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EFFECTS OF CELLULOSE SYNTHASE (CesA) MUTATIONS ON CATALYTIC ACTIVITY, CELL WALL STRUCTURE, AND ISOFORM EXPRESSION PROFILE

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ABSTRACT

In higher plants, cellulose is thought to be produced by cellulose synthase (CesA)-containing complexes. In *Arabidopsis thaliana*, there are 10 CesA isoforms. Six of these isoforms (1, 3, 4, 6, 7, and 8) have been observed within one of two complexes: the primary cell wall CSC (1, 3, 6) and the secondary cell wall CSC (4, 7, 8), however the CesA stoichiometry within these complexes is currently unknown. This study seeks to determine the individual contribution of CesA 4, 7, & 8 catalytic activity on the structure of the secondary cell wall. Catalytically inactivated CesAs were developed using site-directed mutagenesis of a key aspartate residue in each. These mutant genes were then used to complement CesA knockout plants. Spectroscopic analysis of the resultant cell wall structures should yield insight into the number and arrangement of CesA 4, 7, & 8 in the secondary cell wall cellulose synthesis complex.

Additionally, this research plans to address the effect of various CesA mutations on the expression patterns of the remaining isoforms. The CesA promoters were each cloned upstream of the beta-glucuronidase (GUS) gene to create a reporter system. These constructs are then to be introduced into a range of different mutant *Arabidopsis* lines. GUS staining of these lines will reveal any modulation in CesA expression caused by each background mutation, leading to better understanding of the role of each of the 10 CesA isoforms. Currently, the CesA4 and CesA7 reporters are ready for plant transformation and the other reporters are currently in construction.

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1. INTRODUCTION

The synthesis and degradation of cellulose, a major component of wood, is of great interest to scientists seeking renewable energy sources (Guerriero, Fugelstad, & Bulone, 2010). At the microscopic level, cellulose plays an important role in cell protection. Higher plants, as modeled by *Arabidopsis thaliana*, have a primary cell wall that is present on every cell and a thicker secondary cell wall found in vascular tissue to protect the cells from the hydrostatic pressure of the sap transport (Taylor, 2008). It is this secondary cell wall which supplies the rigidity found in woody materials.

Despite being the most abundant biopolymer on earth, the synthesis of cellulose in higher plants is not well understood (Guerriero, Fugelstad, & Bulone, 2010). In *Arabidopsis*, the cellulose synthase (CesA) proteins are thought to be primarily responsible for the production of cellulose. There are 10 genetically distinct versions of CesA in *Arabidopsis*. Six of these CesA proteins have been found to form complexes of unknown stoichiometry dubbed cellulose synthesis complexes (CSCs): CesA 1, 3, and 6 form a complex responsible for primary cell wall synthesis (Desprez et al 2007), while CesA 4, 7, and 8 form a complex which produces the secondary cell wall (Taylor et al. 2003). A recent study has shown, however, that certain CesAs from the primary complex can be associated in the secondary complex and vice versa when promoters are swapped (Carroll, et al., 2012). No studies, to our knowledge have shown this mixing phenomenon under native conditions, so this experiment operates under the assumption that the two CSCs are mutually exclusive. The roles of the other four CesA proteins are not well understood, with the exception of a highly specialized role of CesA9 in seed production (Stork, et al., 2010).

A variety of cellulose-deficient *Arabidopsis* mutants have been discovered in the past two decades including plants with null mutations of the secondary cell wall CesAs (Turner & Somerville, 1997; Turner, Taylor, & Jones, 2001; Taylor, Gardiner, Whiteman, & Turner, 2004; Brown, Zeef, Ellis, Goodacre, & Turner, 2005). Due to the secondary cell wall's role in xylem stabilization, these dwarf mutants are named irregular xylem (irx). The irx mutants are numbered in the order they were discovered, not according to the number corresponding CesA which is affected (See Table 1).

One mutant of particular interest to this investigation is irx1-1 (Turner & Somerville, 1997). This strain contains a missense mutation of an aspartic acids residue (D683N); this residue is one of the three aspartic acids conserved through most processive glycosyltransferases and is thought to serve a catalytic role (Turner, Taylor, & Jones, 2001). This mutation is thought to be sufficient to knock out all catalytic activity from CesA8 and yields a comparable phenotype compared to other irx mutants. While this missense mutation halts catalytic activity of CesA8, it does not hamper the enzyme's ability to participate in the formation of the CSC, as seen by the mutant protein's ability to interact with CesA4 and 7 *in vitro* and *in vivo* (Taylor, Howells, Huttly, Vickers, & Turner, 2003; Gardiner, Taylor, & Turner, 2003). In knockout mutants, elimination of both the catalytic activity of a CesA and its ability to participate in the CSC occurs. This creates a confounding phenotype, as removal of protein-protein interactions with a CesA knockout compromises the whole CSC and thus effects the cellulose synthesis ability of the remaining CesAs. Because irx1-1 and analogous aspartic acid to asparagine mutants in CesA 4 and 7 only take into account the catalytic role of the individual CesA and not the ability of the CSC to form, they should be useful tools in determining each CesA's relative catalytic role in the secondary cell wall CSC. By analyzing the cellulose content and cell wall structure of vascular tissue in

these mutant plants using various spectroscopic and techniques (Harris, Bulone, Ding, & Debolt, 2010), a rough estimate of catalytic unit stoichiometry and orientation of catalytic subunits may be determined. The first half of this investigation centers on the construction of these missense mutants through site-directed mutagenesis and *Arabidopsis* transformation using *Agrobacterium tumefaciens*.

The second half of this project focuses on the detection of CesA expression levels in various organs and at various growth stages within *Arabidopsis* using the GUS reporter. When a gene's promoter is cloned upstream of the GUS gene, GUS is expressed at the time and location within the plant where the native gene is being expressed (Jefferson, 1989). In the presence of X-Gluc, GUS expression leads to a visible blue precipitate, allowing for easy visualization of plant gene expression. Previous studies have used GUS to monitor the expression of certain CesAs in wild-type and primary-cell wall CSC mutant *Arabidopsis* (Desprez, et al., 2007; Persson, et al., 2007). The present investigation will use similar constructs as these experiments, but the expression levels of all 10 CesAs will be monitored in various secondary and primary CSC mutant strains as well as wild-type *Arabidopsis* (See Table 1). By visualizing the expression of each of the CesAs in both mutant and wild-type backgrounds, any compensatory changes in expression level of any of the CesAs due to the mutation of another CesA can be monitored. This may elucidate potential roles of the four CesAs whose purpose is largely unknown (2, 5, 9, and 10).

