Exploring the Function of GT2 in Physcomitrella patens

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Exploring the Function of GT2 in *Physcomitrella patens*

Tess Scavuzzo-Duggan

**Abstract**

The goal of this research project is to investigate the particular function of the gene *GT2* in *Physcomitrella patens* using targeted gene replacement techniques. During the course of this project, mutant lines of *P. patens* were created by removing *GT2* from their genome. The removal of *GT2* was performed by using targeted gene replacement techniques and it was replaced with a gene encoding antibiotic resistance. Proper integration of the vector encoding antibiotic resistance was tested using PCR and agarose gel electrophoresis. A promoter construct has been created and transformation of *P. patens* with the expression clone has been performed to see where *GT2* is expressed. Phenotypic analysis has begun on the knockout lines to gain insight into the function of *GT2*, including rhizoid and caulonemal analysis. No differences in caulonemal development were discovered between wild type and knockout lines.

**Introduction**

Plant cell walls are composed of a variety of carbohydrates, among them cellulose, pectin and hemicellulose. Cellulose is the most abundant carbohydrate in plant cell walls and is deposited in the cell wall as microfibrils via transmembrane cellulose synthesis complexes (CSCs) (Desprez et. al., 2007). These complexes contain the cellulose synthase proteins (*CESAs*) and come in different morphological forms, two of which are rosettes and linear complexes. Rosette shaped cellulose synthesis complexes occur in land plants, whilst linear complexes are commonly found in red algae (Tsekos, 1999). However, some land plants, notably bryophytes (mosses) and seedless vascular plants, contain genes that encode both *CESAs* of the type that form rosette CSCs and also genes similar to *CESAs* found in red algae. The moss *Physcomitrella patens*
contains one gene of the latter type that has been named \textit{GT2} (Harholt et. al., in press). \textit{GT2} may represent a gene that mosses and seedless vascular plants inherited from their algal ancestors and that has been lost in seed plants. This suggests an evolutionary divergence between mosses and seedless vascular plants and seed plants. Although the precise function of the gene is unknown, its similarity to red algal \textit{CESA}s suggests that it plays a part in cellulose microfibril synthesis in the plant cell wall.

In rosette CSCs, the CESAs form a hexameric ring comprised of six subunits of six CESAs each. This 36 CESA complex synthesizes rope-like microfibrils, with theoretically one strand of cellulose synthesized per CESA. Conversely, linear CSCs found in the red algae \textit{Porphyra yeozyensis} contain between eleven and twenty-five CESA subunits, which synthesize flat, ribbon-like microfibrils (Tsekos, 1999). The difference in CSC and microfibril shapes lead to physical differences in the cell walls of land plants and red algae. Although the differences in CSC orientation are known, the precise method of how these CESAs come together and interact is still unknown. This is particularly intriguing in \textit{Physcomitrella patens}, because the rosette shape of its CSCs implies that GT2, while similar in sequence to a CESA found in red algae, must interact with the CESAs commonly associated with land plants in a manner consistent with land plant CESAs or that GT2 forms completely different CSCs, possibly linear, or possibly a cross between rosette and linear CSCs.

This project is a continuation of the research performed by Jilian Boisse under the direction of Dr. Alison Roberts. In the course of her project, Boisse created the \textit{GT2} knockout vector with hygromycin resistance, which was later used to transform wild-type \textit{P. patens} lines. These mutants were then genotyped and subsequently put through phenotype analysis, consisting of both rhizoid and caulonemal analysis. A promoter:GUS expression vector was created with
hygromycin resistance, with the destination vector created by Mai Tran and was used to transform wild-type *P. patens* lines.

**Materials and Methods**

**Expression Vector Preparation**

The promoter:GUS expression vector was created using Invitrogen’s MultiSite Gateway Pro kit (Grand Island, NY, USA) and instructions provided. An entry clone for the promoter was created by first performing a BP recombination reaction using a pDONR P1P5r vector and promoter PCR product (see below) and then transforming competent *E. coli* cells with the entry vector. An expression clone was created by first performing an LR reaction using the promoter entry clone, the GUS entry clone and the destination vector and then transforming competent *E. coli* cells with the expression vector. After the creation of a successfully expression clone, restriction digest was performed on the plasmid using 5 µl of *SwaI* restriction enzyme from New England BioLabs (Ipswich, MA, USA), 30 µl Buffer 3, 3 µl BSA, 100 µl of plasmid DNA and 162 µl ddH$_2$O. The restriction digest incubated at room temperature overnight and was subsequently precipitated with ethanol.

