2017

Characterization of CcMANS1, a Putative Mannan Synthase from Coffea canephora

Janell A. Couperthwaite

Follow this and additional works at: http://commons.emich.edu/honors

Recommended Citation
http://commons.emich.edu/honors/565

This Open Access Senior Honors Thesis is brought to you for free and open access by the Honors College at DigitalCommons@EMU. It has been accepted for inclusion in Senior Honors Theses by an authorized administrator of DigitalCommons@EMU. For more information, please contact libir@emich.edu.
Characterization of CcMANS1, a Putative Mannan Synthase from Coffea canephora

Abstract
Mannans are a type of hemicellulosic plant cell wall polysaccharide that offer structural support and serve as energy reserves for developing plants. Coffee seeds are rich in mannans, and mannan content of coffee seeds impacts brew quality. Mannan backbones are synthesized by CSLA proteins. This study aims to characterize a CSLA enzyme from Coffea canephora (mannan synthase 1; CcMANS1) that has been implicated in the biosynthesis of backbones of coffee seed mannans. This enzyme has not been biochemically characterized and characterizing its activity will help contribute to an understanding of the differences between CSLA proteins that dictate the composition of carbohydrate products. Recombinant CcMANS1 was produced using transgenic Pichia pastoris and Saccharomyces cerevisiae. Despite the use of multiple assays, including biochemical assays, fluorophore assisted carbohydrate electrophoresis, and mass spectroscopy, it was not possible to confirm mannan synthase activity of recombinant CcMANS1. Future studies that characterize additional putative mannan synthases or that focus on identifying the cause(s) of inactivity of CcMANS1 may prove beneficial.

Degree Type
Open Access Senior Honors Thesis

Department
Biology

First Advisor
Aaron Liepman, PhD

Second Advisor
Kristin Judd, PhD

This open access senior honors thesis is available at DigitalCommons@EMU: http://commons.emich.edu/honors/565
CHARACTERIZATION OF CCMANS1, A PUTATIVE MANNAN SYNT HASE FROM COFFEA CANE PHORA

By

Janell A. Couperthwaite

A Senior Thesis Submitted to the

Eastern Michigan University

Honors College

in Partial Fulfillment of the Requirements for Graduation

with Honors in Biology

Approved at Ypsilanti, Michigan, on this date July 28, 2017
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table of Contents</td>
<td>i</td>
</tr>
<tr>
<td>List of Tables</td>
<td>v</td>
</tr>
<tr>
<td>List of Figures</td>
<td>vi</td>
</tr>
<tr>
<td>Abstract</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1. Plant Cell Walls</td>
<td>2</td>
</tr>
<tr>
<td>2. Polysaccharides</td>
<td>2</td>
</tr>
<tr>
<td>3. Mannans</td>
<td>3</td>
</tr>
<tr>
<td>4. Mannan Synthesis</td>
<td>4</td>
</tr>
<tr>
<td>5. Coffee Mannans</td>
<td>10</td>
</tr>
<tr>
<td>6. Importance of CSLA Protein Characterization</td>
<td>11</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>12</td>
</tr>
<tr>
<td>1. Construction of Recombinant DNA Molecules</td>
<td>12</td>
</tr>
<tr>
<td>1.1. PCR Amplification of Vector Fragments and Gene Insert</td>
<td>16</td>
</tr>
</tbody>
</table>
1.2. Purification of PCR Products and Gibson Assembly of Fragments..........18

1.3. Bacterial Transformation........................................................................18

1.4. Restriction Enzyme Digestion & DNA Sequencing....................................19

1.5. Large Scale Plasmid Preparation.................................................................20

1.6. Linearization of DNA for Transformation of *Pichia pastoris*..............21

2. *Pichia pastoris* Transformation..................................................................21

2.1. Preparation of *Pichia pastoris* Competent Cells.....................................21

2.2. Transformation of *Pichia pastoris* Using Electroporation......................22

2.3. Growth of Pilot Cultures and Isolation of Crude Genomic DNA for

    Transgene Screening..................................................................................23

2.4. PCR Amplification of Desired Transgene..................................................25

3. *Saccharomyces cerevisiae* Transformation...............................................23

3.1. Preparation of *Saccharomyces cerevisiae* Competent Cells...................23

3.2. Transformation of *Saccharomyces cerevisiae* Competent Cells Via Heat

    Shock...........................................................................................................24

3.3. Growth of Pilot Cultures and Purification of Plasmid for Transgene

    Screening....................................................................................................25

3.4. PCR Amplification of Desired Transgene..................................................25
4. Recombinant Protein Expression and Microsomal Membrane Isolation from

Saccharomyces cerevisiae and Pichia pastoris.................................................26

5. Protein Concentration Assay and SDS-PAGE............................................28

6. Characterization Attempts of CcMANS1 Activity.......................................29

6.1. Biochemical Assay..................................................................................29

6.2. Sequence Verification of T7-CcMANS1 in Transgenic Pichia pastoris.......30

6.3. FACE.................................................................................................31

6.4. Mass Spectroscopy.................................................................................31

Results.............................................................................................................33

1. Restriction Enzyme Digests to Confirm Proper Gibson Assembly Product......33

2. Large Scale Plasmid Prep and Linearization of DNA for Pichia pastoris

Transformation..............................................................................................35