Table 1: *Arabidopsis* Mutants Used in this Report

Mutant Name	Ecotype	Mutant Gene	Type of Mutation	Zygosity
irx 1-1	Landsberg erecta	CesA8	EMS D683N	Homozygous
irx 1-5	Columbia	CesA8	T-DNA	Homozygous
irx 3-1	Landsberg erecta	CesA7	EMS W859Stop	Homozygous
irx 3-4	Columbia	CesA7	T-DNA	Homozygous
irx 5-4	Columbia	CesA4	T-DNA	Heterozygous
Je5	Columbia	CesA3	G916E	Homozygous
2/5/9 Knock Out	Columbia	CesA 2, 5, and 9	T-DNA	Homozygous

Je5 seeds were supplied as a gift from Dr. Charlie Anderson; the 2/5/9 knock out seeds received as a gift from Dr. Seth Debolt.

2. MATERIALS AND METHODS

2.1 Agarose Gel Electrophoresis

Agarose (.8%) was added to TAE buffer (40 mM Tris, 1 mM EDTA); the suspension was boiled briefly; ethidium bromide (0.5 μg/ml) was added; and the solution was allowed to cool into a gel. Samples were loaded with 5X DNA gel loading buffer (brought to 1X), and the gel was run at 200 V for 15-45 minutes depending on the desired amount of separation. Gels which ran so long that the ethidium bromide evacuated an important area of the gel were soaked in TAE containing 0.5 μg/ml ethidium bromide for 30 minutes to stain the DNA.

2.2 Restriction Endonuclease Digestion

Endonuclease digestions were set up using the buffers suggested by New England Biolabs. All digestions were performed at 37 °C for sufficient time to perform a 10-fold digestion. This time was calculated using the number of units of enzyme, μg of DNA, and the NEB's definition of unit for the enzyme.

2.3 *E. coli* Transformation

XL-1 Blue cells were thawed on ice for 5 minutes, DNA was added, and the cells were mixed by flicking and were placed on ice. After 20-30 minutes, the cells were heat shocked at 42°C for 45-50 seconds. The cells were brought to 1 ml with LB and were allowed to recover, shaking at 37°C for one hour. The eppendorf tube was then spun at 4000 rpm on a table-top microcentrifuge for two minutes to pellet the cells. The cells were resuspended in 40 μl LB with selection antibiotics

(50 μg/ml Kanamycin or 100 μg/ml Ampicillin depending on resistance). The suspension was plated on LB plates containing the same antibiotic and incubated at 37 °C overnight.

2.4 Purification of DNA

Following enzymatic reactions and PCR, DNA was purified by one of two manners. If there was reason to believe contamination by uncut DNA or primers would lead to complications in cloning, the DNA was loaded onto a 0.8% agarose gel and run for 30 minutes at 200 V. The band was then excised from the gel, melted at 50 °C in NT1 buffer (2 μl per mg gel) from the EZ-10 Spin Column DNA Gel Extraction Kit. After 5 minutes, the solution was placed in a spin column. Using a table-top microcentrifuge, the column was spun at 10,000 rpm for 2 minutes, washed with NT3, dried, and eluted into 20-50 μl elution buffer according to the protocol. If the primers or uncut DNA were not threatening to the proceeding steps, the DNA was mixed with NT1 (2:1, NT1: reaction ratio) and added to the spin column. The same steps of spinning, washing, drying and eluting as above were completed. The Qiagen Qiaex II Gel Extraction Kit was used to purify plasmids larger than 9 kb.

2.5 Site-Directed Mutagenesis

Aspartate to Asparagine mutants were created using the Stratagene QuikChange™ II Site-Directed Mutagenesis Kit. The reactions were prepared and run according to the protocol with the exception that mineral oil overlay was not used, as the thermocycler used had a heated lid capability. The polymerization reaction was cycled 12 times with an extension time of 17 minutes. The templates for the reactions were cDNA of CesA 4, 7, and 8. After DpnI digestion, 1 μl of the reaction was transformed into 50 μl XL1-Blue competent cells. Colonies were grown up in LB with cDNA vector-specific antibiotic overnight. The plasmid was isolated from the liquid

culture using the EZ-10 Spin Column Plasmid DNA Kit; the aspartate to asparagine mutation was verified via Sanger sequencing (PennState University Nucleic Acid Facility).

2.6 Cloning of Site-Directed Mutants (SDM) with Native Promoters into pORE-O3

CesA 4, 7, and 8 site-directed mutants (SDM) were amplified using primers (0.5 μM each) with tails containing overlapping recognition sequences as designed for inFusion HD cloning kit. The PCR was set up and run according to the Finnzymes Phusion™ protocol with the exception that 0.23 μl Phusion was used per 50 μl reaction. Similarly, the native promoters, defined as approximately 2.5 kbp upstream of the start codon, of the CesAs were amplified with primers containing inFusion-specific sequences. The promoter template was genomic DNA isolated from Col-0 ecotype *Arabidopsis* according to the protocol previously reported (Kasajima, Ide, Ohkama-Ohtsu, Yoneyama, & Fujiwara, 2004). pORE-O3 was linearized in anticipation of recombination by cutting with Xho1 and BamH1 (New England Biolabs). The inFusion reaction was prepared using the Clontech in-Fusion® HD Cloning Kit User Manual protocol with the exception that each reaction was 3.5 μl total, containing 0.5 μl reaction mix, 1 μl vector, 1 μl promoter, and 1 μl CesA PCR. The reaction was incubated at 50 °C for 20-30 minutes. The reaction was transformed into XL-1 Blue as described above and plated on LB-Kanamycin (Kan) plates. Colonies were screened via colony PCR using the promoter primers. The 20 μl reactions were prepared according to the Promega GoTaq® protocol with the template being replaced with a small amount of *E. coli* cells. This was accomplished by touching the colony with a micropipette tip, tapping an LB-Kan plate six times, and placing the tip in the reaction for 30 seconds, tapping the tip on the reaction tube as it was removed. Colonies positive for promoter DNA were grown up, mini-prepped, and sent for sequencing as described above.

