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The Role of *Cellulose Synthase-like D* Genes in Tip Growth of *Physcomitrella patens*

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Abstract

Physcomitrella patens is a non-vascular plant with a relatively small genome and is amongst the few eukaryotic organisms that have a high rate of homologous recombination. This is valuable in biological research because it allows for targeted genetic modification of the organism. In vascular plants like *Arabidopsis thaliana*, a model organism, *Cellulose Synthase-like D* (*CSLD*) genes have been discovered to be important in tip growth. This type of growth is observed in the pollen tubes and root hairs of these plant types. The *CSLD* genes in *Arabidopsis* were found to play a crucial role in the growth of root hairs and the production of cellulose or cellulose-like β-1,4-glucan chains in root hair tips. The *CSLD* genes have also been recognized to be important in pollen tube growth of vascular plants. *Physcomitrella patens* also contains genes similar to the vascular plant *CSLD*s, but their functions are not yet fully understood. Within the *P. patens* genome there are eight genes that make up the *CSLD* gene family. Additionally, the life cycle of *P. patens* includes a stage that consists primarily of tip growing cells. This growth stage can be optimized in order to study the role of *CSLD* genes in tip growth of *P. patens*.

In an effort to further study the roles of the *CSLD* genes in tip growth of *P. patens*, we constructed a plasmid that expresses the CSLD1 protein with a green fluorescent protein (GFP) tag. This allowed us to visualize the expression of CSLD1 in living cells using fluorescence microscopy. We also constructed plasmids that were designed to remove specific *CSLD* genes from the genome and transformed them into wild type or CLSD1 knockout tissue of *P. patens*. This created single or double knockout mutants that could then be compared to the wild type for changes in the phenotypic characteristics of the plant. These findings will aid in uncovering the roles of the *CSLD* gene family in *P. patens* and may provide insight into the functions of these genes in other plants.

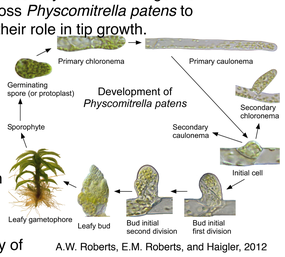
Introduction

Objective:

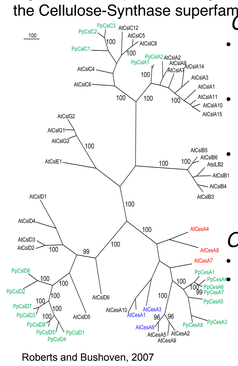
Investigate the role of Cellulose Synthase-like D genes in the development the moss *Physcomitrella patens* to better understand their role in tip growth.

Physcomitrella patens

- Moss
- Non-vascular
- Predominantly lives in haploid phase
  - Tip growth
- High rate of homologous recombination
  - Targeted genetic modification
- Fully sequenced genome
- 8 genes make up the CSLD gene family of the Cellulose-Synthase superfamily



A.W. Roberts, E.M. Roberts, and Haigler, 2012



Roberts and Bushoven, 2007

Cellulose Synthase-like D genes

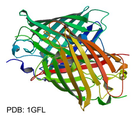
- *CSLD* and *CESA* have higher sequence similarities than other *CSL* gene families (Gu and Nielson, 2013).
- *CESA* is the gene that encodes the catalytic subunit of cellulose synthase (Roberts and Bushoven, 2007).
- Previous studies suggest that CSLDs encode glycosyl transferase enzymes that produce non-crystalline β-glucans (Gu and Nielson, 2013).

*CSLD*s in *Arabidopsis thaliana*

- *CSLD* important in tip growth
- Root tips
  - *CSLD3* required for root hair growth and is a plasma membrane protein
  - Both *CSLD2* and *CSLD3* are important in root hair tip growth
- Pollen tubes
  - *CSLD1* and *CSLD4* mutants have defects in pollen germination (Gu and Nielson, 2013).

