2024

Genotype Analysis of PpCSLD3/4/5/8 KO Mutants

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Genotype Analysis of PpCSLD3/4/5/8 CRISPR/Cas9 Knockout Mutants

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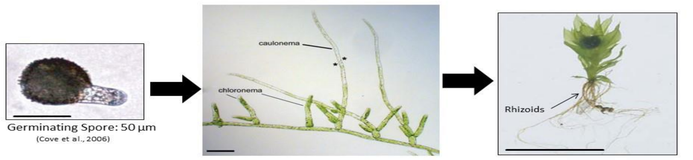
Abstract

The moss *Physcomitrella patens* is an attractive model organism, due to its small genome and dominant haploid phase. *P. patens* has eight genes that form a family known as the *Cellulose Synthase-Like Ds* (*CSLDs*). A previous study using global knockdown of the entire *PpCSLD* family by RNA interference (RNAi) showed a decrease in protonemal tip growth, indicating that *PpCSLDs* play a role in protometal growth. In order to study the roles of specific *CSLDs* in *P. patens* development, potential quadruple KO mutants for *PpCSLD3/4/5/8*? were generated by CRISPR/Cas9 mutagenesis. The goal of this project was to screen potential *ppcsld3/4/5/8*? KO lines for *PpCSLD8* gene deletions using competition-based PCR (cbPCR) and perform sequence analysis on lines positive for gene deletions. A total of 21 potential *ppcsld3/4/5/8*? KO lines were screened and 7/21 were positive for potential *PpCSLD8* deletions. The seven lines with potential deletions were sequenced and it was determined that all lines had the same six base pair insertion located just upstream of the potential deletion site. We hypothesize that this insertion was created due to off-target editing from the CRISPR-Cas9 *PpCSLD4* vector, when the PpCSLD4 gene was knocked out. This insertion most likely prevented the *PpCSLD8* gene from being knocked out using our current strategy.

P. patens



Germinating Spore: 50 µm (Cove et al., 2006) — Protonema: 100 µm (Menand et al., 2006) — Leafy Gametophore: 5 mm (Cove et al., 2006)

- Simple life cycle whose primary mode of growth is polarized tip growth (Schaefer et al., 1997).
- It has a dominant haploid phase (Schaefer et al., 1997).
- Its entire genome has been sequenced (Rensing et al., 2002).
- The CRISPR/Cas9 system can be used to generate knockout mutants (Mallett et al. 2019).

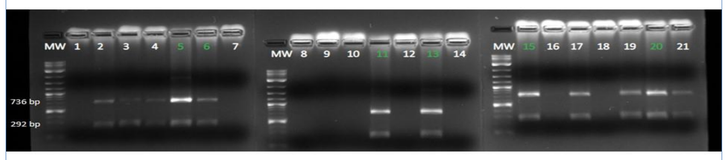
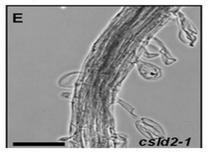


Figure 3. cbPCR for *PpCSLD8* sg1 cutsite: gDNA from twenty-one independent ppcsld3/4/5/8? KO lines were subjected to cbPCR using the D8_cp_sg1_Fout, D8_cp_sg1_Rout, and D8_cp_sg1_Rin primers. Lines 5, 6, 11, 13, 15, 16, and 20 were indel positive (highlighted in green font) and were selected for sequencing.

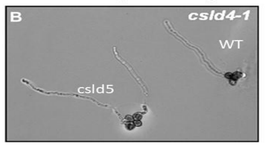
The *CSLD* Gene Family

Insertion mutants of *AtCSLD2* & *3* and *OsCSLD1* lead to ruptured or reduced root hairs.



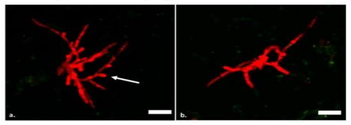
(Bernal et al., 2008)

Insertion mutants of *AtCSLD1* & *4* lead to reduced pollen tube growth.



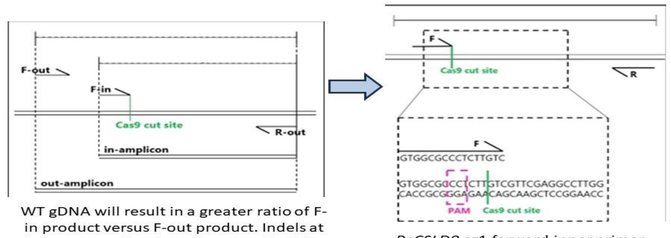
(Bernal et al., 2008)

Global silencing of the entire PpCSLD family by RNAi leads to inhibited protonemal tip growth



(Dimos, 2010)

Competition-Based PCR:



WT gDNA will result in a greater ratio of F-in product versus F-out product. Indels at the cut site will reduce the primer's ability to anneal to the KO gDNA, resulting in the opposite ratio for potential KO lines.

PpCSLD8 sg1 forward inner primer sequence. The forward inner primer overhangs the cut site by three bases.

Adapted from (Harayama et al., 2017).