2.7 Cloning of Promoter-GUS Constructs

Promoters were amplified from Col-0 genomic DNA (Kasajima, Ide, Ohkama-Ohtsu, Yoneyama, & Fujiwara, 2004) according to the Finnzymes Phusion® protocol using primers with tails containing the cut sites for NotI and either SacII (Pro 4 and 7) or XhoI (Pro 1, 2, 3, 5, 6, 8, and 9). pORE-O3 was cut with the same enzymes. Ligation mixtures (200 U T4 ligase, 3:1 insert to vector molar ratio, 5 µl total) were incubated at room temperature for 20 minutes. The ligation (2.5ul) was transformed into XL1-Blue competent cells as described above. The colonies were screened using colony PCR as described above using pORE-O colony PCR primers. The successful promoter-containing plasmid was then cut with Not1 and Kpn1. Gus was amplified from pORE-R1 using primers containing NotI and KpnI sites. GUS was then ligated into the promoter-containing pORE-O3 in the same manner as described above.

2.8 Agrobacterium Transformation

Purified pORE-O3 CesA construct (5ul) was transformed into *Agrobacterium tumefaciens* according to the published protocol (An, Ebert, Mitra, & Ha, 1988). The DNA was added to 50 µl liquid nitrogen-frozen *Agrobacterium*, and the cells were thawed at 37 °C for 5 minutes. The volume was then brought to 1 ml with LB, and the cells were incubated at room temperature for 2 hours. The cells were then spun at 4000 rpm for 2 minutes and plated on LB-Kan plates. The colonies were screened by growing them in LB-Kan liquid media at room temperature for two days, isolating the plasmid by miniprep, and using the plasmid as the template for a diagnostic PCR reaction using promoter-specific primers. The PCR was accomplished according to the Promega GoTaq® protocol.

2.9 *Arabidopsis* Transformation

Arabidopsis plants were grown until they began to flower. Transformed *Agrobacterium* were grown in LB-Kan-Gentamicin for two days, pelleted, and resuspended to an OD_{600}=0.8 in 5% sucrose, 0.02% Silwet L-77. The inflorescences of the plant were then dipped into this solution for 2 seconds. Inflorescences which were too short to be dipped had 10 µl of this suspension applied to their tip using a micropipetter. The plants were returned to the growth in covered trays to recover under high humidity. After 24 hours the covers were removed and the plants were allowed to grow under long day conditions. After two weeks the plants were retransformed with the same *Agrobacterium*.

2.10 *Arabidopsis* growth conditions

Arabidopsis thaliana was grown under 16-hour days at approximately 25 °C in Pro Mix BX with Osmocote 10-10-10 fertilizer (applied according to label). Due to gnat larvae infestation of the soil, a thin layer of soil containing Dr. Pye's Scanmask Beneficial Nematodes was placed on the top of each pot. Once the plants produced mature siliques, they were moved to 24-hour light and allowed to dry out. The inflorescences were then harvested and allowed to dry at least 2 weeks in a paper pouch. The seeds were then harvested by rubbing the pouch against a table to break open the siliques and passing the plant material through a sieve. The seeds were separated from the remaining plant material manually by passing the seeds from one piece of paper to another. Seeds of transformed plants were plated on glufosinate (BASTA) plates (1% Sucrose, 0.5x Murashigi and Skooge, 10 µg/ml BASTA). Successful transformations were then transferred to soil with 1 week under a plastic dome to maintain high humidity while the seedlings established a roothold.

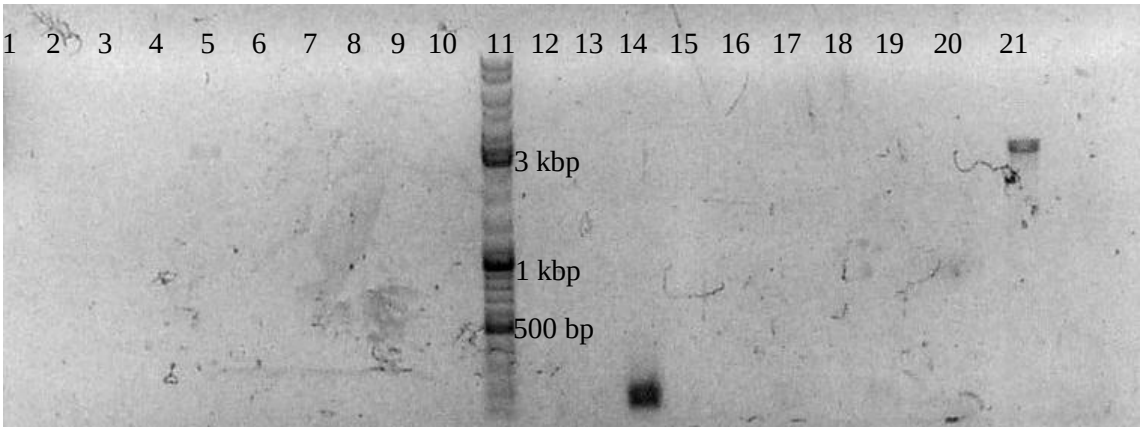
3. RESULTS

3.1 Site-Directed Mutagenesis, Promoter PCR, and inFusion into pORE-O3

Site-directed mutants (SDMs) of CesA 4, 7, and 8 were created using the Stratagene QuikChange™ II system, altering the conserved, catalytic aspartic acid residue to an asparagine (See Apendix B). The mutation was verified via Sanger Sequencing using CesA sequencing primers.

The SDM-CesA plasmids were then used as the template of a PCR reaction to amplify the CesA SDM gene and the CesA 4, 7, and 8 native promoters were PCR amplified from Colombia ecotype *Arabidopsis thaliana*. Equal volumes CesA SDM, corresponding native promoter, and cut pORE-O3 were combined into an inFusion reaction, and transformed into *E. coli* (XL-1 Blue). Colonies were screened using colony PCR with promoter primers. A sample gel of screened CesA4 SDM inFusion colonies is found in Figure 1.