3. Pichia pastoris Transformation Using Electroporation...............................35

4. PCR Amplification of Desired Transgene..................................................36

5. Transformation of Saccharomyces cerevisiae Competent Cells Via Heat Shock.36

6. SDS PAGE/Immunoblot of S. cerevisiae and P. pastoris Samples...............37

7. Biochemical Assay.....................................................................................39

8. Sequencing of Pichia gDNA....................................................................43
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Characteristics of the vectors used</td>
<td>13</td>
</tr>
<tr>
<td>2. Primers used</td>
<td>14</td>
</tr>
<tr>
<td>3. Features of the pPICZ-CcMANS1 and the pYES-CcMANS1 expression constructs</td>
<td>19</td>
</tr>
<tr>
<td>4. Composition of reagents used in this study</td>
<td>33</td>
</tr>
<tr>
<td>5. Restriction enzymes used</td>
<td>34</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>1.</td>
<td>Biosynthesis of Pure Mannans and Glucomannans</td>
</tr>
<tr>
<td>2.</td>
<td>Molecular phylogeny of CSLA protein sequences</td>
</tr>
<tr>
<td>3.</td>
<td>Theories of mannan backbone synthesis</td>
</tr>
<tr>
<td>4.</td>
<td>Modified pPICZ-G418 vector map</td>
</tr>
<tr>
<td>5.</td>
<td>pYES vector map</td>
</tr>
<tr>
<td>6.</td>
<td>Structure of T7-CcMANS1 GBlock</td>
</tr>
<tr>
<td>7.</td>
<td>KpnI and HindIII digests</td>
</tr>
<tr>
<td>8.</td>
<td>SacI linearization digests</td>
</tr>
<tr>
<td>9.</td>
<td>Gel electrophoresis of pPICZ-CcMANS1 transformed <em>Pichia</em></td>
</tr>
<tr>
<td>10.</td>
<td>Gel electrophoresis of pYES-CcMANS1 transformed <em>Saccharomyces</em></td>
</tr>
<tr>
<td>11.</td>
<td>Immunoblot of T7-tagged CcMANS1 in <em>Saccharomyces</em></td>
</tr>
<tr>
<td>12.</td>
<td>Immunoblot of T7-tagged CcMANS1 in <em>Pichia</em></td>
</tr>
<tr>
<td>13.</td>
<td>Mannan synthase assay of <em>Pichia</em> expressed CcMANS1</td>
</tr>
<tr>
<td>14.</td>
<td>Mannan synthase assay of <em>Saccharomyces</em> expressed CcMANS1</td>
</tr>
<tr>
<td>15.</td>
<td>Glucomannan synthase assay of <em>Pichia</em> expressed CcMANS1</td>
</tr>
<tr>
<td>16.</td>
<td>Glucomannan synthase assay of <em>Saccharomyces</em> expressed CcMANS1</td>
</tr>
</tbody>
</table>
17. FACE analysis of Guar galactomannans, Konjac glucomannans and pure mannans from Carob digested with BMACJ...........................44

18. FACE analysis of Guar galactomannans, Konjac glucomannans and pure mannans from Carob digested with BMABS............................................45

19. FACE analysis of carbohydrates from transgenic *Pichia pastoris*..........................46

20. Mass spectroscopy results of X33 and carbohydrate standards...............................47

21. Mass spectroscopy results of all samples.............................................................48
Abstract

Mannans are a type of hemicellulosic plant cell wall polysaccharide that offer structural support and serve as energy reserves for developing plants. Coffee seeds are rich in mannans, and mannan content of coffee seeds impacts brew quality. Mannan backbones are synthesized by CSLA proteins. This study aims to characterize a CSLA enzyme from *Coffea canephora* (mannan synthase 1; CcMANS1) that has been implicated in the biosynthesis of backbones of coffee seed mannans. This enzyme has not been biochemically characterized and characterizing its activity will help contribute to an understanding of the differences between CSLA proteins that dictate the composition of carbohydrate products. Recombinant CcMANS1 was produced using transgenic *Pichia pastoris* and *Saccharomyces cerevisiae*. Despite the use of multiple assays, including biochemical assays, fluorophore assisted carbohydrate electrophoresis, and mass spectroscopy, it was not possible to confirm mannan synthase activity of recombinant CcMANS1. Future studies that characterize additional putative mannan synthases or that focus on identifying the cause(s) of inactivity of CcMANS1 may prove beneficial.
Introduction

Plant Cell Walls

Plant cell walls are the most abundant source of renewable biomass (Liepman & Cavalier 2012). There are many ways in which we use materials derived from plant cell walls in our daily lives. For example, plant cell walls are an important part of wood products, paper, textile fibers, animal food and human food (Yin et al. 2010). Plant cell walls are a largely untapped resource that could be used as a source of biofuels, and though abundant, only ~2% of plant cell walls are used by humans (Pauly and Keegstra 2008). Research into how plant cell walls are synthesized will contribute to an understanding of plant biomass characteristics and may eventually lead to the ability to engineer plant materials better suited for specialized applications.

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). These polysaccharides offer structural support and serve as energy reserves 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 class of hemicellulosic plant cell wall polysaccharides that include 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 the composition of backbone chains. Pure mannans are composed of 1,4-ß linked backbone chains of mannosyl residues, while glucomannans have backbone chains composed of a mixture of 1,4-ß linked mannosyl and glucosyl residues (Fig. 1; Pré et al. 2008). The composition of mannans differs among plants, 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)](image)

Outside of their natural functions in plant cell walls, humans have various uses for mannans. Applications of mannans range from food preparation and consistency to pharmaceutical uses. Galactomannans such as guar gum have many uses in food industries as thickeners and food additives 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 also are used in vaccines as a DNA coating to efficiently deliver the DNA vaccine to antigen presenting cells, inducing 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 such as *Coffea canephora* (robusta coffee) and *Trigonella foenum-graecum* (fenugreek) synthesize mannans with pure mannan backbones, others such as *Arabidopsis thaliana*, *Pinus taeda* (loblolly pine) and *Amorphophallus konjac* (voodoo lily) are also capable of synthesizing glucomannans. Pure mannans are synthesized from the nucleotide sugar GDP-mannose and glucomannans from GDP-mannose and GDP-glucose (Gille et al. 2011, Joet et al. 2013). 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 an enzyme that adds galactosyl sidechains to the pure mannose backbones via 1,6-α linkages, forming galactomannans and to the glucose/mannose backbones forming galactoglucomannans (Pré et al. 2008).

To identify enzymes that play a role in mannan synthesis, studies have used transcriptional profiling to identify genes highly expressed during developmental stages when mannans are deposited (Dhugga et al. 2004, Pré et al. 2008). Putative mannan
synthases were identified in *Coffea canephora* by comparing them to known CSLA proteins (Pré et al. 2008). These possible synthases have high expression levels while mannan deposition in the endosperm is maximal (Pré et al. 2008). Though transcriptional profiling studies provide evidence of CcMANS1 probable function, biochemical proof of mannan synthase activity was not provided. Identifying these enzymes leads to subsequent research avenues where these putative mannan synthases are characterized to confirm activity. These types of identification studies lay the foundation for studies that focus on individualized protein analysis such as in this study.

