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Phenotyping Cesa4KO Mutants in *P. patens*

Kristina Bischak

Introduction

In order to successfully study the cellulose deposition mechanisms of plants like the moss *Physcomitrella patens*, understanding the function of the Cesa genes is crucial. The Cesa genes, better known as the Cellulose Synthase gene superfamily, are responsible for the formation of cellulose in developing plants. Cellulose is an important biological molecule necessary for proper plant growth, particularly in non-vascular plants such as *P. patens*. There are seven known Cesa genes in *P. patens*: Cesa 3, 4, 5, 6, 7, 8, and 10. The effects of these cellulose synthase genes on the developing plant can be observed by removing specific Cesa genes from the moss genome. This can be done by engineering a “knockout” vector that will essentially remove the target gene (in the case of my project, Cesa4) from the chromosome. My overall goal for this project is to successfully phenotype specific Cesa4 KO mutant moss strains. The Cesa4 KO mutants were first isolated and genotyped by Dr. Norris and Daniella Carrasco in September of 2013. However, the only successfully isolated and genotyped mutant was Cesa4 13A. Although other mutants were isolated, efforts to genotype the 3' end of the Cesa4 KO vector in these knockout lines have not been successful. In my project I analyze the phenotypes of three Cesa4 KO mutant lines- 13A, 12B, and 14B- and compare them to the wild type (normal) Gd11 line.

Methods

To see whether the absent Cesa gene has an effect on cellulose formation and deposition during plant development, the rhizoids and caulonemal filaments are compared to those of the wild type Gd11 moss using a dissection microscope. If abnormalities are observed, then the phenotype of the moss has been altered and the knocked-out Cesa gene has an effect on moss development. First, the desired strains of genetically modified moss must be selected from the existing Cesa 4 KO moss cultures. All *P. patens* cultures will be grown under constant light at 25°C, and will be subcultured weekly. The subculturing procedure is as follows:

Start with unvented Petri plates containing BCDAT medium. Place sterile cellophane disks onto each plate and flatten if necessary. In the sterile centrifuge tube, add about 2 mL of sterile water. Scrape the moss from the starter culture into a mound and transfer it to the centrifuge tube; about ¼ of a plate of moss should be used to inoculate each new plate. To break up the moss, use a sterile homogenizer at medium power for 5-10 seconds or until the moss is uniformly dispersed without clumping. Next, pipette about 2 ml of moss suspension onto the surface of each plate and spread evenly. The new plates should be placed unsealed in the 25°C incubator with constant illumination (at about 50 µmol m⁻²). For my experiment, at least one plate of each cell line- the Gd11 wild type and the three Cesa4KO lines- will be subcultured each week, with four additional plates for caulonemal and rhizoid assays when necessary.

To phenotype *P. patens* rhizoids, moss cultures of the Cesa4KO and Gd11 lines are prepared using the above procedure. After seven days, the chloronemal tissue is spotted at 0.5 cm² on BCD medium with 1 µm/L auxin for 2 weeks with a replicate of 3 for each cell line. The genotype KO and wild type lines can be plated on the same plate for comparison, with a total of 3 plates. After sufficient growth, the results can be observed using the Leica dissecting microscope.

To prepare a caulonema growth assay, the above moss culturing procedure is again followed to produce 7 day old moss tissue. With a replicate of 3 from each cell line, approximately 0.5 cm² of tissue is spotted on 35 mM sucrose BCD plates. Genotype KO lines and wild type lines can be plated on the same plate for comparison with a total of three plates. The cultures are grown under constant illumination for 7 days, after which the plates are transferred vertically for 2 weeks. The caulonemal filaments are then ready for phenotyping using a dissecting microscope.