Figure1: Agarose Gel of Colony PCR of CesA4 SDM inFusion



Bands at approximately 3.5 kb such as that found in lanes 5 and 21 represent the presence of the CesA gene and therefore were considered positive results. Lane 14 which contained a thicker smear of DNA around 150 bp was far too small to be an amplification of the CesA gene and therefore was considered a negative result, as was an empty lane.

Those colonies which harbored the CesA-containing plasmids were grown in liquid culture, and their plasmid DNA was isolated via mini-prep. It was found, through Sanger Sequencing with colony and all 6 CesA sequencing primers primers, that CesA 4, 7, and 8 SDM constructs with corresponding native promoters were successfully incorporated into pORE-O3.

3.2 *Agrobacterium* and *Arabidopsis* Transformation of SDM Constructs

Agrobacterium tumefaciens was transformed with the native promoter SDM CesA pORE-O3 constructs. Colonies found to include the native-promoter CesA SDM pORE-O3 construct were used to transform *Arabidopsis* plants according to the modified Floral Dip method (Clough & Bent, 1998). A summary of which mutant lines were transformed is found in Table 2.

Table 2: *Arabidopsis* Strains Transformed with CesA SDM pORE-O3 Constructs

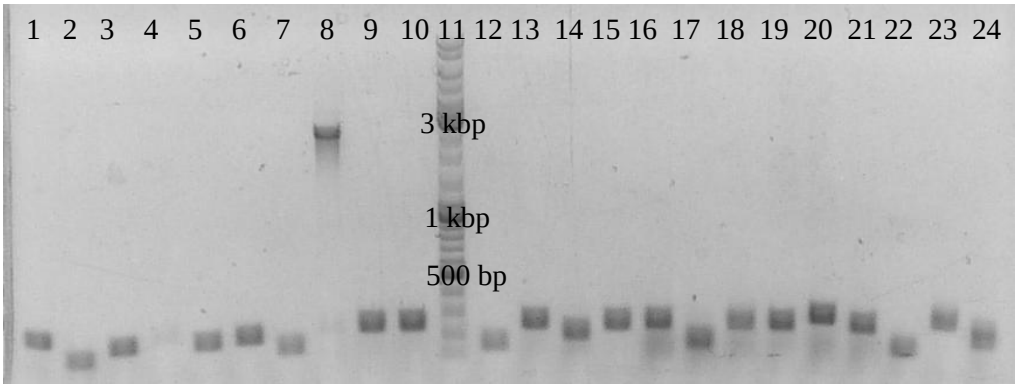
Mutant name	**Ecotype**	**Pro:CesA SDM Constructs Transformed**
Col-0 (WT)	Columbia	4, 7, 8
Ler-0 (WT)	Landsberg erecta	4, 7, 8
irx 1-5	Columbia	8
irx 3-1	Landsberg erecta	7
irx 3-4	Columbia	7
irx 5-4	Columbia	4

At the time of this report, none of the transformed plants are ready to be harvested; therefore none of the seeds have been screened on glufosinate plates.

3.3 Cloning of Promoter, GUS Reporter Constructs

Native CesA promoters 1-9 were amplified from Columbia ecotype *Arabidopsis* using PCR successfully; attempts to amplify the CesA10 promoter were unsuccessful. The PCR products were purified and cut with their corresponding restriction enzymes. The promoters were then ligated into linearized pORE-O3. The ligation reaction was transformed into XL-1 Blue, and colonies were screened using colony PCR with colony primers. A sample agarose gel of the colony PCR is shown in Figure 2.

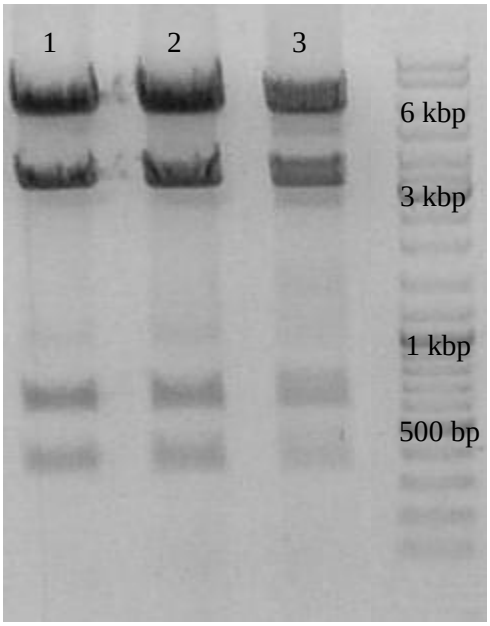
Figure 2 Colony PCR of Pro 4 pORE-O3 Ligation.



A band at approximately 2.5 kb, such as that in lane 8 represents a positive result; a band less than 500, such as in all lanes except 8, signifies a negative result.

Currently, only promoters 4 and 7 have been successfully ligated into pORE-O3. The GUS gene was then PCR amplified and cloned into these two pORE-O3-Promoter constructs. The agarose gel of the colony PCR reactions yielded no bands, but due to the high number of colonies on the ligation plates compared to the vector controls (roughly 10:1, data not shown), colonies were grown up, mini-prepped and analyzed with by diagnostic restriction digestion with BgII. An example BgII digest is found in Figure 3.

Figure 3: Diagnostic BglII Digestion of Suspect Promoter 7 GUS pORE-O3 Construct



pORE-O3 containing both promoter 7 and the GUS gene in the correct orientation would produce bands approximately 6, 3.4, 0.6, and 0.4 kbp in size (See Appendix 3). Lanes 1, 2, and 3 all contain this band pattern and were therefore considered positive for GUS ligation.

Samples with positive diagnostic BglII digestion were sent for Sanger sequencing, and the GUS gene was found to have been ligated in the correct position. GUS was successfully cloned into pORE-O3 constructs containing CesA promoters 4 and 7.

4. DISCUSSION

4.1 Summary

The goal of this research was to produce useful novel *Arabidopsis thaliana* mutants for spectroscopic and visual analysis. The first set of mutants created were secondary cell wall CSC CesA knock-out mutants complimented with catalytically inactive versions of the CesA which can still participate in the formation of the CSC. To this end, site-directed mutants (SDMs) of CesA 4, 7, and 8 were created, changing a critical aspartic acid to asparagine. These mutant genes were then placed downstream of their native promoters in pORE-O3 via an inFusion reaction. These SDM CesA expression constructs were then transformed into *Arabidopsis* knock-out mutants as well as wild-type plants.