The *CELLULOSE SYNTHASE LIKE-A (CSLA)* genes encode proteins responsible for the synthesis of both pure mannan and glucomannan backbones (Liepman & Cavalier 2012). Many studies have characterized activity of CSLA proteins, but much is still unknown about what determines the differences in the product composition of these CSLA proteins. Some genes encode proteins that synthesize glucomannans, while other genes are thought to encode proteins that synthesize only pure mannans (Fig. 2). Some CSLA proteins synthesize both glucomannans and pure mannans (Liepman & Cavalier 2012). Mannan and/or glucomannan synthase activity is thought to be a conserved trait among CSLA proteins (Liepman et al. 2007). 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). A CSLA protein from *Arabidopsis thaliana*, AtCSLA7, has also been identified as playing an important role in embryo development and it may play a role in cell signaling (Goubet et al. 2003).
An example of a characterized CSLA protein is TfMANS, an enzyme responsible for mannan synthesis (*Trigonella foenum-graecum*; Wang et al. 2012). This enzyme exhibits high levels of mannan synthase activity and preferentially uses GDP-mannose, even when both GDP-mannose and GDP-glucose are present (Wang et al. 2012). Galactomannans are found in abundance in the developing endosperm of fenugreek and glucose is not found in the backbone of the fenugreek mannans (Wang et al. 2012).

Another identified CSLA protein is AkCSLA3, an enzyme responsible for glucomannan synthesis in *Amorphophallus konjac* (Gille et al. 2011). Corms of *A. konjac* contain ~49-60% glucomannans and these carbohydrates serve as energy stores (Gille et al. 2011). Glucomannan and pure mannan synthase activity of recombinant AkCSLA3, expressed in yeast, was confirmed using radiochemical assays (Gille et al. 2011). Even though these two CSLA enzymes have been shown to synthesize mannan and glucomannan backbones, not much is known about features of these enzymes that determine the differences in the composition of their products.

There have been many studies that use mannan synthases from microsomal membrane fractions isolated from plant tissue, such as from *Trigonella foenum-graecum* and *Cyamopsis tetragonoloba* (guar), to analyze *in vitro* galactomannan synthesis (Edwards et al. 1989). Studies of mannan synthase activity isolated from plant tissue are difficult because the amount of protein that one can isolate from tissue is much less than what can be obtained from recombinant yeast expression. Attempting to analyze enzymes from plant tissue extracts may also be complicated by the many different proteins present,
which may include multiple CSLA proteins that may be responsible for the observed synthase activity. For example, there are nine CSLA genes present in Arabidopsis thaliana (Liepman et al. 2007). Recombinant protein expression in yeast is a powerful approach for studying CSL protein functions but it also has its limits. Other proteins from plant tissue may be needed for proper mannan synthase activity, and with recombinant yeast expression, these would be absent (Wang et al. 2013).

A molecular phylogeny of CSLA proteins shows apparent segregation of the CSLA proteins thought to catalyze the synthesis of pure mannans, such as those from Trigonella foenum-graecum, Coffea canephora, and Cyamopsis tetragonoloba (Chatterjee et al. 1981, Dhugga et al. 2004, Pré et al. 2008, Liepman 2015, Fig. 2) from those proteins 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). This segregation of CSLA protein sequences suggests that there may be structural differences among these proteins that determine whether a CSLA synthesizes pure mannans and whether it is capable of synthesizing glucomannans as well (Liepman 2012). It is hypothesized that structural features of CSLA proteins determine the composition of the mannan backbones they produce, however characterization of additional CSLA proteins is necessary to test this hypothesis. Two hypotheses as to how and why different carbohydrate backbones are synthesized are the enzyme specificity model and the substrate availability model (Liepman, Personal Communication 2016, Fig. 3). The enzyme specificity model proposes that there are two substrates, GDP-
mannose and GDP-glucose, available in the cell but biochemical characteristics of various CSLA enzymes result in some that use only GDP-mannose (thereby synthesizing pure mannans) and others that use GDP-mannose and GDP-glucose (thereby synthesizing glucomannans). The substrate availability model, on the other hand, presents the theory that if both substrates are available in the cell, a CSLA enzyme will use both substrates. According to this hypothesis, pure mannans would be synthesized if only GDP-mannose, and no GDP-glucose was present. It is possible that the carbohydrate products of CSLA proteins may be affected by both the substrate availability model, as well as the enzyme specificity model.
Figure 2 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. Figure modified from Liepman (2015).
Coffee Mannans

In coffee seeds, galactomannans are abundant in the cell walls of the endosperm and make up over 50% of seed dry mass (Joet et al. 2013). These polysaccharides greatly influence the production of different coffee products (Pré et al. 2008) and the solubility of coffee mannans is a key factor that determines the yield of soluble coffee (Redgwell & Fischer 2006, Pré et al. 2008). The degree of galactosylation of coffee galactomannans is thought to play a role in the solubility of the galactomannans, with higher solubility related to a higher degree of galactosylation (Redgwell & Fischer 2006). Increasing the solubility of galactomannans is a focus of commercial coffee research because this could
lead to an increased yield of soluble coffee powder (Redgwell et al. 2003). Coffee is one of the most popular drinks worldwide and the coffee industry accounts for approximately $10 billion annually (Pré et al. 2008, Butt et al. 2011). Identifying factors that influence coffee solubility could allow producers to select or engineer varieties of coffee plants that produce more easily extractable coffee, increasing yield and profits (Pré et al. 2008). Being able to influence the biological process of a plant that is the focus of such a widespread industry could have a major impact on how coffee is processed around the world.