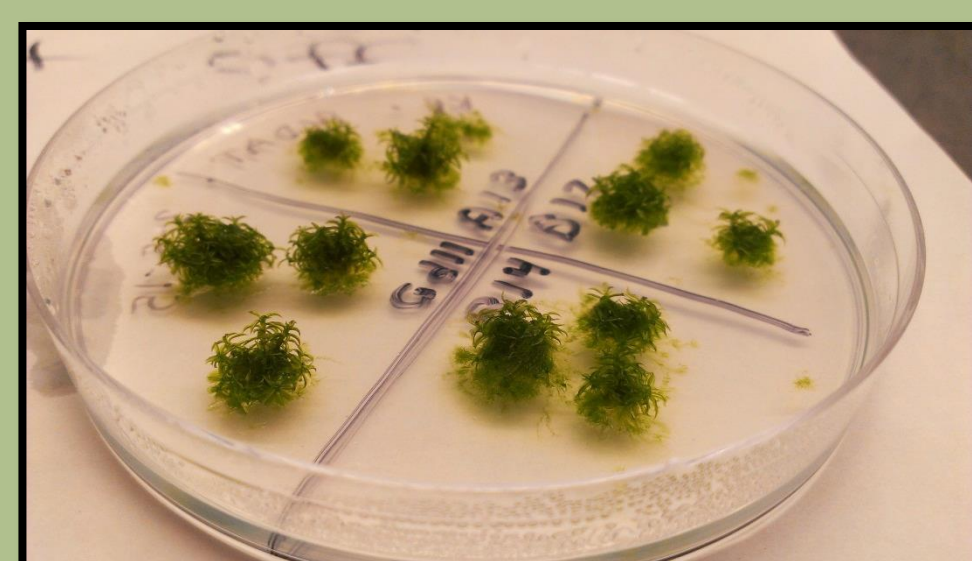
Create Cesa4 knockout mutant moss cells

Grow mutant moss colonies

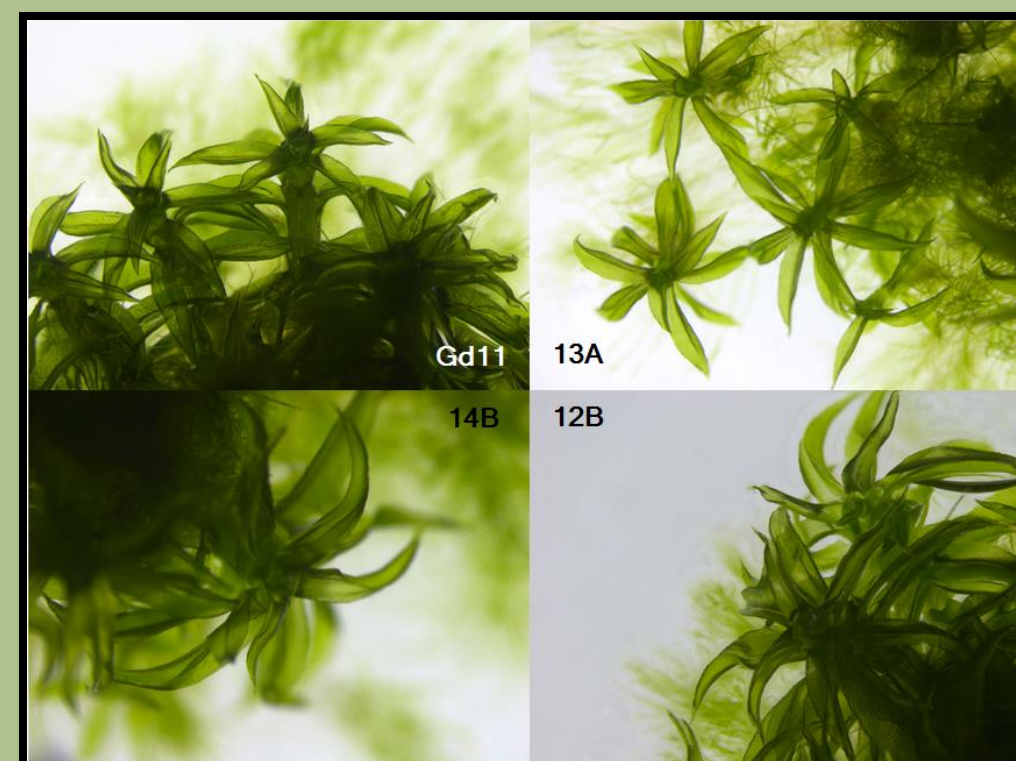
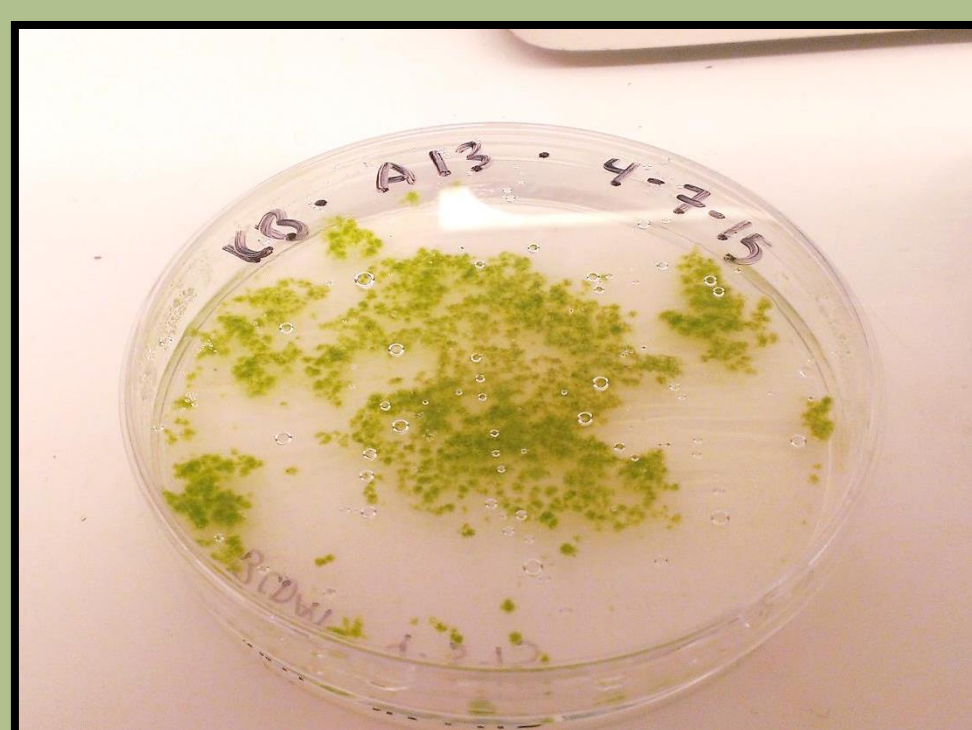
Subculture moss

