5-2013

Exploring the role of CESA5 in the synthesis of cellulose using Physcomitrella patens

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Abstract

Cellulose is very essential to plants because it determines the shape of cells, protects them from pathogens, and helps retain water that is needed for plant functions. It is also the major component of wood, cotton, and paper, which are items we use on a daily basis. Also, it can be used for the synthesis of biofuels. However, cellulose exists as strong fibers, which make it hard to breakdown for biofuel synthesis. If we can understand how cellulose is synthesized we can manipulate its fibers to make them stronger, more flexible, more absorbent, or easier to break down for use as biofuels. Cellulose synthase complexes are observed by electron microscopy in the plasma membrane and Golgi vesicles of algae and plants. However, the different CESA proteins cannot be identified using this technique. Therefore, the number of CESAs in a complex is still unknown as well as their stoichiometry. Regulation of the activity and assembly of the complex are also unknown. A general idea is that there are certain CESAs that are responsible for the formation of the primary cell wall, and others that are responsible for the secondary cell wall in vascular plants. But, the moss *Physcomitrella patens* has only primary cell walls and still has seven different CESAs. The purpose of this project is to study the properties and characteristics of cellulose synthase 5 (CESA5). Understanding the role of CESA5 in *P. patens* can lead to a better understanding of the evolution of the cellulose synthase complex and the formation of cellulose. This can ultimately enable us to break down cellulose for biofuels and use it as a possible solution to global warming. During the course of this

project, a miniSOG-tagged CESA 5 expression clone was successfully created using Gateway cloning. Polymerase chain reaction and a BP reaction were used to make a tandem-miniSOG entry clone and an LR reaction was used to insert the entry clones into the destination vector. The destination vector was transformed into the moss. Phenotypic analysis will be performed on the transformed lines to gain insight onto the function of CESA 5 in the synthesis of cellulose.

Introduction:

Plant cell walls are vital for plant development. Cellulose is the major component in plant cell walls, and it is the world's most abundant biopolymer. Cellulose is very essential to plants because it determines the shapes of the cells, protects it from pathogens, and it helps retain water that is needed for plant functions.

Cellulose contains linear chains of glucose residues, but these chains aggregate to form impeccably strong microfibrils, which are deposited into outside the plasma membrane by transmembrane proteins protein complexes known as Cellulose Synthase Complexes (CSC) (Sommerville et. al, 2006). These complexes contain cellulose synthase proteins (CESA) and come in different morphological forms, two of which are the rosette and linear forms. In seed plants there are certain CESA proteins that are responsible for the formation of the primary cell walls and others that are responsible for synthesis of the secondary cell wall. Genome sequencing has shown that there are 7 CESA genes and 3 pseudogenes in *Physcomitrella patens* (Roberts and Bushoven, 2007). Additionally, freeze fracture

electron microscopy has shown that *P.patens* has rosette shaped CSCs that polymerize the beta-1-4-glucan chains that assemble into cellulose microfibrils (Roberts et. al, 2013). *Physcomitrella patens* has only a primary cell wall and still has the seven different CESAs. Understanding the roles of each of the CESAs in *P. patens* can lead to a better understanding of the evolution of the CSC and the formation of cellulose.

To enable better visualization, a genetically encoded tag can be used to image specific proteins within cells and organisms. The miniSOG tag used in this project enables the highest spatial resolution in protein localization via electron microscopy. MiniSOG is a fluorescent flavoprotein made from *Arabidopsis* phototropin 2. "Illumination of miniSOG does not destroy the cell, it allows for ultra structural preservation and 3-D protein localization" (Shu et. al, 2011). Electron microscopy allows for higher magnification ultimately allowing for better visualization. In this project, a miniSOG-CESA5 expression vector was created and then transformed into the moss *Physcomitrella patens* for genotypic and phenotypic analysis.

Methods:

Polymerase Chain Reaction (PCR):

The purpose of the PCR was to amplify the miniSOG DNA template; it creates the product with the attB sites that recombine with the attP site on the pDONR vector to successfully create the entry clone. In this project, TdSOG and N1SOG templates were amplified to produce the appropriate entry clone using the Phusion enzyme according to manufacturers instructions. The forward primers used for TdSOG and N1SOG templates were miniSOGTd-att1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACCATGGAGAAGAGCTTCGTGAT) and mSOG-att1 (GGGGACAAGTTTGTACAAAAAAGCAGGCTCAACCATGGAGAAAAGTTTCGTGAT),

respectively, and the reverse primer used was mSOG-5r (GGGGACAACTTTTGTATACAAAGTTGTTCCATCCAGCTGCACTCCGAT):

BP Recombination Reaction:

