Characterization of CcMANS1 from *Coffea canephora*

PLANT CELL WALLS

Plant cell walls are the most abundant source of renewable biomass (Liepman 2012). There are many ways in which we use materials derived from plant cell walls in our daily lives. Plant cell walls are an important part of wood products, paper, textile fibers, animal food as well as human food (Yin et al. 2010). Though abundant, only ~2% of plant cell walls are being used by humans (Pauly and Keegstra 2008). Plant cell walls are a largely untapped resource that researchers believe could be used as a source of biofuel (Pauly and Keegstra 2008). Research into how plant cell walls are synthesized is important in helping biologists understand how unused plant cell walls could be used in practical applications and it could help in the future engineering of plant biomass that is better suited for the needs of particular industries.

POLYSACCHARIDES

Plant cell walls are mainly composed of polysaccharides. There are three types of polysaccharides commonly found in plant cell walls: cellulose, hemicelluloses, and pectins (Pré et al. 2008, Yin et al. 2010, O-Villarreal et al. 2012). Polysaccharides in plant cell walls offer structural support and energy for the developing plant. Cell wall storage polysaccharides are thought to play an important role in seed hardiness and resistance to mechanical damage, seed water buffering, and radicle protrusion (Joet et al. 2013).

MANNANS

Mannans are a type of hemicellulosic plant cell wall polysaccharide. Mannans are polymers consisting of monosaccharides such as D-Mannose, D-Galactose, and D-Glucose (Moreira and Filho 2008). Polysaccharides in the mannan family are structurally similar, but can vary in terms of the composition of backbone chains. Pure mannans are composed of a pure 1,4-

β linked backbone chain of mannosyl residues, while glucomannans have backbone chains composed of a mixture of 1,4-β linked mannosyl and glucosyl residues (Fig. 1; Pre et al. 2008). The composition of mannans differs from plant to plant but functionally, they provide structural support and some plants use mannans as energy reserves (Moreira and Filho 2008, Liepman & Cavalier 2012).

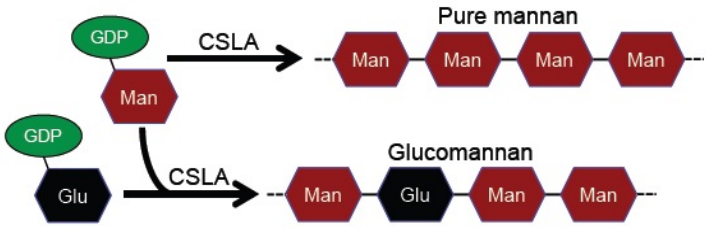

Figure 1. Biosynthesis of Pure Mannans and Glucomannans using nucleotide sugar substrates, GDP-Mannose and GDP-Glucose. Figure from Liepman (2015)

Mannans have various uses outside of their natural functions in the plant cell wall. The application of mannans in our daily life ranges from food preparation and consistency, to pharmaceutical uses. Galactomannans such as guar gum have many uses in food industries as a thickener and food additive because of their rheological properties (Yildiz and Oner 2014). Mannans have been studied as a valuable alternative to petroleum based products because of their biodegradability and their ability to form films (Yildiz and Oner 2014). Mannans are being used in vaccines as a DNA coating. Using mannans as a coating helps to successfully and efficiently deliver the DNA vaccine to antigen presenting cells and has shown that it induces a much stronger immune response in test mice, compared to DNA immunizations without the mannan coating (Yildiz and Oner 2014).

MANNAN SYNTHESIS

While some plants synthesize mannans with a pure mannan backbone, others are also capable of synthesizing glucomannans. Pure mannans are synthesized from the activated nucleotide sugar GDP-mannose and glucomannans from GDP-mannose and GDP-glucose (Joet

et al. 2013, Gille et al. 2011). The enzyme 1,4-β-mannan synthase (ManS) is responsible for the synthesis of pure mannans with a 1,4-β-mannose backbone (Liepman & Cavalier 2012). GMGT (galactomannan galactosyltransferase) is the name of the enzyme that adds galactosyl sidechains to the pure mannose backbones, making galactomannans and to the glucose/mannose backbones making galactoglucomannans (Pré et al. 2008).