Rhizoid Assay

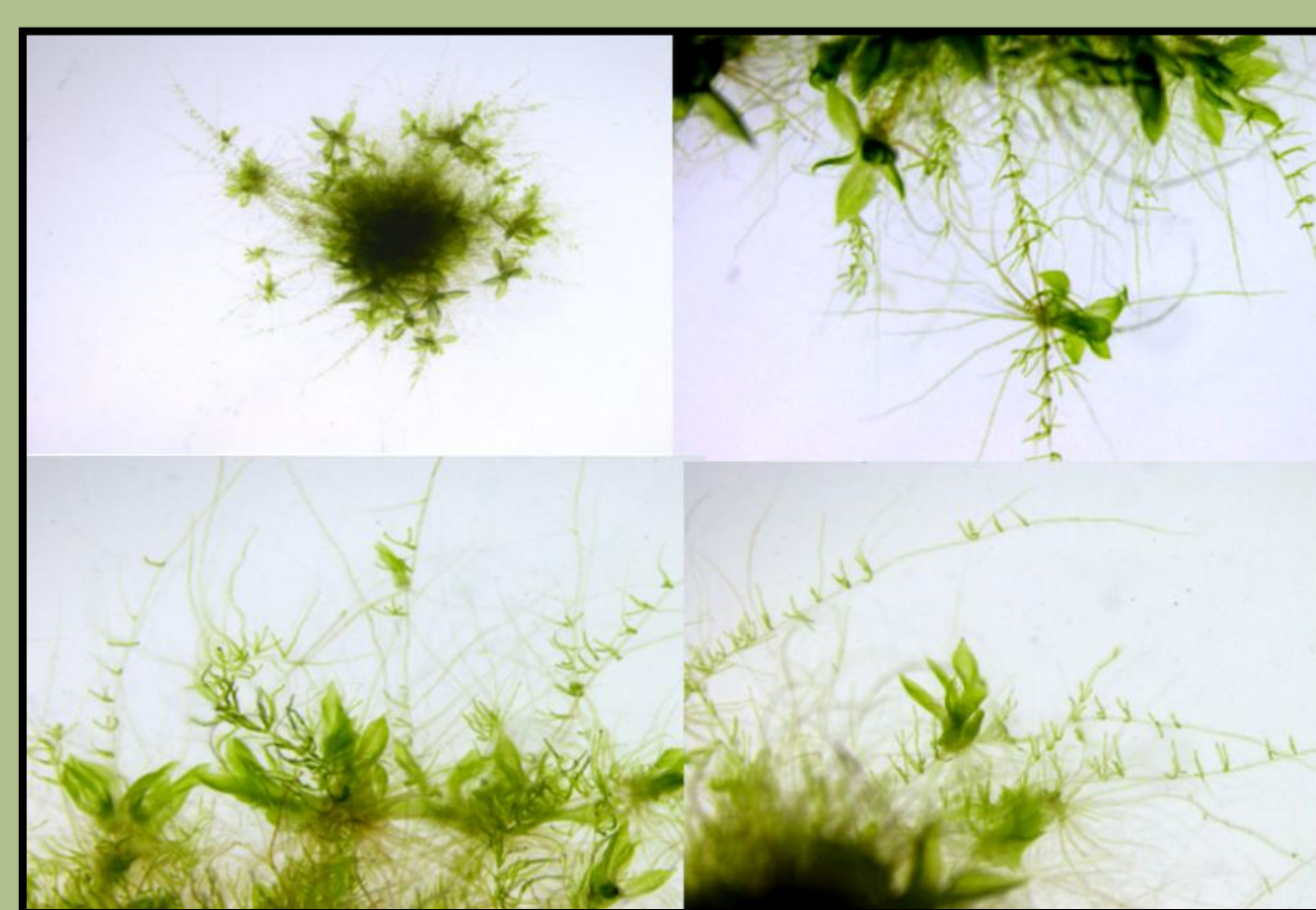
Caulonemal Assay



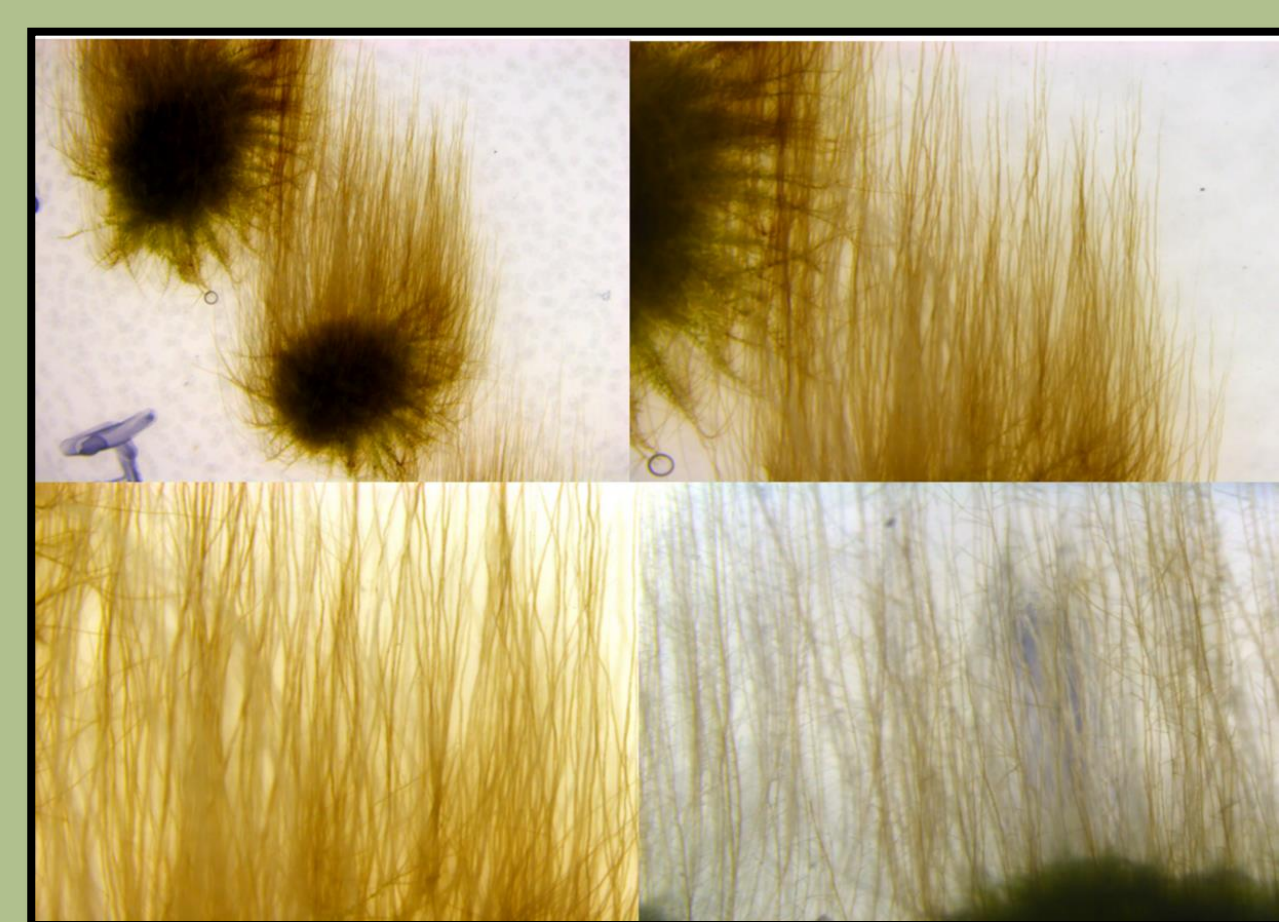
Top image: example of moss colonies grown on BCDAT media. Bottom image: subcultured moss