A second set of transgenic plants was created to monitor the temporal and spatial expression of CesAs within different mutant plant lines. To accomplish this, the GUS reporter gene was placed downstream of the native CesA promoter in pORE-O3. This was accomplished with CesA4 and 7 promoters. These two constructs are ready for *Arabidopsis* transformation once plants reach maturity.

4.2 Analysis of Site-Directed Mutagenesis and Assembly of SDM Constructs

The site-directed mutagenesis of the cDNA-containing plasmids proved to be a fairly straight-forward process. CesA7 and 8 SDM were successfully created on their first try; CesA4 SDM, after the synthesis of new, longer primers, was also successful.

The more time-consuming section of this portion of the experiment proved to be cloning the CesA SDM gene with its native promoter into a plant expression vector. Originally, the addition

of CesA SDM gene was attempted using traditional cloning in the vector pORE-O2. This was met with limited success, and it was at this time that we realized pORE-O2's kanamycin resistance cassette was incompatible with some of the plant lines we were planning to use. To rectify this problem, we switched to pORE-O3 which instead contains the *pat* gene for glufosinate resistance; consequently, cloning had to be restarted. After several months of failed traditional cloning, we switched to the inFusion recombination system. This proved to be a more successful system.

4.3 Analysis of *Agrobacterium* and *Arabidopsis* Transformation of SDM Constructs

Screening *Agrobacterium* colonies proved to not be as simple as screening *E. coli* colonies during cloning. Colony PCR with *Agrobacterium* colonies was not successfully accomplished; instead, colonies were grown in liquid media and mini-prepped. The concentration of purified plasmid was too low for the DNA to be visualized on an agarose gel. The plasmid was instead used as the template of a diagnostic PCR using promoter primers. This proved to be an effective method of screening colonies; however, it takes several days for the culture to grow to a sufficient density to form a pellet, so this procedure is more time-consuming than colony PCR.

Following transformation, plants recovered relatively quickly without signs of damage due to the transfection. Previous studies warn of the possibility of silwet poisoning, but the plants appeared healthy even four weeks after transformation.

4.4 Analysis of the Cloning of Native Promoter GUS Constructs

Besides suffering the same setback due to change in vector as described in section 4.2, the cloning of the native promoter GUS constructs was met with the problem of a high background during the ligation of promoters into pORE-O3. The pORE-O3 was cut with NotI and SacII for a 10-fold digestion and gel purified, but there would still be colonies containing empty vector after

the transformation into *E. coli*. In an attempt to rectify this, the pORE-O3 was also cut with XhoI prior to ligation. The Xho1 cut site is located between that of NotI and SacII in the multiple cloning site. Despite this additional digestion, the vast majority of colonies screened still contained empty pORE-O3. This problem was not resolved as of the time of this report, but may be solved by using different restriction endonucleases (see 4.5 Future Projects).

4.5 Future Projects

As this project was not successfully completed in its entirety within the time constraint of this report, the first future work would be to complete the originally proposed project. To accomplish this, the promoters (other than 4 and 7) would be cloned into pORE-O3. There were a high number of colonies present on the vector control plate whenever pORE-O3 was cut with Not1 and SacII, suggesting incomplete digestion of the vector leading to re-ligation. To rectify this, alternative enzymes will be used for cloning, such as XhoI. Once the promoters are cloned into pORE-O3, the next step would be to clone in GUS, which in the past has not been difficult to accomplish. These new constructs, along with the promoter 4 and 7 GUS constructs already made would be transformed into *Agrobacterium tumefaciens* and subsequently into *Arabidopsis*. Once the transformants were grown and their progeny screened with glufosinate, the plants which prove to have been transformed can be examined under light microscopy and the blue precipitate caused by the GUS expression would yield data about CesA expression.

In order to finish the CesA SDM project, the plants which have been dipped and are currently growing to maturity will have their seeds screened with glufosinate. Those seeds which germinate would then be grown, and their vascular tissue would be tested for cellulose content using various methods (Harris, Bulone, Ding, & Debolt, 2010). The stems of the new mutants could also be examined using sum-frequency generation spectroscopy which could compare the cellulose

crystallinity of the novel mutants with that of wild type *Arabidopsis* as well as CesA knock-out mutants. In addition, to demonstrate the efficacy of the promoter and CesA pOREO3 constructs in *Agrobacterium*-mediated plant transformation, wild type CesA should be placed downstream of its native promoter using the same method as the SDMs. This wild-type construct should then be able to rescue knock-out mutants of that CesA; if there is complete complementation, the method of gene introduction can be considered effective.

Appendix A: Primers

Restriction endonuclease cut sites and in-Fusion recognition sites are in lower-case text.

CesA1ProSacIIF;	AccgcggAAGTCCACTGAGGAGCCC
CesA1ProNotR;	ACgcggccgcCGCAGCCACCGACAC
CesA2ProSacIIF;	AccgcggGCTAGTAGCTACCGACAG
CesA2ProNotR;	ACgcggccgcGATGTCTTCTACACCGAA
CesA3ProSacIIF;	AccgcggGAAACTTAAGTCTGAAAGC
CesA3ProNotR;	CCgcggccgcTTGTCACTTAGTTGCTTC
CesA4ProSacIIF;	AccgcggACCCATACATGTGATGCTA
CesA4ProNotR;	CAgcggccgcGGCGAGGTACACTGAGCT
CesA5ProSacIIF;	AccgcggCTGCTAGCTTATTACCTGT
CesA5ProNotR;	ACgcggccgcAGTACCGACAAAACAGTAG
CesA5ProSacIIF2;	AccgcggCTGCTAGCTTATTACCTGTCT
CesA5ProNotR2;	ACgcggccgcAGTACCGACAAAACAGTAGAC
CesA6ProSacIIF;	AccgcggATAGCTGTGGTATGATCG
CesA6ProNotR;	CAgcggccgcATTTGTCTGAAAACAGACA
CesA7ProSacIIF;	AccgcggGACGAGGCAAGAGTATGTTG
CesA7ProNotR;	ACgcggccgcAGGGACGGCCGGAGATTA
CesA8ProSacIIF;	AccgcggGAGGTTGCTTTGATCGGA
CesA8ProNotR;	CTgcggccgcCTTCGAATTCCCCTGTTT
CesA9ProSacIIF;	AccgcggCGATGATTGAGTTACGAG
CesA9ProNotR;	CAgcggccgcGATTCACTAGTCCGATCA
CesA10ProSacIIF;	TccgcggTGATGACTTTCTTCAAATCTT
CesA10ProNotR;	CTgcggccgcGCCGGCGTTTTGCTGC
CesA10ProSacIIF2;	TccgcggGAGTATGAATCCAATCATCCC
CesA10ProNotR2;	GTgcggccGCCGGCGTTTTGCTGC