The *CELLULOSE SYNTHASE LIKE-A* (CSLA) genes encode proteins responsible for the synthesis of both pure mannan and glucomannan backbones. Numerous studies have shown that members of the CSLA family from different plant species are involved in the synthesis of 1,4-β mannan and glucomannan backbones (Liepman & Cavalier 2012). Even though CSLA enzymes have been shown to synthesize mannan and glucomannan backbones, very little is known about factors governing the enzymatic activity and products formed by the CSLA proteins.

It is hypothesized that structural features of CSLA proteins determine the composition of the mannan backbone. Figure 2 (Liepman 2015) shows a molecular phylogeny of CSLA proteins that are associated with the synthesis of glucomannans and pure mannans. As demonstrated by Figure 2, there appears to be a clear segregation of the CSLA protein sequences that are thought to catalyze the synthesis of pure mannans, such as those from *Trigonella foenum-graecum, and Coffea canephora* (Chatterjee et al. 1981, Pré et al. 2008) and those sequences that appear to catalyze the synthesis of glucomannans, such as those from *Arabidopsis thaliana*, *Pinus taeda* and *Amorphophallus konjac* (Liepman et al. 2007, Liepman & Cavalier 2012). As represented by the phylogeny, it appears that the CSLA protein sequences segregate to form two distinct groups. This clear segregation of CSLA protein sequences suggests that there may be structural differences that determine whether a CSLA protein synthesizes pure mannans and whether it is capable of synthesizing glucomannans as well (Liepman 2012).

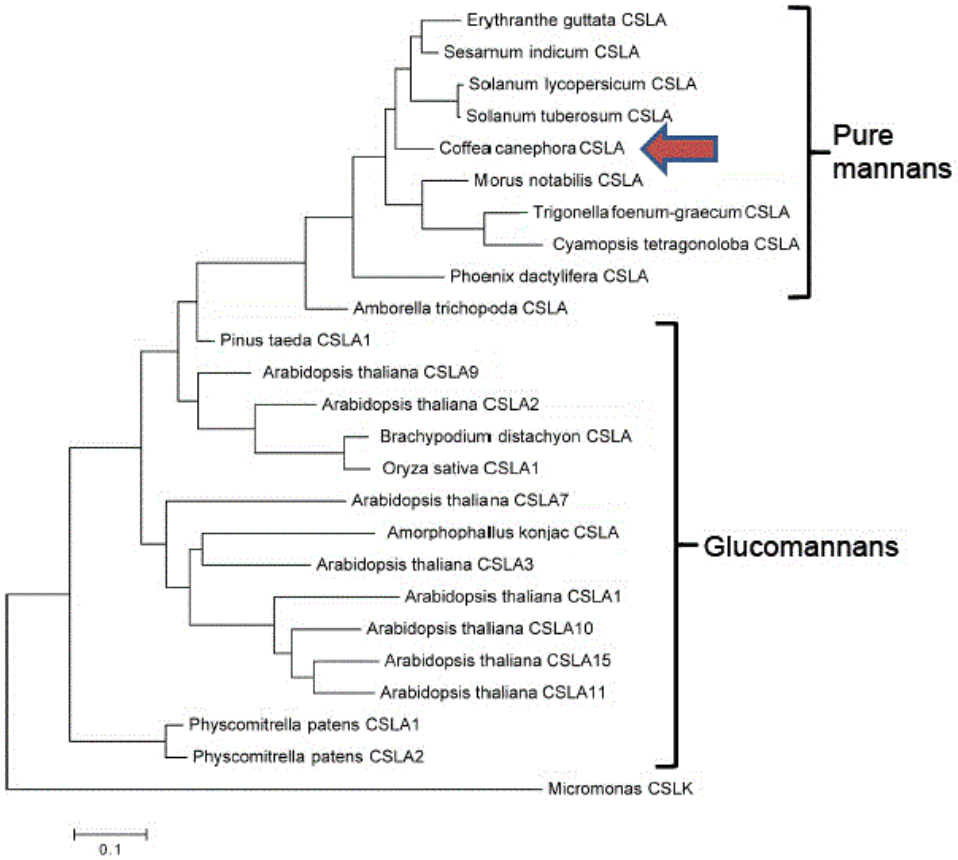

Figure 2. A molecular phylogeny of CSLA protein sequences. The type of mannans thought to be synthesized by members of each clade is indicated. The red arrow indicates the CcMANS1 protein. It is hypothesized that the CSLA proteins in the upper group lost the ability to biosynthesize glucomannans. Figure modified from Liepman (2015).