**Importance of CSLA Protein Characterization**

Though members of the CSLA protein family have been studied and it has been determined that they biosynthesize the backbones of mannan polysaccharides in plants (Liepman & Cavalier 2012), not much is known about the particular factors that govern mannan backbone composition. Research identifying the factors that influence plant cell wall polysaccharide composition could help increase future use of plant cell wall polysaccharides. Understanding the factors that influence the biosynthesis of polysaccharides may enable manipulation of carbohydrate composition in plants to improve the suitability of plant materials for various downstream uses. Potential future uses include the use of plant cell walls for biofuel production, or in the case of Coffea canephora, higher yields or improved coffee varieties in the coffee industry.
This study aimed to characterize a CSLA protein from *Coffea canephora* (CcMANS1) that has been implicated in the biosynthesis of pure mannann backbone of galactomannans present in coffee seeds (Pré et al. 2008). Based on the position of the CcMANS1 sequence in the phylogeny, as well as the composition of mannans in the developing coffee seed, it was hypothesized that this protein synthesizes mannans with a pure mannose backbone (Fig. 2). It is not known whether the production of pure mannans by CcMANS1 is a result of the enzyme specificity and/or substrate availability model. To evaluate mannan synthase and glucomannan synthase activity of the CcMANS1 protein, recombinant CcMANS1 was compared with other CSLA proteins known to produce pure mannans (e.g., Fenugreek MANS, TfMANS) and glucomannans (e.g., Arabidopsis CSLA9, AtCSLA9).

**Materials and Methods**

To study the characteristics of the CcMANS1 protein, a source of this protein was needed. Recombinant protein expression in two different types of yeast, *Saccharomyces cerevisiae* and *Pichia pastoris*, was used to produce this protein.

**Construction of Recombinant DNA Molecules**

Recombinant CcMANS1 protein was expressed in two different species of yeast, *Saccharomyces cerevisiae* and *Pichia pastoris*. This study started with the construction and verification of recombinant DNA molecules consisting of the *Coffea canephora*
mannan synthase (CcMANS1) coding sequence (Genbank accession number EU568115) in two different yeast expression vectors. pPICZ-G418 was used for CcMANS1 expression in *Pichia pastoris* and pYES2 was used for its expression in *Saccharomyces cerevisiae* (Fig. 4, Fig 5). Key characteristics of these vectors are outlined in Table 1.

**Table 1** Characteristics of the vectors used in this study, allowing for optimized recombinant protein expression.

<table>
<thead>
<tr>
<th>pYES-AtCSLA9 (S. cerevisiae)</th>
<th>pPICZ-G418 (P. pastoris)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Characteristic</strong></td>
<td><strong>Function</strong></td>
</tr>
<tr>
<td>GAL1 promoter</td>
<td>Initiates expression of CcMANS1 in the presence of Dextrose or Raffinose as a carbon source</td>
</tr>
<tr>
<td>T7-Tag</td>
<td>Epitope tag for detection of recombinant protein</td>
</tr>
<tr>
<td>URA3</td>
<td>Auxotrophic marker that allows for selection of <em>Saccharomyces</em> carrying the plasmid</td>
</tr>
<tr>
<td>AmpR gene</td>
<td>Allows for selection of <em>E. coli</em> carrying the plasmid using the antibiotic ampicillin.</td>
</tr>
</tbody>
</table>
## Table 2 Primers used in this study

<table>
<thead>
<tr>
<th>PRIMER NAME</th>
<th>5'–3' Sequence</th>
<th>Tm</th>
</tr>
</thead>
<tbody>
<tr>
<td>CcMANS1 Seq P1</td>
<td>GTTGGATCGAAAAGGGCGTCAATG</td>
<td>58.8 °C</td>
</tr>
<tr>
<td>CcMANS1 Seq P2</td>
<td>CCCTCCACTTTCAAGGCTTACAG</td>
<td>59.5 °C</td>
</tr>
<tr>
<td>CcMANS1 Seq P3</td>
<td>GTGGCAGGTATCATACTATGACTAG</td>
<td>57 °C</td>
</tr>
<tr>
<td>CcMANS1 Seq P4</td>
<td>CCTCCATAGGAAATCTTCTITCTGG</td>
<td>56.3 °C</td>
</tr>
<tr>
<td>GAL1 fwd</td>
<td>GTAATAATACCTCTATAATTTAAGGTACAGGC</td>
<td>54.9 °C</td>
</tr>
<tr>
<td>pYES T7-CcMANS1 vector fwd</td>
<td>CGGTACGATCTGAACAGTATGAGTTAAACCGATCGATCC</td>
<td>63 °C</td>
</tr>
<tr>
<td>pYES T7-CSLA vector rev</td>
<td>CCACCTGTCATCGAAGCCTTTTGTAGCTTATATTCTCTATAGTGG</td>
<td>61 °C</td>
</tr>
<tr>
<td>CYC1 rev</td>
<td>GCCTGAATGTAAGGCTGAC</td>
<td>54.3 °C</td>
</tr>
<tr>
<td>T7-CcMANS1 ORF for pYES fwd</td>
<td>CTCAATAGGAATTTAAGCTTACATACAAATGCTTCCCATGACAGGTTG</td>
<td>75 °C</td>
</tr>
<tr>
<td>T7-CcMANS1 ORF rev primer for pYES</td>
<td>GGATCAGCGGTTTAAACTCATTACGTTACATCGTACCG</td>
<td>77 °C</td>
</tr>
<tr>
<td>5’ AOX1 primer</td>
<td>GACTGGTTCCAATTGACAAGC</td>
<td>54.3 °C</td>
</tr>
<tr>
<td>3’ AOX1 primer</td>
<td>CGTTAACGTTAGACTGAGG</td>
<td>54.8 °C</td>
</tr>
<tr>
<td>T7-CSLA ORF for pPICZ fwd</td>
<td>CGACAAATCTGAGAAGTCAAAAAATGGCTTCCATGACAGGTTG</td>
<td>75 °C</td>
</tr>
<tr>
<td>T7-CcMANS1 ORF rev primer for pPICZ</td>
<td>GCTGGGACACTGTTTTACGCTTGACATCGGACC</td>
<td>78 °C</td>
</tr>
<tr>
<td>pPICZ-CcMANS1 vector fwd</td>
<td>GGATCAGCGGTTTAAACTCATTACGTTACATCGTACCG</td>
<td>64 °C</td>
</tr>
<tr>
<td>pPICZ-T7-CSLA vector rev</td>
<td>CACCTGTACATGGAAGGCTTTTCTCAATGTTGTCG</td>
<td>59 °C</td>
</tr>
<tr>
<td>Janell CcMANS1 P2</td>
<td>TTACGGTTTGGTGATCGATCTACGATCGTATATCC</td>
<td>58.8 °C</td>
</tr>
<tr>
<td>Janell CcMANS1 P1</td>
<td>ATGGCTTCCAATGACAGGGGAC</td>
<td>59.5 °C</td>
</tr>
</tbody>
</table>
Figure 4 Map of modified pPICZ-G418 vector used for recombinant protein production in *Pichia pastoris*.
Figure 5 Map of the pYES vector that was used for recombinant protein production in *Saccharomyces cerevisiae*.

