs obtained from PEP, it was possible to obtain a putative amino acid sequence for *P. patens* mature PsbW. The proposed primary structure is given in Figure 2 and included comparisons with PsbW amino acid sequences available. The proposed amino acid sequence for *P. patens* shows 40% homology to that for *Chlamydomonas reinhartii* and 60% homology to that found in the higher plants *Arabidopsis thaliana* and *Spinacia oleracea*.

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P. patens          LVDERLSTEGTGLGLGISNTKLTWILVGVTALIWTLYFTY   40

C. reinhardtii     LVDERMNGDGTGRPFGVNDPVLGWVLLGVFGTMWAIWFIG   40
A. thaliana        LVDERMSTEGTGLPFGLSNNLLGWILFGVFGLIWTFFFAY   40
S. oleracea        LVDERMSTEGTGLPFGLSNNLLGWILFGVFGLIWALYFVY   40
T. aestivum        LVDERMSTEGTGLSLGLSNN                       20


P. patens          SPTLPEG-DDDSGLDL                           55

C. reinhardtii     QKDLGDFEDADDGLKL                           56
A. thaliana        TSSLEE--DEESGLSL                           54
S. oleracea        ASGLEE--DEESGLSL                           54
T. Aestivum                                                   20
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Figure 2. Alignment of *P. patens* putative PsbW mature protein sequence with previously available sequences. Conserved residues are shaded in grey.

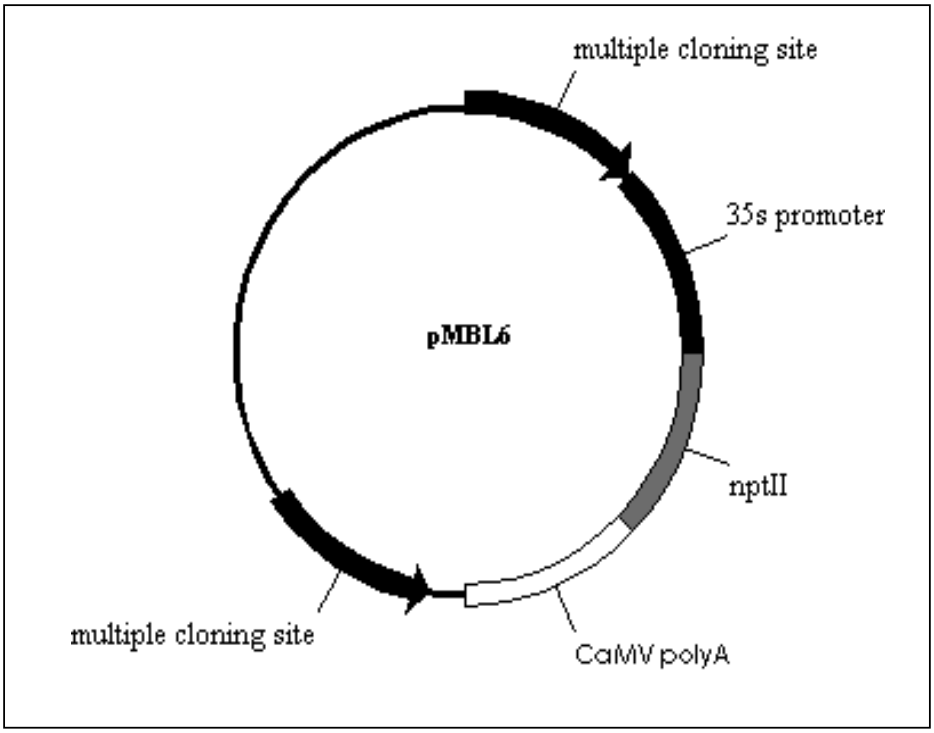

Figure 3. Map of the moss vector pMBL6, used for gene targeting.

Ten positive λ fix II plaques were chosen after initial library screening. The DNA extracted from these was subjected to restriction enzyme and southern analysis. Work is currently being undertaken to clone the *psbW* gene into a moss vector, pMBL6 (Figure 3) and sequence the clone.

Discussion

The aim of our work is to create targeted mutations in the nuclear genes involved in PSII structure and function. The PsbW protein is situated towards the core of the complex and

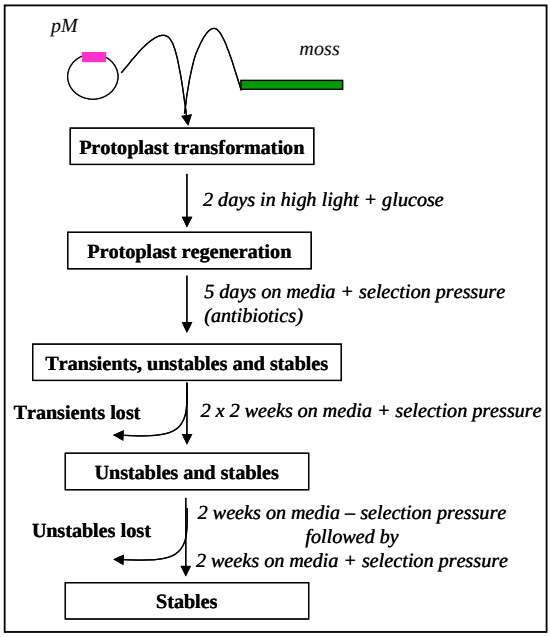

Figure 4. The selection procedure for transformants.
Three different types are transformants are produced:
1) Transient transformants, which do not retain antibiotic resistance for more than one week
2) Unstable transformants, which do not retain antibiotic resistance after being transferred to non-selective media.
Both these types of transformants are though to arise from non-integrated, extra-chromosomal plasmids
3) Stable transformants, which retain antibiotic resistance even after several months on non-selective media.
The selection procedure eliminates transient and unstable transformants, leaving only the positive, stable ones

therefore is potentially of importance to PSII structure and function. It is also an ideal protein to tag for rapid purification of the complex. We have obtained *psbW* ESTs from PEP and shown that these are likely to code for a protein that has 60% homology to the PsbW protein found in *Arabidopsis* and Spinach.

As a first step, we have started to clone the gene from a genomic library. Once the gene has been sequenced and cloned into the moss gene replacement vector, pMBL6, it will then be transformed into *P. patens* protoplasts using a PEG-mediated system. Transformants will then be taken through a selection procedure to determine positive, stable transformants (Figure 4).

This procedure will also be used to produce histidine tagged and site directed mutations in the *psbW* gene.

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