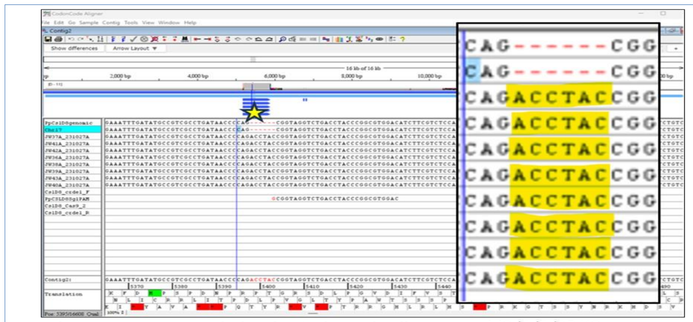
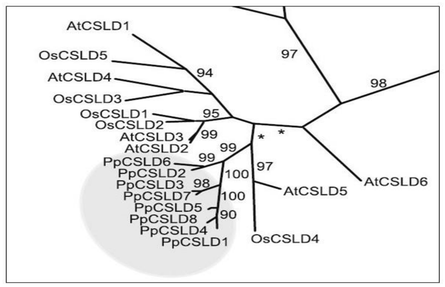


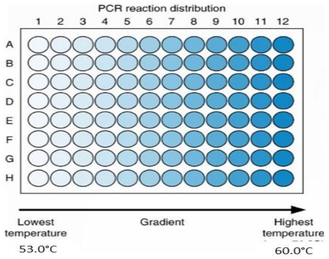
Figure 4. Sequence analysis using CodonCode Aligner: ppcsld3/4/5/8? KO lines 5, 6, 11, 13, 15, and 20 were sequence. A 6-point insertion mutation (highlighted) was found at the beginning of each *PpCSLD8* sg1 cut site.

Physcomitrella patens CSLD Gene Family



(Roberts et al., 2007)

Gradient PCR:



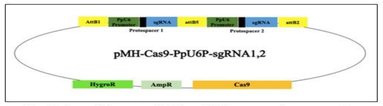
(He et al., 2018)

- A range of annealing temperatures is established slightly below and above the estimated optimal annealing temperature.
- The thermocycler is program to create a temperature gradient with in the established range.
- PCR reactions with identical components (template DNA, primers, polymerase, nucleotides, buffer, etc.) are set up in multiple reaction tubes.
- Each PCR reaction is run at a different annealing temperature within the gradient.
- The intensity and specificity of PCR products are compared across the gradient. The annealing temperature that produces the strongest and most specific amplification bands with minimal non-specific products is considered optimal.

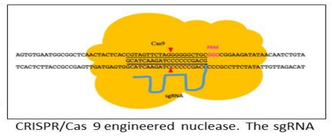
Summary

- Temperature Optimization PCR determined the ideal annealing temperature for outer primers (Fout & Rout) was in the 55.1 to 52.5 °C range & inner primer (Rin) was a range close to 53.0°C
- Competition PCR was used to identify the presence of 6 mutants that likely had indels: 5, 6, 11, 13, 15, and 20.
- These 6 samples were sent for sequencing after purification was performed.
- Sequencing data showed a 6 bp insertion upstream of the sg 1 cut site (ACCTAC).
- The same 6 bp insertion was detected in all 6 samples.
- We hypothesize that the *PpCSLD3/4/5* KO line that was transformed with the CRISPR-Cas9 *PpCSLD8* vector already contained the 6 bp insertion, which was due to off-target editing by the CRISPR-Cas9 *PpCSLD4* vector.
- The 6 bp insertion interrupts the cut site, most likely preventing CRISPR/Cas9 from knocking out the *PpCSLD8* gene.

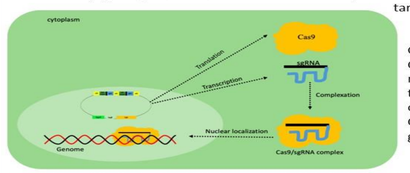
CRISPR/Cas9 Target-specific Modification of the *P. patens* Genome (Yuan, 2020)



Final plasmid map with 2 sgRNA expression cassettes, Cas9 expression cassette, and antibiotic selection cassettes (hygromycin, G418, and zeocin resistance)



CRISPR/Cas 9 engineered nuclease. The sgRNA works with the Cas9 endonuclease to ensure target-specific modification of the genome.



Gene editing by the CRISPR/Cas9 system. A CRISPR/Cas9 plasmid vector is used for PEG-mediated protoplast transformation. Inside the protoplast, the vector drives expression of the Cas9 protein and sgRNA, and the Cas9/sgRNA complex targets the moss genome for editing.

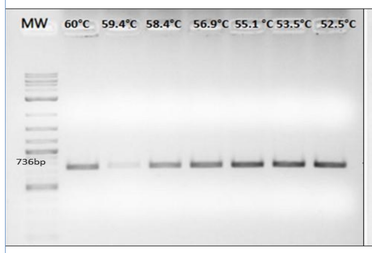


Figure 1. *PpCSLD8* cbPCR Outer Primer Gradient PCR- PCR products from amplification of Wildtype gDNA using the D8_cp_sg1_Fout and D8_cp_sg1_Rout primers from 53.0°C to 60.0°C.

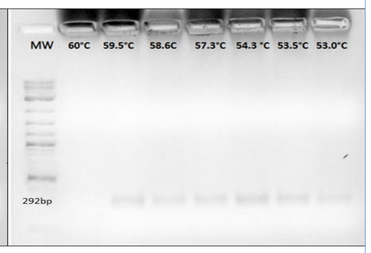


Figure 2. *PpCSLD8* cbPCR Inner Primer Gradient PCR- PCR products from amplification of Wildtype gDNA using the D8_cp_sg1_Fout and D8_cp_sg1_Rin primers from 53.0°C to 60.0°C.

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Acknowledgments

This project is supported by National Science Foundation Grant NSF 21-506 Division of Integrative Organismal Systems Core Programs.

Dr. Alison Roberts, University of Rhode Island

Ariel Chaves, University of Rhode Island

Dr. Michael Budziszek, Johnson & Wales University