**Protoplast Transformation**

Transformation followed procedures outlined in "Knocking Out the Wall: Protocols for Gene Targeting in Physcomitrella patens" (Roberts et. al., 2011) for both the *GT2 KO* vector and the promoter:GUS vector. Chloronemal tissue was scraped from a Petri plate and added to a Petri plate containing 9 ml of 8.5% mannitol and 3 ml of 2% Driselase. The plate was shaken at 60 rpm for one hour at 25°C. Protoplast suspension was then pipetted through a nylon filter into a 50 ml centrifuge tube. This filtered suspension was then put into a 15 ml centrifuge tube and centrifuged at 5,000 x g for seven minutes. After discarding the supernatant, the protoplasts were
resuspended in 10 ml of 8.5% mannitol and centrifuged at 5,000 x g for seven minutes. The supernatant was again discarded and the previous step repeated in order to remove any traces of 2% Driselase. After resuspension in 10 ml of 8.5% mannitol, 10 µl of the protoplast suspension was loaded into the hemocytometer to count protoplasts in order to estimate density. Supernatant was again discarded and the protoplasts were resuspended in 3M at a density of approximately 2 x 10^6 protoplast/ml. 0.3 ml of protoplast suspension and 0.3 ml of PEG were added to 15 µl of vector DNA in a 15 ml centrifuge tube and incubated at room temperature for ten minutes. Protoplast suspension was then heat shocked at 45ºC for three minutes and then transferred to a water bath at 25ºC for ten minutes. Protoplasts were resuspended in 5 ml 45ºC PRMT and poured onto plates containing PRMB overlain with cellophane. After five days of incubation, the protoplasts underwent antibiotic selection on BCDAT medium with 15 µg/ml of hygromycin and incubated for seven days. The protoplasts were subsequently incubated on BCDAT medium for seven days after which the protoplasts underwent the second round of antibiotic selection.

DNA Extraction

DNA was isolated from GT2 KO lines in accordance with the procedures outline in "Knocking Out the Wall: Protocols for Gene Targeting in Physcomitrella patens," (Roberts et. al., 2001). Fresh moss tissue (approximately the size of an Arabidopsis leaf) was ground in a 1.5 ml microcentrifuge tube by Kontes pestle motor (Kimble Kontes, Vineland, NJ, USA). The ground up tissue was suspended in 0.4 ml of extraction buffer (0.2 M Tris-HCl, pH 9.0; 0.4 M LiCl; 25 mM EDTA; 1% SDS) and centrifuged for five minutes at 14,000 x g. 0.35 ml of the supernatant was transferred to a 1.5 ml microcentrifuge tube containing 0.35 ml isopropanol and mixed by inversion. The microcentrifuge tube was then centrifuged for ten minutes at 14,000 x g. After centrifugation, the supernatant was poured out and the pellet was allowed to air dry with the
tubes arranged upside down on a paper towel to remove any remaining alcohol. Once dry, the pellet was resuspended in 0.4 ml of TE by initially vortexing and subsequently shaking at room temperature for 30 minutes.

PCR and Agarose Gel Electrophoresis
Genotyping for GT2 KO P. patens transformants was performed using Polymerase Chain Reaction. Primers were created to amplify the 5’ and 3’ integration sites as well within the vector sequence. Primers were also created to amplify the promoter sequence of GT2 (Table 1). Tests for proper 5’ and 3’ integration were performed using the Stratagene Paq5000 kit (Cedar Creek, TX, USA) as directed. The thermocycler was set using a Hot Start of 94°C, and initial denaturation of 94°C for two minutes, and 30 cycles of denaturation at 94°C for 45 seconds, 57°C annealing for 45 seconds and 72°C extension for two minutes ending with a final extension period of 72°C for five minutes. Tests for proper integration within the vector sequence were performed using the Taq PCR Kit from New England BioLabs (240 Country Road, Ipswich, MA, USA) as directed. The thermocycler was set using a Hot Start of 94°C, and initial denaturation of 94°C for two minutes, and 30 cycles of denaturation at 94°C for 45 seconds, 57°C annealing for 45 seconds and 72°C extension for two minutes ending with a final extension period of 72°C for five minutes. For promoter amplification, Phusion DNA polymerase by Finnzymes (Rastatie 2, 01620, Vantaa, Finland) was used. The thermocycler was set using a Hot Start of 98°C, and initial denaturation of 98°C for 30 seconds, and 30 cycles of denaturation at 98°C for ten seconds, 60°C annealing for 45 seconds and 72°C extension for two minutes ending with a final extension period of 72°C for five minutes. PCR products were run on a 1% TAE agarose gel with 0.4 µg/ml ethidium bromide for 30 minutes.
<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence</th>
<th>Expected Amplicon Size</th>
<th>Amplified Region</th>
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<tr>
<td>BHRRR</td>
<td>TCCGAGGCAAAGAAATAGA CAAGACCACATTGTGCCATC</td>
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<td>5’ integration site</td>
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<td>GT2PROattB5r</td>
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<td>GGGGACAAGTTTTGTACAAAAAGCAGGC TCAACACCTGAAAACATATGGGACCAGGT</td>
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**Table 1.** Primers used for PCR.