**PCR Amplification of Vector Fragments and Gene Insert**

The primers pYES T7-CcMANS1 vector forward and pYES T7-CSLA vector reverse were used to amplify the pYES2 vector, and the primers pPICZ-CcMANS1 vector forward and pPICZ-T7CSLA vector reverse were used to amplify the pPICZ-G418 vector. A PCR reaction of the two vectors was conducted using the following concentrations of reagents; 1X NEB Q5 master mix, 500 nM of each primer, and 1 ng of
vector template in a total reaction volume of 25 µl. The pPICZ PCR was carried out using the following thermocycling conditions:

1. 98°C for 30 seconds,
2. 98°C for 10 seconds
3. 62°C for 30 seconds
4. 72°C for 2 minutes
5. repeat steps 2-4 29 more times
6. 72°C for 2 minutes.

The pYES2 PCR was carried out using the same thermocycler conditions as the pPICZ PCR reaction with step 3 changed to 64°C for 30 seconds. Products of PCR reactions were verified using a 0.8% agarose gel in 1X TAE with 1X gel red. Each sample was prepared using 1X DNA loading dye and was run at 110 V for approximately one hour. A synthetic DNA sequence (GBlock, Fig. 6), containing the T7-CcMANS1 coding sequence, codon optimized for recombinant protein expression in yeast, was obtained from Integrated DNA Technologies (IDT). The GBlock is 1631 base pairs long and includes a yeast Kozak consensus sequence followed by the T7 epitope-tag encoding sequence followed by the CcMANS1 open reading frame. Codon optimization was conducted using the IDT Codon Optimization Tool (www.idtdna.com/CodonOpt). The GBlock was not amplified prior to Gibson Assembly.
Figure 6 Structure of T7-CcMANS1 GBlock. 1631 base pairs long, including a yeast Kozak consensus sequence, the T7 epitope-tag, and the CcMANS1 open reading frame.

Purification of PCR Products and Gibson Assembly of Fragments

PCR products were purified using the Promega Wizard SV Gel and PCR Clean-Up System. Purified PCR products were quantitated using a Nanodrop UV/vis spectrophotometer. The T7-CcMANS1 GBlock insert was assembled into each vector, using Gibson Assembly. For the Gibson Assembly, 1X NEBuilder master mix, 0.02 pmol of the pPICZ or pYES vector PCR products, and 0.04 pmol (vector to insert ratio of 1:2) of the T7-CcMANS1 gene insert were incubated in a final volume of 20 µl at 50°C for 60 minutes according to manufacturer’s recommended conditions (New England Biolabs). These assembled products were stored at -20°C until needed.

Bacterial Transformation

Competent E. coli cells were transformed by adding 4 µl of the chilled Gibson Assembly product to chilled competent cells. This mixture was gently mixed and incubated on ice for 30 minutes, heat shocked at 42°C for 30 seconds, and incubated on
ice for 2 minutes. 950 µl of Super Optimal broth with Catabolite repression (SOC) was added to the tube and the mixture was incubated at 37°C for 60 minutes while shaking at 250 RPM. Cells were plated on Luria-Bertani (LB) plates with the corresponding antibiotics (Table 3), at a final concentration of 50 µg/ml. These plates were incubated for approximately 24 hours at 37°C.

**Table 3** Features of the pPICZ-CcMANS1 and the pYES-CcMANS1 expression constructs that allowed for selection of the specific *E. coli* and yeast cells containing the respective constructs.

<table>
<thead>
<tr>
<th>Expression Construct</th>
<th>Compatible yeast</th>
<th><em>E. coli</em> Resistance marker</th>
<th>Yeast identification marker</th>
</tr>
</thead>
<tbody>
<tr>
<td>pPICZ-CcMANS1</td>
<td><em>Pichia pastoris</em></td>
<td>Kanamycin</td>
<td>G418</td>
</tr>
<tr>
<td>pYES-CcMANS1</td>
<td><em>Saccharomyces cerevisiae</em></td>
<td>Ampicillin</td>
<td>URA3</td>
</tr>
</tbody>
</table>

Liquid cultures of individual colonies were started in 4 ml of LB + respective antibiotic [50 µg/ml]. Liquid cultures were shaken at 37°C for approximately 48 hours. Plasmid DNA from these cultures was purified using the Promega Wizard SV Miniprep system and quantified using a Nanodrop spectrophotometer.

**Restriction Enzyme Digestion & DNA Sequencing**

To verify successful plasmid construction, restriction digests were carried out. Each digest was carried out using the following final concentrations: corresponding optimized buffer stocks 1X, restriction enzyme 3 units/rxn, and DNA 150 ng/rxn, final volume of 20 µl, per reaction. Each digest was incubated at 37°C for 1 hour. The
products of the restriction digests were analyzed by gel electrophoresis. The digest products in 1X loading dye were run on a 0.8% gel with 1X TBE and 1X gel red.

Samples that yielded the expected restriction digest band sizes were sent for DNA sequencing analysis at the University of Michigan DNA Sequencing Core. The samples were prepared according to the guidelines on the University of Michigan DNA Sequencing Core Homepage (https://seqcore.brcf.med.umich.edu/). Sequencing primers used for CcMANS1 were Seq P1, Seq P2, Seq P3, and Seq P4 (Table 2). Resulting sequences were aligned to a reference CcMANS1 sequence using MacVector and error-free constructs were carried forward.