*CSLD*s in *P. patens*

- Possible involvement in the biosynthesis of crystalline or non-crystalline cellulose and polarized tip growth.
- Single knockout *CSLD* mutants do not have a phenotype.
- RNA interference (RNAi) studies where all of the *CSLD* genes of *P. patens* were silenced gave a phenotype in which overall growth was affected (Dimos, 2010).
- Exact role of *CSLD* genes not yet fully understood



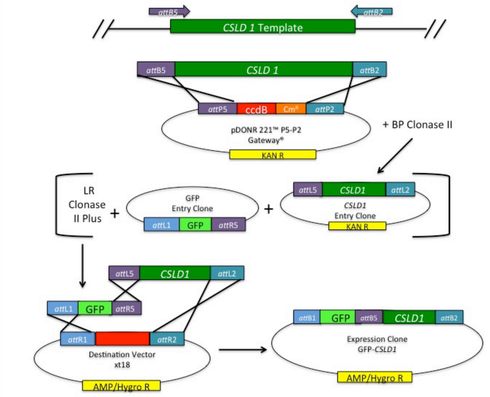
PDB: 1GFL

Green Fluorescent Protein (GFP)

- Protein isolated from the jellyfish *Aequorea victoria*
- ~27 kDa
- Composed of beta sheets that form a beta-barrel
- Absorbs blue light and emits green light
- Commonly used as a reporter in genetic studies

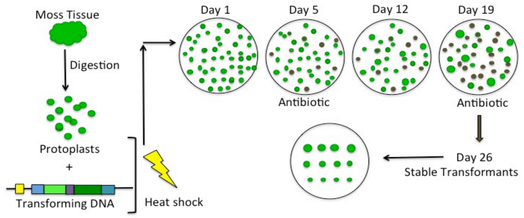
Methods

- PCR: A Polymerase Chain Reaction was done using *att*B primers and *CSLD1* template. This amplified *att*B flanked *CSLD1* necessary for plasmid construction.
- Plasmid Construction: We conducted a BP and an LR reaction, illustrated below, to construct the GFP-*CSLD1* expression clone. This plasmid DNA was cut using a SWAI restriction digest and transformed into wild type *P. patens* subcultured tissue.

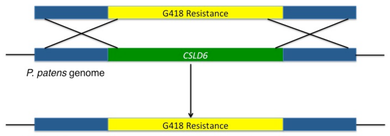


Modified plasmid map of Invitrogen Gateway® pDONR™ 221 Vector

- Transformation and Antibiotic Selection: The GFP-*CSLD1* plasmid DNA was transformed into Gd11 (wild type) moss tissue (Roberts, 2011). Successful recombinants will have hygromycin resistance. The regenerating protoplasts were transferred to hygromycin BCDAT medium 5 days after the transformation. 7 days later they were transferred onto BCDAT without the antibiotic and after 7 days they were transferred to the second round of hygromycin selection. After 7 days on the second round of selection, stable transformants were spot plated onto BCDAT.



- Fluorescence Microscopy: GFP-*CSLD1* tissue samples were microscopically observed under fluorescence.
- *CSLD* knockout mutant construction: The construction of the *CSLD* knockout vectors was done by Christos Sotirios Dimos. The knockout plasmids were constructed the same way as the GFP-*CSLD1* expression clone with some exceptions. Instead of inserting a fluorescent tag entry clone, the targeted gene (the gene to be knocked out) was removed by homologous recombination and replaced with a G418 selection cassette. We transformed *CSLD4, CSLD5,* and *CSLD6* knockout vectors into Gd11 and *CSLD1* knockout moss tissue in an effort to produce single and double knockout mutants that have G418 antibiotic resistance. Genotyping must be executed to confirm that the gene of interest was removed from the genome of the transformants before phenotypic analyses can be done.

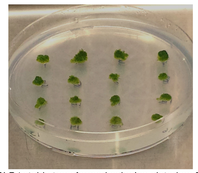


Modified *P. patens* genome with target gene knocked out

Results

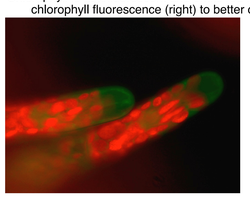
GFP-*CSLD1* Results

- 16 stable transformants of GFP-*CSLD1* were obtained from transformation after second round on selection medium
- GFP fluorescence of *CSLD1* observed in the tips of *P. patens*
- GFP-*CSLD1* was found to be localized in vesicles and cell membranes of the tips of *P. patens* (below)



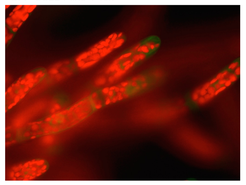
16 GFP-*CSLD1* stably transformed colonies plated on BCDAT

Chlorophyll is autofluorescent and emits red light (left). A filter was applied to remove the chlorophyll fluorescence (right) to better observe the GFP fluorescence (green).