Caulonemal Analysis

Caulonemal analysis was performed as described by “The use of mosses for the study of cell polarity,” (Cove and Quatrano, 2004). Protonemal tissue was placed along the diameters of Petri plates containing BCDAT medium with 1.2% agar and 35 mM sucrose. Plates were incubated at 25°C with constant illumination at 50-80 μmol/m²/s for one week and then placed vertically and incubated in the dark at 25°C for fourteen days.
Rhizoid Analysis

Rhizoid analysis was performed as described by "Involvement of auxin and a homeodomain-leucine zipper I gene in rhizoid development of the moss Physcomitrella patens," (Sakakibara et al., 2003). Protonemal tissue was placed on BCD medium with 1 µM naphthalacetic acid and incubated at 25°C with constant illumination at 50-80 µmol/m²/s for fourteen days.

Results

GT2 KO Transformation and Selection

Six mutant lines were by transformation of wild type *P. patens* with the *GT2 KO* vector. These six lines were shown by PCR to have properly integrated *GT2 KO* at the 5’ and 3’ regions in addition to having deleted the *GT2* gene sequence. (Figure 1A-F).

**Figure 1.** PCR Screening for proper integration of *GT2 KO* vector. **A-C.** First round of transformants. **A.** Integration of 5’ region. **B.** Integration of 3’ region. **C.** Integration of *GT2* sequence – C. is the positive control. **D-F.** Second round of transformants. In the interest of space, only positive lines are labeled. **D.** Integration of 5’ region. **E.** Integration of 3’ region. **F.** Integration of *GT2* sequence – C. is the positive control.
Caulonemal Analysis

After two weeks of incubation in the dark, the mutant lines from the second round of transformants showed no deviation from the wild-type *P. patens* in caulonemal development (Figure 2). All lines produced red-brown negatively gravitropic caulonema. The only visible deviation is from line 27, in which tissue was taken from gametophores instead of protonema as no protonema was available.

![Caulonema assays](image)

**Figure 2.** Caulonema assays. **A.** Assays with tissue from wild-type *P. patens* and *GT2 KO* lines 5 and 21. The physical differences between 21 and the other lines are due to the use of gametophore tissue instead of protonema owing to the lack of protonemal tissue for line 21 at the time. **B.** Assays with tissue from wild-type *P. patens* and *GT2 KO* lines 24 and 27.

Rhizoid Analysis

Rhizoid assays are currently underway with wild-type *P. patens* and *GT2 KO* lines 5, 21, 24 and 27. They are not yet ready for analysis.
Promoter:GUS Transformation and Selection

*P. patens* has undergone transformation and is currently on the first round of antibiotic selection. While there are developing protoplasts on the hygromycin medium, it is too soon to accurately tell if the transformation was successful.

**Discussion**

This project intended to discern the function of *GT2* in *Physcomitrella patens*. Although successful knock out mutants were created, no phenotype has thus far been found. Despite these preliminary results, much progress has been made in elucidating *GT2*’s potential function. Continued research of *GT2* in completion of the rhizoid assay and the promoter:GUS assay may further understanding of this gene’s function. Additionally, a phenotypic comparison between wild-type *Physcomitrella patens* and *GT2 KO* mutants along the development continuum from protoplasts to gametophytes may yield results as well.
References