GUS Primers:

pOREcGusNotF;	ACgcggccgcCATGTTACGTCCTGTAG
pOREcGusKpnR;	AAggtaccTCATTGTTT

Site Directed Mutagenesis of CesA4/7/8:

CesA4DtoNF;	GGATCAGTAACAGAAAATATCCTCACTGGATTTAG
CesA4DtoNR;	CTAAATCCAGTGAGGATATTTTCTGTTACTGATCC
CesA7DtoNF;	GCTCTATCACAGAGAATATTTTGACGGGATTCAAG
CesA7DtoNR;	CTTGAATCCCGTCAAAATATTCTCTGTGATAGAGC
CesA8DtoNF;	GTCGATCACCGAAAACATTTTGACGGGTTTC
CesA8DtoNR;	GAAACCCGTCAAAATGTTTTCGGTGATCGAC

CesA4DtoNLongF;
GATTTATGGATCAGTAACAGAAAATATCCTCACTGGATTTAG
CesA4DtoNLongR;
CTAAATCCAGTGAGGATATTTTCTGTTACTGATCCATAAATC

Colony PCR Primers:

ColonyRpORE-OE2;	GCGTAGCGGATAACAATTTC
ColonyFpORE-OR;	TAAACTGAAGGCGGGAAA

Sequencing Primers:

T7;	TAATACGACTCACTATAGGG
T7-term;	GCTAGTTATTGCTCAGCGG
CesASeq1F;	ATMTGYGARATYTGTTYGC
CesASeq1R;	AACAYTTVGGRAACTGATC
CesASeq2F;	GTYATWAGYTGYGGWTAYGA
CesASeq2R;	CRTACCANADAGGACWRTG
CesASeqEndF;	TAYCCDTTCYTBAARGG
CesASeqBegR;	YMTBDTCDCYACADATBTBRCA

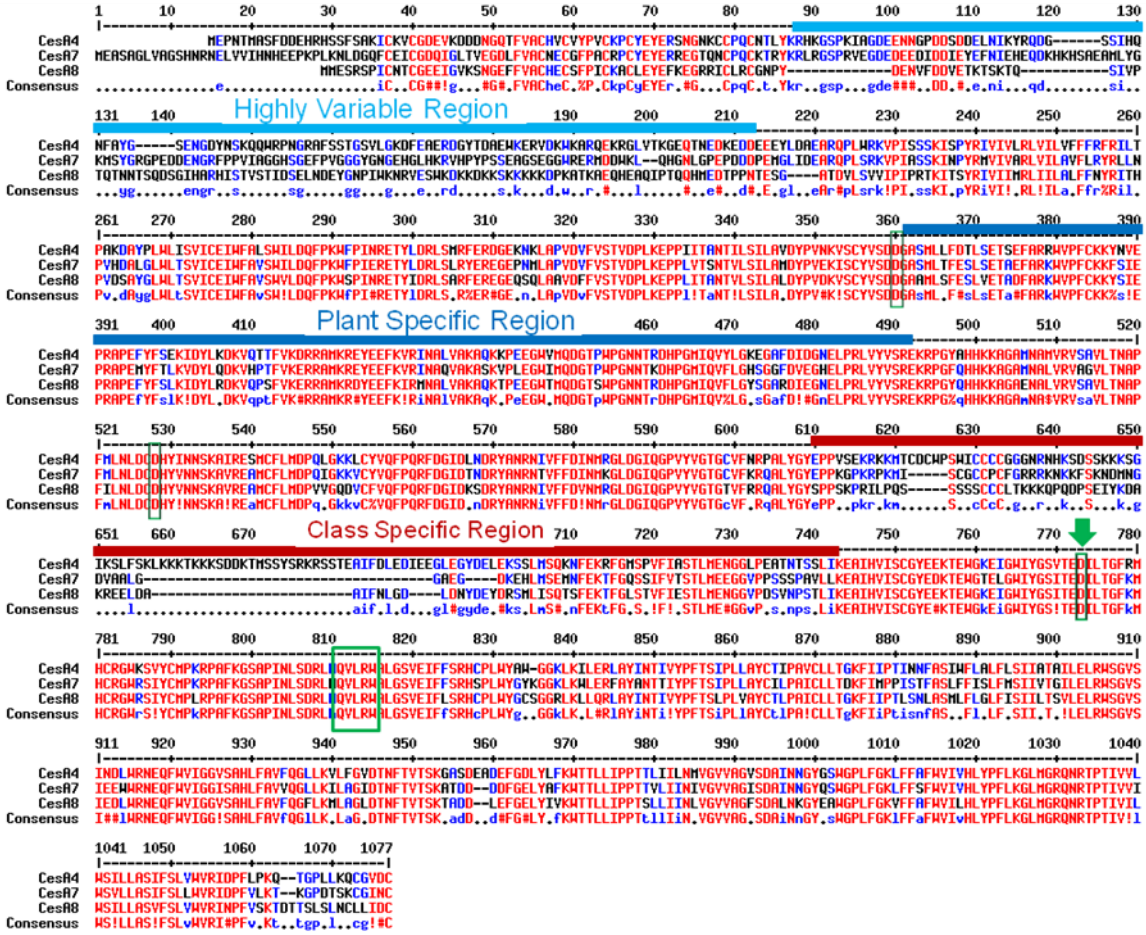
In-Fusion Primers:

CesA4ProInFuF;	gggcccaacgttctcgaACCCATACATGTGATGCTA
CesA4ProInFuR;	ttggttccatGGCGAGGTACACTGAGCT
CesA4OREInFuF;	gtacctcgccATGGAACCAAACACCATG
CesA4OREInFuR;	attctcccgggtggatcTTAACAGTCGACGCCAC
CesA7ProInFuF;	gggcccaacgttctcgaGACGAGGCAAGAGTATGTTG
CesA7ProInFuR;	tagcttccatAGGGACGGCCGGAGATTA
CesA7OREInFuF;	ggccgtccctATGGAAGCTAGCGCCG
CesA7OREInFuR;	attctcccgggtggatcTCAGCAGTTGATGCCACA
CesA8ProInFuF;	gggcccaacgttctcgaGAGGTTGCTTTGATCGGA

CesA8ProInFuR;	actccatcatCTTCGAATTCCCCTGTTT
CesA8OREInFuF;	gaattcgaagATGATGGAGTCTAGGTCTCC
CesA8OREInFuR;	ccttctagaaaaactcgaTTAGCAATCGATCAAAAGAC
CesA8OREInFuR2;	attctcccgggtggatcTTAGCAATCGATCAAAAGAC

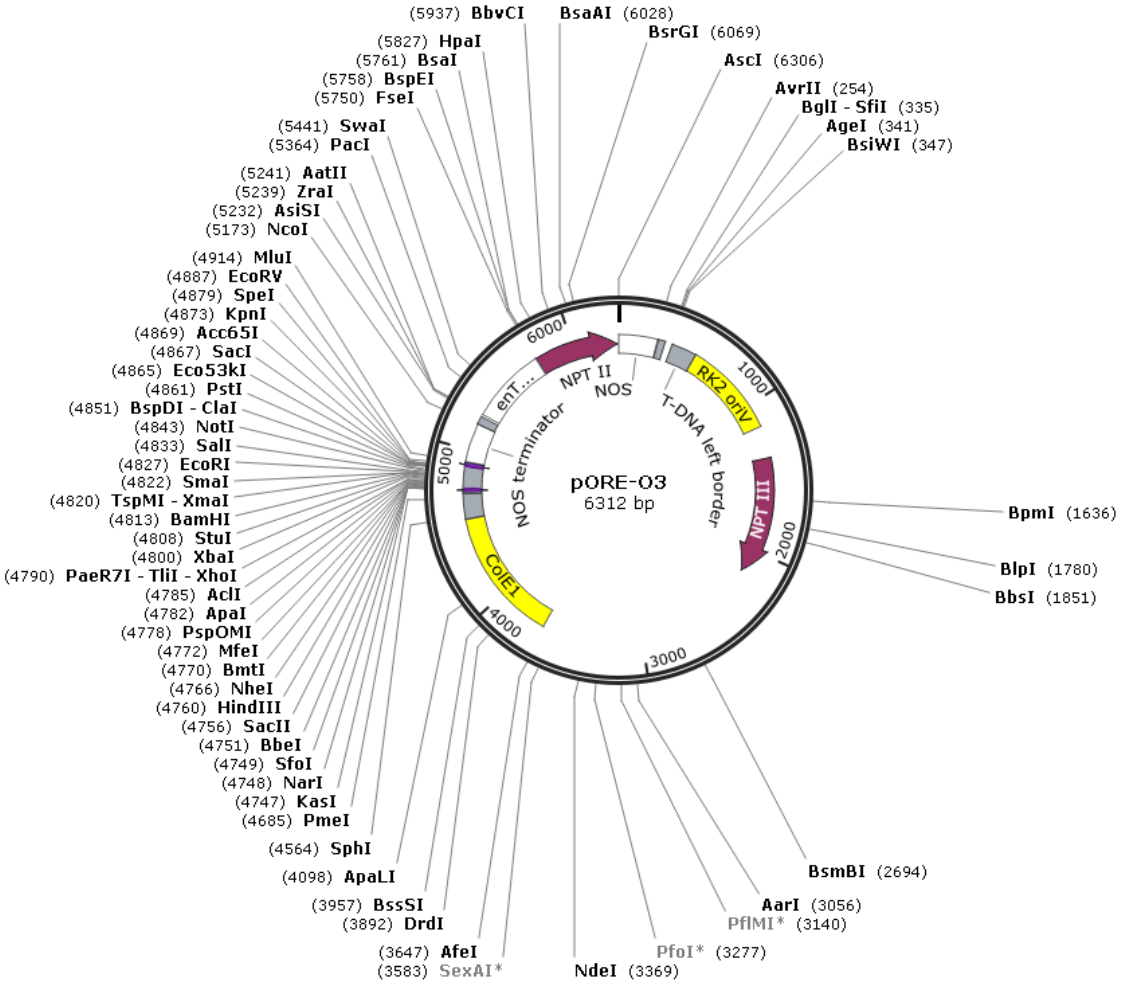
Appendix B: CesA Sequence Mulit-Alignment

Highlighted are the three named regions: the highly variable region, the plant specific region, and the class specific region. Boxed are the three conserved aspartic acid residues and QxxRW motif found all processive glycosyltransferases. The arrow is pointing to the aspartate that is converted to asparagine in the site-directed mutagenesis experiment.