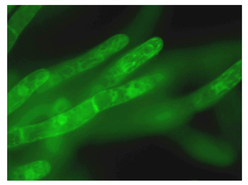


GFP-*CSLD1* line 4 40x

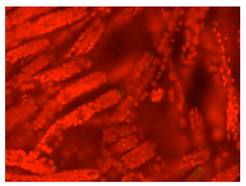
GFP-*CSLD1* line 11 40x



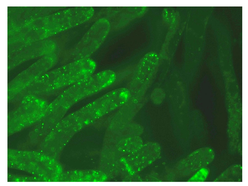
GFP-*CSLD1* line 4 20x



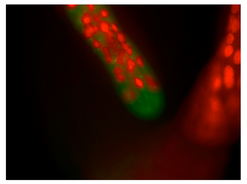
GFP-*CSLD1* line 4 20x



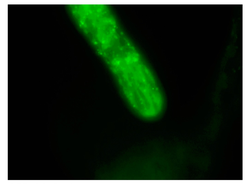
GFP-CSLD1 line 5 20x



GFP-CSLD1 line 5 20x



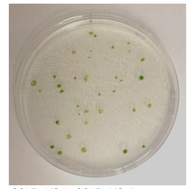
GFP-*CSLD1* line 5 40x



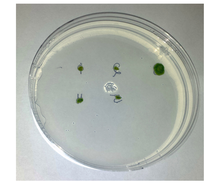
GFP-*CSLD1* line 5 40x

CSLD knockout mutant results

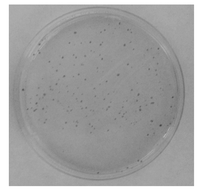
- *CSLD4*, *CSLD5*, and *CSLD6* knockout transformations were successful in producing stably transformed colonies.
- 3 *CSLD4* KO, 2 *CSLD5* KO, and 5 *CSLD6* KO stable transformants were obtained after transformation into *CSLD1* KO moss tissue. This created *CSLD4/1*, *CSLD5/1*, and *CSLD6/1* double knockout mutants.
- *CSLD4, CSLD5*, and *CSLD6* transformations into wild type (Gd11) moss tissue were performed but are not yet completed.



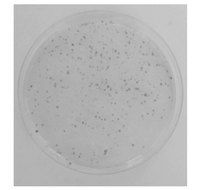
CSLD6 KO in *CSLD1* KO tissue before last round of selection on G418



CSLD6-1 KO stable transformant colonies, spot plated onto BCDAT



CSLD4 KO in Gd11 after the first round on G418 selection media



CSLD6 KO in Gd11 after the first round on G418 selection media

Discussion

- The results of this study can increase the understanding of the role of the *Cellulose Synthase-Like D* genes in *Physcomitrella patens*.
- These data suggest that the CSLD1 protein is localized in growing tips of *P. patens* and may contribute to proper tip growth, consistent with previous studies.
- The results of the *CSLD* knockout mutant portion of the study are not complete.
- Double and single *CSLD* KO mutants need to be genotyped in order to confirm successful knockout before comparing the knockouts to the wild type in a phenotypic analysis.
- Phenotype analysis of the double knockout mutants will help reveal the roles of the individual *CSLD*s in cellulose biosynthesis and tip growth.

Literature Cited

Christos Sotirios Dimos (2010) Functional Analysis of the *Cellulose Synthase-Like D (CSLD)* Gene Family in *Physcomitrella patens*. *Univ. of Rhode Island*.

Gu and Nielson (2013) Targeting and Regulation of Cell Wall Synthesis During Tip Growth in Plants. *Journal of Integrative Plant Biology*. 55 (9), 835-846.

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Roberts and Bushoven (2007) The Cellulose Synthase (CESA) gene superfamily of the moss *Physcomitrella patens*. *Plant Mol Biol*. 63 (2), 207-219.

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