COFFEE MANNANS

Coffee is one of the most popular drinks among people over the world (Pre et al. 2008). In the coffee seed, mannans are found in abundance in the cell walls of the endosperm and make up over 50% of seed dry mass (Joet et al. 2013). Galactomannans compose up to 25% of the biomass of the mature coffee grain and greatly influence the production of different coffee products (Pré et al. 2008). The solubility of coffee mannans is a key factor that determines the yield of soluble coffee (Redgwell & Fischer 2006, Pré et al. 2008). Being able to identify factors that influence coffee solubility allows producers to select varieties of coffee plants that may produce more easily extractable coffee, increasing the yield and in turn increasing profits (Pré et al. 2008). The coffee industry is one of the largest and most profitable in the world. Being able to

influence the biological process of a plant that is the focus of such a wide spread industry would have a major impact on how coffee is processed around the world.

WHY THIS STUDY?

Though members of the CSLA protein family have been studied and it has been determined that they play a role in biosynthesis of mannan polysaccharides in plants (Liepman & Cavalier 2012), not much is known about the particular factors that govern mannan backbone composition. There is a developing need for increased plant biomass (Pré et al. 2008). This type of study, which aims to identify the factors that influence plant cell wall polysaccharide composition, could help increase future use of plant cell wall polysaccharides. This future use could include the use of plant cell walls as a biofuel, or in the case of *Coffea canephora*, higher yields in the coffee industry which would in turn increase profits.

Understanding the factors that influence the biosynthesis of polysaccharides may help industries manipulate these different structures to better suit their various needs. This study will focus on the biosynthesis of pure mannans with a 1,4-β-mannose backbone and will aim to ultimately answer the question; are coffee mannan synthase enzymes capable of synthesizing glucomannans? This study aims to characterize a CSLA protein from *Coffea canephora* (CcMANS1) that has been implicated in the biosynthesis of pure mannan backbones of galactomannans present in coffee seeds (Pré et al. 2008). Based on the position of the CcMANS1 protein in the phylogeny in figure 2, it is hypothesized that this protein synthesizes mannans with a pure mannose backbone. Mannan synthase and glucomannan synthase activity of the recombinant CcMANS1 protein will be compared with other CSLA proteins known to produce pure mannans (e.g., Fenugreek MANS, TfMANS) and glucomannans (e.g., Arabidopsis CSLA9, AtCSLA9).

METHODS

This study will be conducted in four different phases with each phase consisting of subsections as outlined in figure 3. To study the characteristics of the CcMANS1 protein, we need a source of this protein. I will use recombinant protein expression in two different types of yeast, *Saccharomyces cerevisiae* and *Pichia pastoris*, to produce this protein.