Large Scale Plasmid Preparation

Large scale cultures, of one pPICZ-CcMANS1 line and one pYES-CcMANS1 line containing the plasmid with the correct sequence were started in 250 ml LB with appropriate antibiotic at a final concentration of 50 µg/ml. Glycerol stocks (15% v/v glycerol) of these samples were prepared and stored in a -80°C for future use. The plasmid DNA was purified from the 250 ml cultures using the Qiagen Maxi-prep system and quantified using a Nanodrop spectrophotometer. These samples were sequenced at the University of Michigan DNA Sequencing Core and verified to be error-free.
Linearization of DNA for Transformation of Pichia pastoris

Purified plasmid was obtained from the large-scale cultures and linearized using SacI HF so it could be transformed into the *Pichia pastoris*. The digest was carried out using the following final concentrations: 1X cutsmart buffer, SacI HF 3 units/rxn, and DNA 10 µg/rxn, in a final volume of 25 µl. The digest was incubated at 37°C for 1 hour. 500 ng of the products of the digest were analyzed by gel electrophoresis. The products were combined with 1X loading dye, which was run on a 0.8% gel with 1X TBE to confirm correctly linearized plasmid DNA. After initial confirmation, a second digest was performed using the same parameters. This product was purified using Wizard SV Gel and PCR Clean-up System and used for transformation.

Pichia pastoris Transformation

Preparation of Pichia pastoris Competent Cells

*Pichia pastoris* transformation and expression was conducted as outlined in the EasySelect *Pichia* Expression Kit (Version G, 122701 25-0172) by Invitrogen. YPD + ampicillin (50 µg/ml) plates were streaked for isolation with *Pichia pastoris* X33 cells from a glycerol stock. These plated cells were incubated for approximately 72 hours at 30°C. 10 ml cultures of YPD + ampicillin were inoculated with single colonies of X33 and grown approximately 24 hours with shaking at 250 rpm at 30°C. Two 1 L flasks of 250 ml YPD containing ampicillin were inoculated with 150 µl and 75 µl of cells from the X33 liquid cultures. Cultures were grown, shaking at 250 RPM at 28°C for
approximately 18 hours. The cultures were combined and centrifuged in 250 ml plastic bottles at 2,500 x g for 10 minutes at 4°C. Cell pellets were washed by resuspension in 150 ml each of ice cold sterile water and spun at 2,500 x g for 10 minutes at 4°C. The subsequent pellets were resuspended in 62.5 ml of ice cold sterile water each and spun at 2,500 x g for 10 minutes at 4°C. The following pellets were resuspended in 10 ml each of ice cold sterile water and combined and spun at 1,500 x g at 4°C for 5 minutes. The resulting pellet of washed *P. pastoris* cells was resuspended in 0.75 ml of sterile 1 M sorbitol and these competent cells were kept on ice.

*Transformation of Pichia pastoris Using Electroporation*

The 10 µg linearized pPICZ-CcMANS1 were dried using a SpeedVac to approximately 10 µl. 80 µl of the freshly prepared *P. pastoris* competent cells was added to the tube containing linearized DNA tube and the mixture was transferred to a pre-chilled BioRad cuvette, with a 0.4 cm electrode (Product # 1652088), and incubated on ice for 5 minutes. The samples were electroporated using the BioRad Gene Pulser, with the following settings: 1.5 volts, 400 OHMS, and 25 µFD. Following electroporation, 1 ml of ice cold sterile 1 M sorbitol was mixed with the yeast sample in the cuvette, the mixture was transferred to a 15 ml conical tube and incubated for 2 hours at 30°C with gentle agitation by flicking every 20 minutes. 200 µl of each sample was plated on YPDS +Amp +G418 solid media. Remaining cells in the sample were centrifuged at 2,000 x g
for 2 minutes and the concentrated sample was spread on a plate. These plates were incubated for approximately 3 days at 30°C for colony growth.

**Growth of Pilot Cultures and Isolation of Crude Genomic DNA for Transgene Screening**

Tubes containing liquid YPD with G418 (500 µg/ml) and ampicillin (25 µg/ml) were inoculated with single transformed colonies. These cultures were grown approximately 24 hours at 30°C shaking at 300 RPM. A small sample of each culture was used for preparation of glycerol stocks. 80% glycerol thoroughly mixed into pilot cultures to achieve a final concentration of 15%, these were then stored at -80°C. DNA from the remaining yeast cultures was purified using a boil/freeze procedure. 250 µl of each sample was spun at 14,000 x g for 2 minutes and the pellet was resuspended in 250 µl of water. These samples were boiled at 100°C for 10 minutes. The samples were frozen at -80°C for approximately 24 hours. Samples were thawed and spun at 14,000 x g for 10 minutes and a sample of the supernatant was collected.

**Saccharomyces cerevisiae Transformation**

**Preparation of Saccharomyces cerevisiae Competent Cells**

The protocol for the transformation of *Saccharomyces cerevisiae* was based upon a procedure outlined in Methods in Enzymology Volume 194 (Guthrie and Fink 1991).
Two 3 ml cultures of *Saccharomyces cerevisiae* InvSc1 strain, auxotrophic for Ura, were incubated and shaken at ~300 RPM at 30°C for approximately 24 hours. 50 ml of YPD was inoculated with 1 ml of the overnight culture. When the OD₆₀₀ of each culture was between 0.2 and 0.25 the cultures were grown for an additional 3 hours, shaking at 30°C. Each culture was transferred to a 50 ml conical tube and centrifuged at 5,000 x g for 5 minutes at room temperature. The supernatant was discarded and the pellet was resuspended in 1 ml of sterile distilled water. This sample was centrifuged at approximately 14,000 x g in a tabletop microcentrifuge for 30 seconds. The supernatant was discarded and the pellet as resuspended in 250 µl of sterile 1X TE/1X LiAc.