The BP reaction creates the entry clones by inserting the PCR fragments into the pDONR vector. The number of fmoles of DNA is converted to nanograms to determine the amount of DNA needed for the reaction. A 4 µl reaction containing 11 ng of attb PCR product (TdSOG), 1 µl of pDONR vector, and 1X TE buffer was placed in a tube, and then 1µl of BP Clonase II was added to catalyze the reaction. The reaction was incubated at 25^{0}C for 1 hour. After the BP reaction was complete, competent bacterial cells were transformed with the plasmid. In the transformation reaction, 2 µl of the DNA was added to the *E. coli* cells and incubating them for 20 minutes at room temperature and heat shocking them at 42^{o}C for 30 seconds. Then 250 µl of S.O.C medium was added to the vial, which was incubated for 1 hour at 37^{o}C. During that LB kanamycin antibiotic plates were made. When the plates cooled, the transformation product was smeared on two LB kanamycin plates and incubated overnight for bacterial colonies to develop.

Restriction Analysis:

The Qiaprep Spin Miniprep Kit was used to isolate bacterial plasmids from the 4 bacterial colonies that were selected from the transformation reaction. The restriction analysis required 10x Buffer 4, and 0.25 µl of EcoRV to cut the insert out of the plasmid. Following the analysis, an agarose electrophoresis gel was prepared to run the samples of plasmids to determine the sizes of the fragments.

LR Reaction:

The LR reaction was used to insert our CESA5 and TdSOG entry clones into the xt18 destination vector using LR clonase enzyme to catalyze the reaction. The tube containing the tdSOG, CESA5 and xt18, TE buffer, and the LR clonase enzyme were incubated for 16 hours at room temperature. Competent bacterial cells were transformed with the plasmid as described for the BP reaction.

Genetic transformation of *P. patens*:

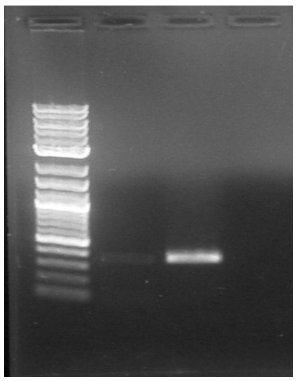
The expression vector, which consisted of CesA5-miniSOG, was transformed into the moss. After transformation and antibiotic selection, the genotypes of the antibiotic resistant moss were analyzed.

Results:

For the first PCR we used TdSOG and N1SOG templates and mSOG-att1 and mSOG-att5r primers. The gel revealed that TdSOG, which we expected to amplify as a 727 bp double miniSOG, amplified as a 382 bp single miniSOG (Figure 1). Restriction digest analysis and sequencing revealed that the single miniSOG was successfully cloned (Figure 2). Unfortunately, the LR reaction was not successful. The restriction digests of the plasmids did not show the expected 10177 and 2915 bp bands (Figure 3a). In order to test the remaining colonies, we performed a bacterial screening test with a positive control. The positive control was compared to colony screens to determine if any of them contained the right product. The gel from the screening revealed an absence of the expected band from all other samples therefore our LR

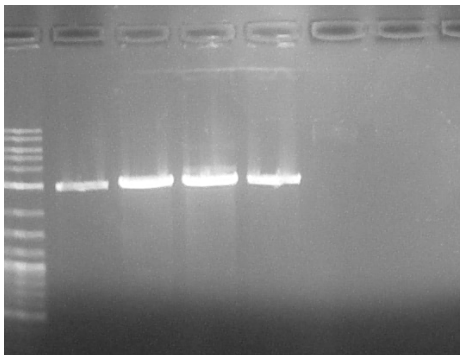
was unsuccessful (Figure 3b). PCR of TdSOG using the miniSOGTd-att1 and mSOG-att5r produced the expected 727 bp band, and when the plasmid was sequenced, results showed that the TdSOG sequence was correct. Another LR reaction was performed using the Td-miniSOG-P1 5r and miniSOG- P15r and the xt18 as the destination vector. After performing a transformation reaction and restriction digest, the gel electrophoresis confirmed the expected 10177 and 2915 bp bands (Figure 4).

Protoplasts isolated from a *P. patens cesa*5 knockout line were transformed with the expression vector. After antibiotic selection was complete there were 5 moss colonies, and 3 out of the 5 *cesa*5 knockouts produced leafy gametophores (Figure 5). Results from the PCR-based genotyping for the 5' to 3' integration of the vector produced two bright bands from colonies 4 and 5 confirming the 3' integration (Figure 5).