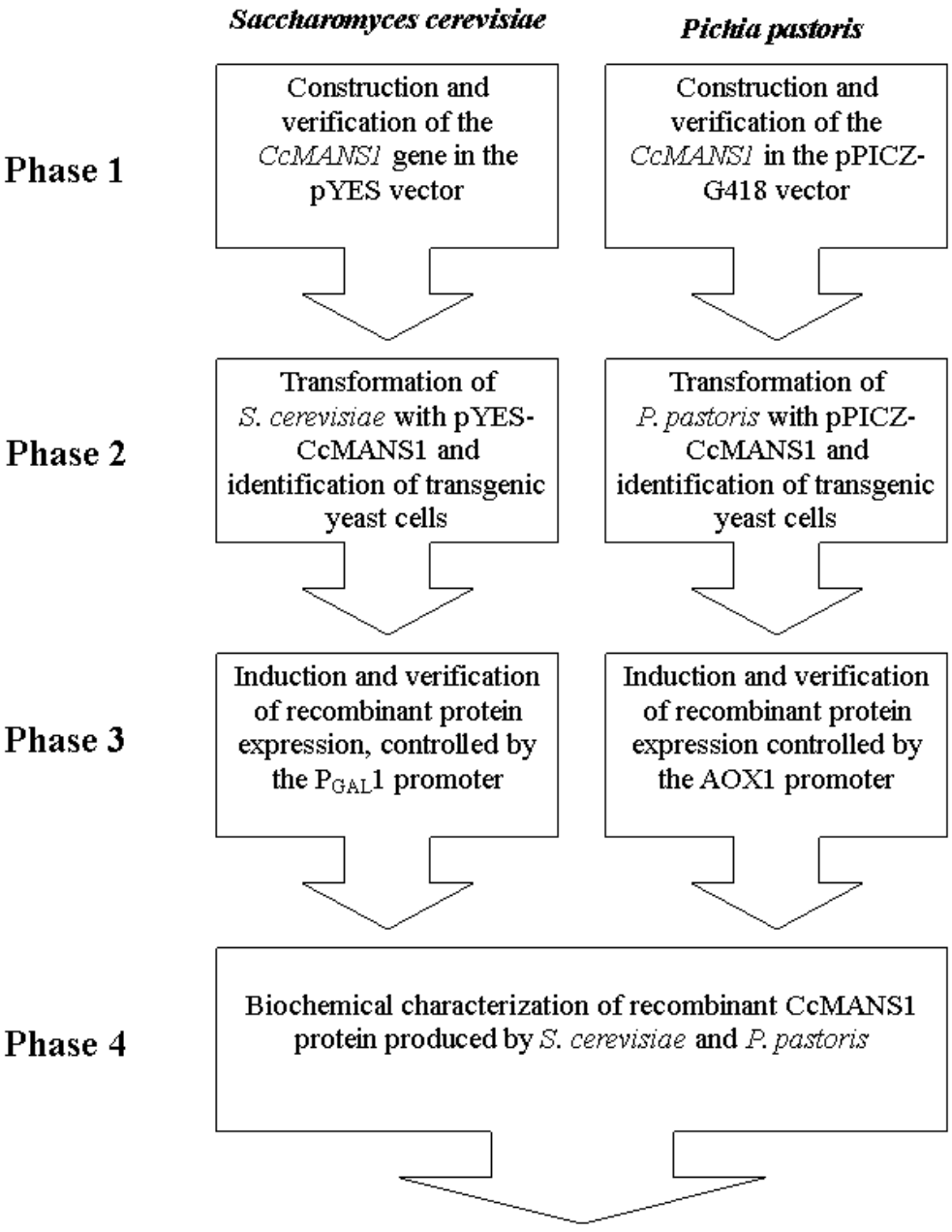

Figure 3. A diagram outlining the different phases of the study.

Phase 1 will consist of construction and verification of recombinant DNA molecules consisting of the *Coffea canephora*

mannan synthase (CcMANS1) coding sequence in two different yeast expression vectors. pPICZ-G418 will be used for CcMANS1 expression in *Pichia pastoris* and pYES will be used for its expression in *Saccharomyces cerevisiae*. Figures 4 and 5 show the maps of vectors that will be used in this study. The two recombinant DNA molecules that will be constructed are pPICZ-CcMANS1 and pYES-CcMANS1. These two molecules will be constructed using the Gibson assembly procedure (New England Biolabs).