Close-up of mutant gametophores compared to wild type gametophores



Top left: colony grown in rhizoid assay. Counter clockwise from top right: magnified images of rhizoids. Images courtesy of Bailey Mallon.



Top left: Close-up of colonies grown in caulonemal assay. Top right: 12x magnification of colony. Bottom left: 20x magnification of same colony.

Conclusion and Future Directions

Although I was not able to fully phenotype the Cesa4 knockouts, progress was made in terms of avoiding contamination and performing caulonemal filament and rhizoid assays on the mutant moss. Also, the data I have gathered from the Cesa4KO mutants during my project will contribute to the information known about Clade II cellulose synthase genes and their evolutionary history. In order to fully analyze phenotypic differences and to gather statistically significant data, much more work needs to be done on this project. Caulonemal and rhizoid assays need to be repeated, and measurements taken to quantify any differences between wild type and mutant gametophores.

Despite my limited success in completing the project, I have learned many valuable skills in the process. Throughout the course of this project I have learned sterile tissue culture techniques, experiment planning and management skills, and the importance of patience when dealing with biological materials.

Discussion

Most of my time working on this project was used to get the moss growing properly on cellophane. Unfortunately this means that I was not able to collect much data, although I do have data from two caulonemal growth assays and one rhizoid assay. Based on the first caulonemal assays, there are a few noticeable differences between wild type and mutant moss caulonemal filaments. There was no noticeable difference in average filament length between the wild type and mutants, but a few of the filaments grown from the 12B cell lines appeared to have branched filaments. All other filaments varied from slightly wavy to straight, but no others appeared to exhibit branching.

A general observation of colonies grown from each mutant line also produced interesting results. The shape of the mutant gametophores varies from the wild type gametophores in subtle ways. For example, the Gd11 gametophores exhibit a very normal structure. The leaves have a gentle sloping curve, and each gametophore typically has around 5-6 leaves per stalk. Looking at 13A gametophores, we see that the leaves appear to be somewhat curly compared to the Gd11 leaves. Also, the gametophores are not as thickly distributed, and appear to be longer than the Gd11 gametophores. 13A mutants also seem to have slight difficulty growing compared to the other mutants. Similar variations were seen on the clumps of the other mutants: 12B and 14B were slightly less densely populated compared to Gd11 clumps, and the gametophore leaves also had slightly different structures. 12B and 14B mutants have leaves that appear to be slightly thinner than the wild type, although not wavy like the 13A mutants. More mutant colonies would have to be grown, and measurements for gametophore leaf width and gametophore height would have to be taken in order to determine whether these differences have statistical significance.

Acknowledgements

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