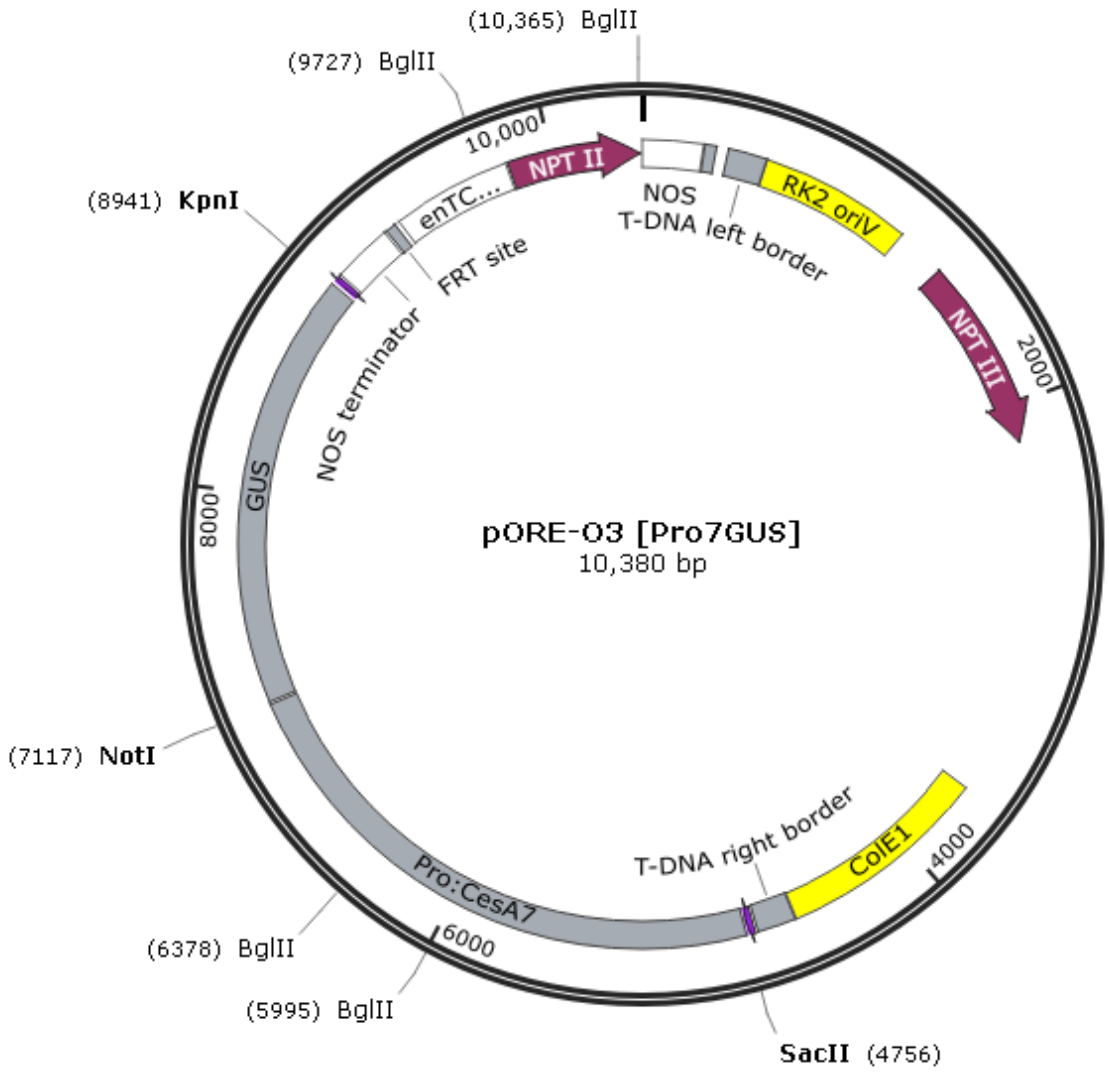


Appendix C: Vector and Construct Maps

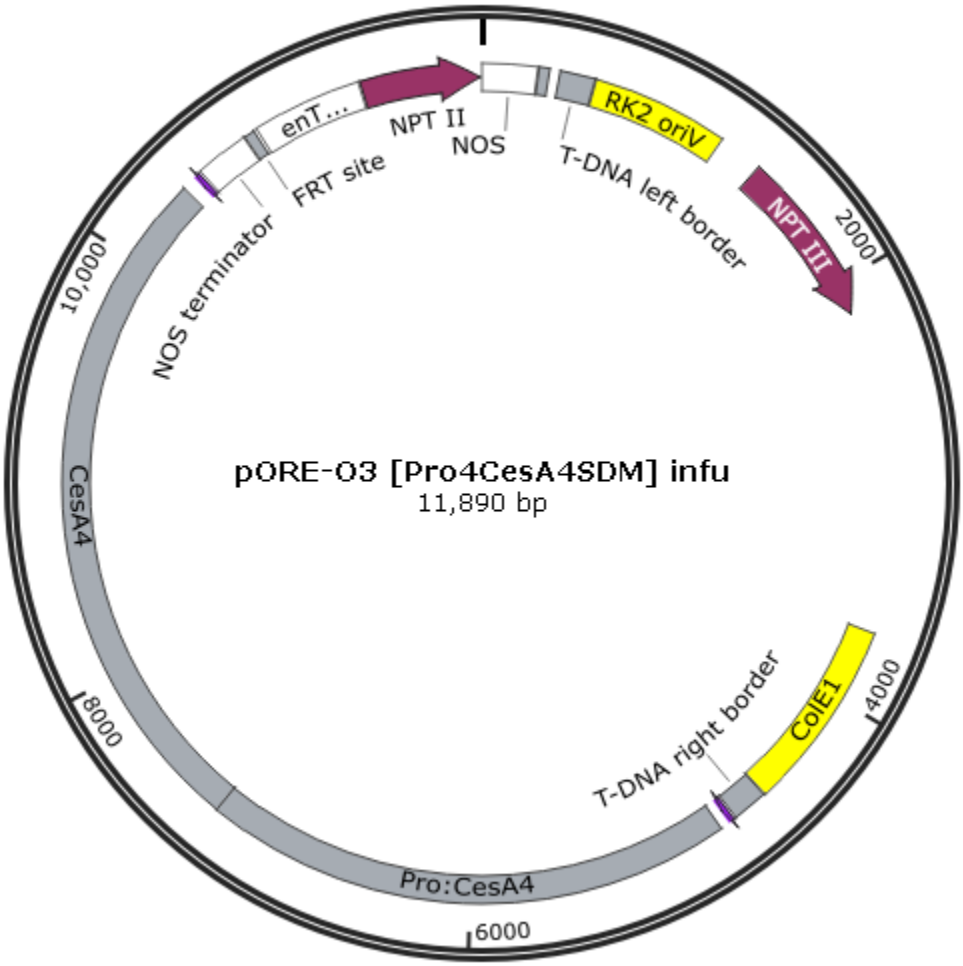
pORE-O3: the primary vector used.



Sample pORE-O3 CesA Promoter (7) GUS construct: BglII sites included to show DNA fragment size for diagnostic BglII digestion of ligation products.



Sample CesA SDM Native Promoter pORE-O3 Construct: CesA4. CesA7 and 8 were incorporated in the same location.



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