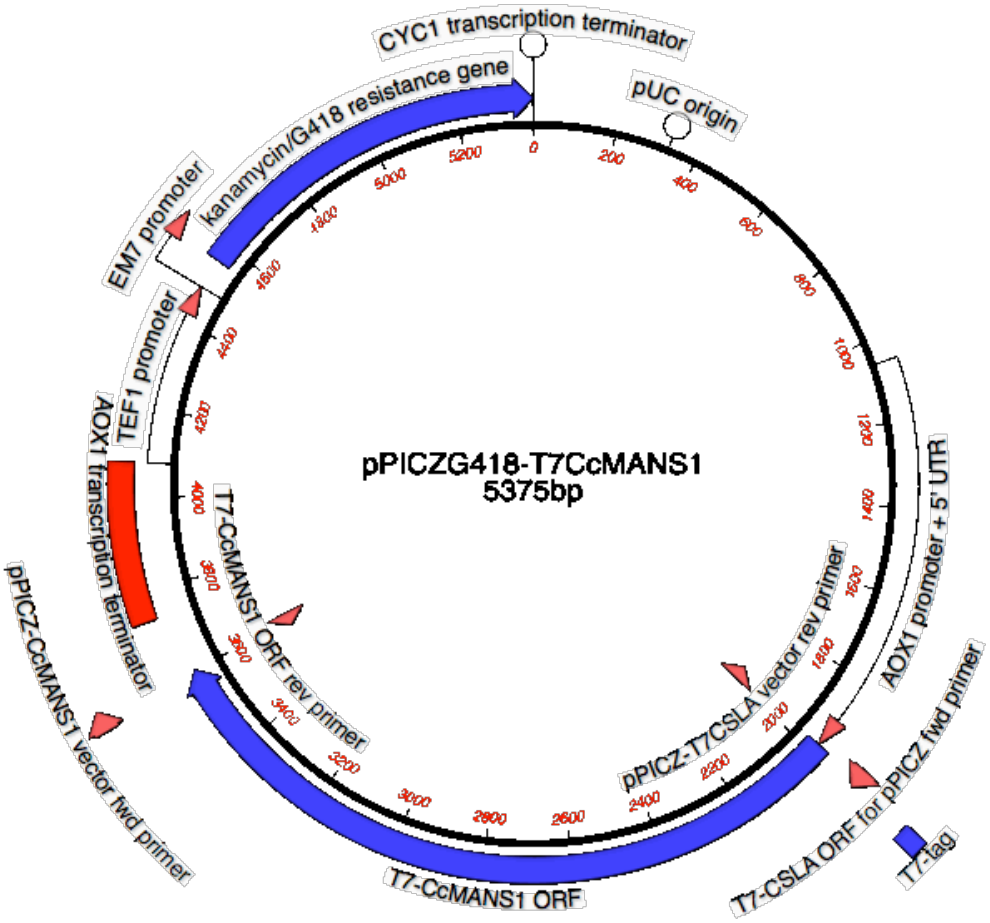

Figure 4. Map of modified pPICZ-G418 vector for recombinant protein production in *Pichia pastoris*.

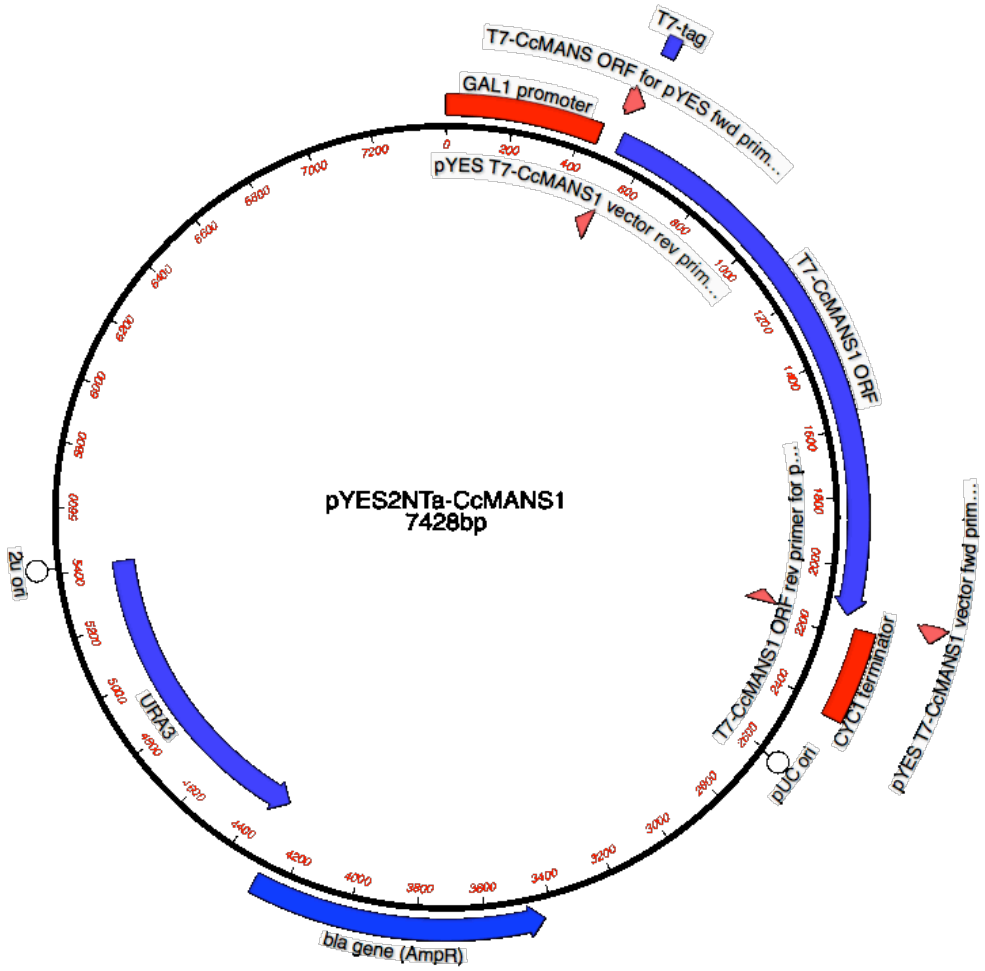

Figure 5. Map of the pYES vector that will be used for recombinant protein production in *Saccharomyces cerevisiae*.