*Transformation of Saccharomyces cerevisiae Competent Cells Via Heat Shock*

2500 ng of the pYES-CcMANS1 plasmid was mixed with 10 µl of 10 µg/µl boiled salmon sperm carrier DNA. 100 µl of the yeast competent cells in 1X TE/1X LiAc was added and briefly vortexed. 600 µl of PEG/1X LiAc was added to each sample and vortexed to mix. Transformations were shaken at 200 RPM at 30°C for 30 minutes. After incubation, 70 µl of DMSO was added and gently inverted to mix. The cells were heat shocked for 15 minutes at 42°C and cooled on ice for 1-2 minutes. Transformation reactions were centrifuged for 5 minutes at 14,000 x g in a tabletop microcentrifuge at room temperature. The supernatant was discarded and the pellet was resuspended in 150 µl of sterile 1X TE and plated on YPD +G418 -Ura plates at varying concentrations of
antibiotics: undiluted, 1:10, and 1:100. These plates were incubated at 30°C for approximately 3 days, until colonies were observed.

**Growth of Pilot Cultures and Purification of Plasmid for Transgene Screening**

Colonies of transformed *S. cerevisiae* were picked and grown in 3 ml of SD -ura as a selection marker. A small sample of these cultures was stored as glycerol stocks in a -80°C freezer for future use and the remaining sample was purified using a boil/freeze prep as described for the *Pichia pastoris* samples.

**PCR Amplification of Desired Transgene**

To confirm transgene presence in yeast lines, a PCR-based verification was used. For *Pichia pastoris*, a PCR of the boil/freeze prep DNA was conducted using CcMANS1 P1 and CcMANS1 P3 primers. 1 µl of DNA was used as template in a 20 µl reaction containing 1X Taq master mix, 0.2 µm each of forward and reverse primers. The PCR was carried out using the following parameters:

1. 94°C for 30 seconds
2. 94°C for 30 seconds
3. 56°C for 30 seconds
4. 68°C for 60 seconds
5. repeat steps 2-4 29 times
6. 68°C for 5 minutes.
The *Saccharomyces cerevisiae* PCR of the boil/freeze prep DNA was carried out using GAL1 and CcMANS1 Seq P4 as the forward and reverse primers, using the same reagent concentrations and thermocycler guidelines as the PCR for the amplification of the desired transgene in the *Pichia pastoris* samples, only differing in step 3, which was carried out at 55°C for 30 seconds. PCR products from both reactions were analyzed on a 1.5% (w/v) agarose gel in 1X TBE with 1X gel red to confirm correct product size.

**Recombinant Protein Expression and Microsomal Membrane Isolation from *Saccharomyces cerevisiae* and *Pichia pastoris***

*Saccharomyces cerevisiae* samples that screened positive for the transgene using PCR were grown in 15 ml cultures of SR -Ura, shaking at 30°C for approximately 18 hours. Cultures were grown for ~24 hours. The liquid cultures were used to prepare microsomes. Cells from liquid cultures were collected by centrifugation at 5,000 x g for 5 minutes and the resulting pellet was washed with 30 ml of sterile water. This was spun again at 5,000 x g for 5 minutes and the resulting pellet was resuspended in 4 ml of extraction buffer and added to a 30 ml glass tube containing approximately 3 g of sterile beads (425-600 µm). To lyse yeast cells, tubes were vortexed vigorously for eight 30-second bursts, resting on ice for 30 seconds between each vortex. To prepare microsomes, samples were transferred to a 15 ml conical tube and spun at 1,000 x g for 5 minutes. The supernatant from this centrifugation was saved and then spun again at 12,000 x g for 15 minutes. The supernatant from this step was transferred to a
polycarbonate ultracentrifuge tube and spun at 100,000 x g for 60 minutes, using a Sorvall T-865.1 ultracentrifuge rotor. The resulting pellet was resuspended in 350 µl of extraction buffer and the solution was homogenized using a glass homogenizer. The resulting microsomal membrane fractions were stored at -80°C.

Multiple lines of transformed *Pichia pastoris* were grown at 30°C on plates with increasing concentrations of G418. The concentrations used were 500 µg/ml, 2,000 µg/ml, 5,000 µg/ml, and 10,000 µg/ml. These were compared to identify lines that appeared to have a higher resistance to G418. These more resistant lines of *Pichia pastoris* were selected from the 10,000 µg/ml plate, for expression to possibly identify a relationship between resistance and protein yield. Lines were selected and patches were started, growing for approximately 48 hours at 30°C on YPD +Amp +G418. Patched cells were spread into lawns and left to grow until full, thick lawns were observed, approximately 72 hours.

Cells were harvested and liquid cultures were started in 30 ml of the expression media BMMY, containing 50 µg/ml ampicillin to minimize bacterial contamination. Expression cultures were grown for approximately 20 hours, shaking at 300 rpm at 30°C. The cultures were spiked with 150 µl of 100% methanol after 20 hours of growth and shaken at 30°C for an additional 3 hours. Microsomes were prepared from *P. pastoris* cells as described above for the *S. cerevisiae*. 
Protein Concentration Assay and SDS-PAGE

To determine protein concentration, a Pierce BCA protein assay was done using a 96-well microtiter plate with varying dilutions of samples and BSA standards.

SDS-PAGE of microsomes and the corresponding cell pellets was run using 40 µg total protein in each lane. A 10% resolving gel and a 4% stacking gel was used and was run at a constant current of 30 mA.

The proteins from the gel were transferred for approximately 18 hours at a constant voltage of 40 V to an Immobilon-P membrane. The membrane was briefly washed with agitation in 1X TBS-Tween and blocked in 5% nonfat dry milk (NFDM) in TBS-Tween for 1 hour. The membrane was incubated with a 1:5,000 dilution of a horseradish peroxidase-conjugated anti-T7 monoclonal antibody (EMD/Millipore) in 1X TBS-Tween containing 5% NFDM for 1 hour with gentle rocking, flipping the membrane after 30 minutes. The blocked membrane was vigorously washed in 1X TBS-Tween four times for 15 minutes each. After the final wash, the membrane was blotted dry and incubated in Bio-Rad Clarity ECL detection reagent mixture for one minute. The membrane was blotted dry and imaged using the Gel Doc™ XR+ Gel Documentation System.
Characterization Attempts of CcMANS1 Activity