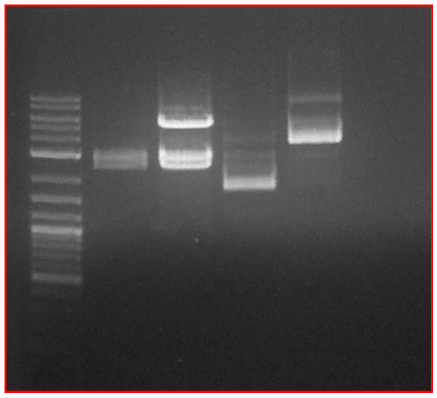
L 1 2

Figure 1 PCR OF TdSOG (Lane 1) and N1SOG (Lane 2) templates with mSOG-att1 m-SOGatt5r primers. The bright band in lane 1 represents the TdSOG that amplified as a single template.

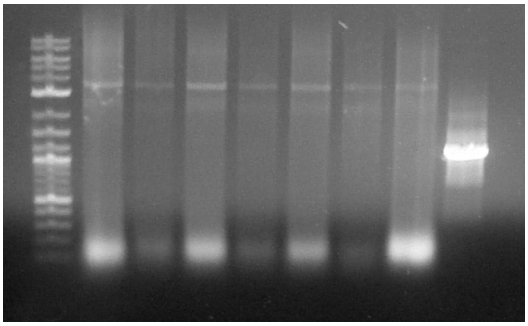


L 1 2 3 4

Figure 2: Restriction digest of plasmids from BP Reaction: Electrophoresis of the plasmid DNA isolated from the colonies that resulted from a BP recombination reaction cut with EcoRV.



L 1 2 3 4



1 2 3 4 5 6 7 8 9 10

Figure 3: (A) Restriction digest of plasmids from LR reaction. Electrophoresis of the plasmid DNA isolated from the colonies that resulted from an LR recombination reaction cut with SwaI. None of the plasmids produced the expected 10177 and 2915 bp fragments. (B) PCR of bacterial colonies that resulted from an LR reaction (6 colonies per lane). The bright band in lane 10 represents the positive control. This band was absent in the other lanes confirming that the LR was unsuccessful.

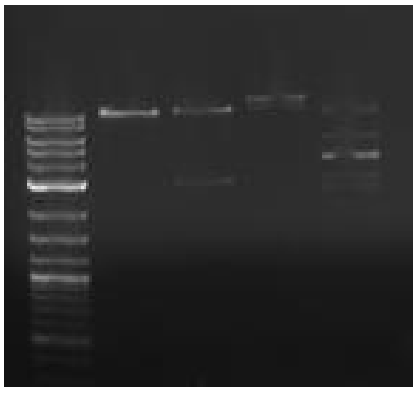


Figure 4: LR reaction of TD-miniSOG-P15r and miniSOG- P15r, using xt-18 as the destination vector. The bands represent in lane 2 confirm the expected 10177 and 2915 bp bands.

Figure 5: This photo shows the CESA5-KO that produced leafy gametophores.

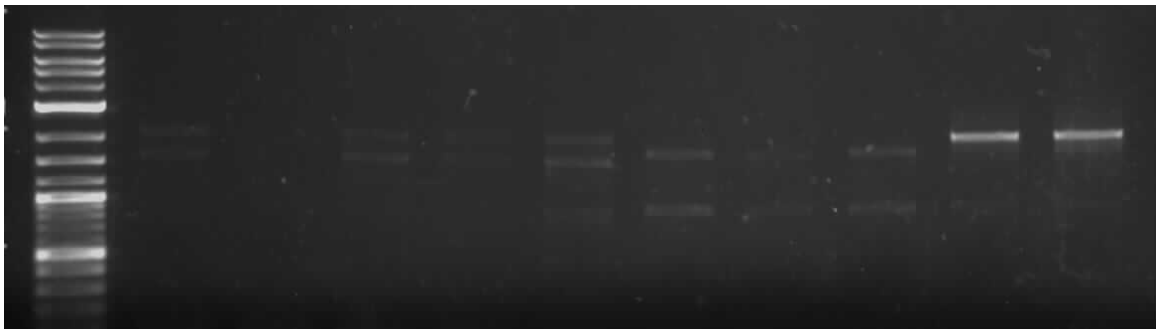


Figure 6: Gel electrophoreses from the PCR-based genotyping of moss transformants. The two bright bands indicate successful 3' integration of the vector from colonies 4 and 5.

Discussion:

The main goal of this project was to create an expression vector containing CESA5 and TdSOG and use it to transform a *P. patens cesA5* knockout mutant. After several tries the entry clones where successfully created and the moss was successfully transformed. The transformation produced 2 lines (#4 and #5) with the vector successfully integrated by double homologous recombination based on PCR genotyping. The wild type phenotype was restored in both of these lines. This shows that the miniSOG-tagged CESA5 protein is functional. More research will be

conducted on CESA5 to determine its role in the formation of CSCs, including how CESA5 interacts with other CESAs and how it affects the synthesis of cellulose.

Reference:

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