These two particular yeasts are used because of their success with recombinant protein expression (Ahmad et al. 2014). Recombinant protein yield from each yeast will be compared. With the pYES-CcMANS1 vector, *E. coli* that have successfully taken up the desired DNA will also contain the ampicillin resistance marker. We will then select the correct *E. coli* cells by plating them on a media containing ampicillin. The cells containing the ampicillin resistance gene, along with the pYES-CcMANS1 construct will survive, while untransformed *E. coli* cells will not. The ampicillin resistant *E. coli* cells carrying the pYES-CcMANS1 recombinant DNA molecule will be cultured and the plasmid will be extracted and prepared for yeast transformation

in phase two. The sequence of each recombinant DNA molecule will be verified by DNA sequencing.

The pPICZ-G418 vector contains the G418 resistance marker. The G418 resistance marker will enable selection using kanamycin for the *E. coli* that have successfully taken up our DNA vector. Just as with the pYES vector, the *E. coli* cells containing the pPICZ-CcMANS1 construct will be cultured and the plasmids will be extracted and prepared for stage two after the sequence of each recombinant DNA molecule is verified by DNA sequencing.

Phase 2 will consist of the transformation of yeasts with respective yeast expression constructs. We will then identify the transgenic yeast cells containing the desired DNA sequences. Recombinant *Saccharomyces cerevisiae* cells containing the pYES-CcMANS1 vector will be identified using the URA3 marker, which is an auxotrophic marker that complements the mutant INVSc1 host cells (ura-/ura- genotype) that will be used. Following transformation, INVSc1 cells will be plated on media lacking uracil. The cells that have successfully taken up our desired vector should be the only yeast cells that will be able to survive due to complementation of uracil auxotrophy by the URA3 gene of the pYES-CcMANS1 vector. *Pichia pastoris* cells carrying the pPICZ-CcMANS1 vector will be selected on media containing G418. Table 1 shows the specific parameters for each construct that will be used in this study.

Expression Construct	Compatible yeast	E. coli Resistance marker	Yeast identification marker
pPICZ-CcMANS1	*Pichia pastoris*	Kanamycin	G418
pYES-CcMANS1	*Saccharomyces cerevisiae*	Ampicillin	URA3

Table 1. shows the different features in the pPICZ-CcMANS1 and the pYES-CcMANS1 expression constructs that will allow for selection of the specific E. coli and yeast cells containing the respective constructs.

In **Phase 3** of this study, we will induce recombinant protein expression in the transgenic yeast cultures. In *Saccharomyces cerevisiae*, induction of CcMANS1 protein expression will be controlled by the GAL1 promoter. Induction of the protein expression will be repressed while glucose is present; when glucose is absent and galactose is present as a carbon source, transcription will occur (Invitrogen 2012).

In *Pichia pastoris*, induction of CcMANS1 protein expression will be controlled by the AOX1 promotor. Induction is repressed when these cells are grown on in the absence of methanol. Protein expression will be induced when transferred to a medium containing methanol (Invitrogen 2001).

Following induction of CcMANS1 in both types of yeast (*S. cerevisiae* and *P. pastoris*), we will verify expression of the recombinant CcMANS1 protein. This will be done by mechanically breaking open the yeast cells, isolating Golgi-enriched membranes using differential centrifugation, and then using SDS-PAGE and immunoblot to detect the epitope tagged recombinant CcMANS1 protein (Invitrogen 2001, Invitrogen 2012).

Phase 4 of this study will focus on the enzymatic function of the CcMANS1 protein. Recombinant CcMANS1 will be biochemically compared, using in vitro radiometric enzyme assays, to other CSLA proteins identified, and we will attempt to characterize its ability to synthesize pure mannans and glucomannans (Liepman 2005). Table 2 shows the proposed timeline for each phase of this study.

Milestones	Timeline			
	Winter 2016	Summer 2016	Fall 2017	Winter 2017
Phase 1 - Cloning of DNA				
Phase 2 -Transformation of Yeast				
Phase 3 - Recombinant Protein Expression				
Phase 4 - Characterization of recombinant CcMANS1				
Complete Thesis				
Present at UG Symposium				

Table 2. The proposed timeline for each phase of this study.

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