Biochemical Assay

Samples were selected for analysis and a mannan synthase assay was prepared with the reagents in the following concentrations for 30 µl reactions: 1X ManS assay buffer, 17.5 µM GDP-mannose, 2.5 µM GDP-[14C]-mannose (Table 4). 100 µg of each microsomal membrane protein sample was used for each reaction. Glucomannan synthase activity assays were also conducted, using 1X ManS AB, 10 µM of GDP-mannose, 2.5 µM of GDP-glucose, and 2.5 µM of GDP-[14C]-glucose. Reactions were incubated for 15 minutes at room temperature. After incubation, the reactions were terminated by adding 1 ml of cold 70% ethanol containing 2 mM EDTA and 10 µl of 1% (w/v) guar galactomannan. Terminated reactions were incubated at -20°C for approximately 24 hours. Using a vacuum manifold fitted with Whatman glass fiber filters, samples were filtered and washed with 15 ml of 70% ethanol containing 2 mM EDTA. Washed filters were transferred to scintillation vials to which Eco-Safe scintillation fluid was added and the radioactivity was counted using a scintillation counter. TfMANS and AkCSLA were used as positive controls for *Pichia pastoris* samples. Untransformed InvSc1 and X33 were used as the negative controls for the assays. Two replicates of each assay were performed to ensure consistent results.
Sequence Verification of T7-CcMANS1 in Transgenic Pichia pastoris

Genomic DNA was isolated from Pichia pastoris using a procedure described by Lõoke et al. (2011). Approximately 8 colonies of yeast cells streaked from a single glycerol stock and grown on YPD + ampicillin were suspended in 100 µl of 200 µM LiOAc / 1% SDS solution. This solution was incubated at 70°C for 15 minutes, 300 µl of 95% ethanol was added and samples were vortexed to mix. The mixture was centrifuged at approximately 15,000 x g for 3 minutes and the sedimented material was resuspended in 100 µl of TE. This mixture was centrifuged at approximately 15,000 x g for 1 minute. The resulting supernatant containing the yeast gDNA was stored at -80°C.

A PCR amplification of the isolated gDNA was run with the following reagents: 1X Q5 master mix, 50 µM forward primer (Janell P1), 50 µM reverse primer (Janell P2), and 1 ng/rxn of purified yeast DNA. The reaction was carried out using the following thermocycler settings:

1. 98°C for 30 seconds
2. 98°C for 10 seconds
3. 58°C for 30 seconds
4. 72°C for 2 minutes
5. repeat steps 2-4 29 times
6. 72°C for 2 minutes

The correct PCR product was verified using a 0.8% (w/v) agarose gel in 1X TBE with 1X gel red. After confirmation of desired product, the PCR product was purified with the
Wizard SV Gel & PCR Clean-Up System and sent to the University of Michigan DNA Sequencing Core for sequence confirmation.

**FACE**

Fluorophore assisted carbohydrate electrophoresis (FACE) was run using 1,4-β-mannooligosaccharide standards (Megazyme) in order to optimize the procedure to analyze yeast-produced carbohydrates. Samples were digested and labeled according to procedure described by Goubet et al. (2002) and the FACE gel was prepared according to the protocol outlined in Gao and Lehrman (2003).

BMABS and BMACJ, two different 1,4-β-mannanases (Megazyme) were used to digest carbohydrates present in yeast samples. These digests were carried out using 1 µl of 1% (w/v) carbohydrate standards or 1 µl of yeast cells, 0.1 M enzyme specific buffer, and 0.39 units of BMABS or 0.5 units of BMACJ. Each reaction was incubated at 42°C for 1 hour and reactions were terminated by boiling for 5 minutes. These digests were compared and the most versatile enzyme (BMABS) was used in the digestion of X33, AtCSLA and CcMANS1 samples. The labeling compound used in this study was 7-amino-1,3-naphthalenedisulfonic acid (ANDS).

**Mass Spectroscopy**

To obtain the soluble and insoluble cell fraction for mass spectrometry analysis, the pellet of *Pichia pastoris* cells expressing the desired protein was resuspended in 4 ml
of 50 mM Hepes KOH pH 7.0. This cell suspension was put into a 30 ml glass tube containing approximately 3 g of sterile beads (425-600 µm). Tubes were then vortexed vigorously for eight 30-second bursts, resting on ice for 30 seconds between each vortex. Each sample was left to rest on ice for 5 minutes to let bubbles settle. The broken cell mixture was transferred to a 15 ml conical tube and centrifuged at 1,000 x g for 5 minutes. The supernatant (soluble fraction) was transferred to a clean 15 ml tube and the pellet (insoluble fraction) was saved for further preparation. The soluble fraction and the sample was boiled for 5 minutes. The solution was then centrifuged at 9,000 x g for 15 minutes. The remaining supernatant was saved for digestion. The insoluble fraction was resuspended in 10 ml of 70% EtOH by vortexing and incubated at 65 °C for 15 minutes with agitation by inversion every 5 minutes. The sample was centrifuged for 5 minutes at 3,000 x g. The resulting pellet was then resuspended in 10 ml of 70% EtOH and the incubation and wash was repeated for a total of three times. The final washed pellet was resuspended in approximately the same amount of water as the resulting soluble fraction was. These were frozen overnight at -80°C. X33 samples, AtCSLA9 samples, Guar standards and Konjac standards were all digested with BMABS in 0.1 M glycine buffer at a pH of 8.8. 100 µl of each yeast cell fraction whether soluble or insoluble, were used for each digest reaction. Each X33 sample with additional carbohydrate standards contained 1 µl of 1% (w/v) Guar galactomannan or 1 µl of 1% (w/v) Konjac glucomannan, depending on the sample. The digests were carried out using the following reagents: 40 µl of buffer specific for each enzyme, 165 µl of sterile water, 2 µl of enzyme and 100 µl
of yeast cell fraction. For the Guar and Konjac carbohydrate standards, 1 µl of 1% (w/v) carbohydrate standard was combined with 20 µl of buffer, 82.5 µl of sterile water and 1 µl of BMABS. Each BMABS yeast fraction digest was incubated at 42°C for 9 hours and each E-CELTR digest was incubated at 37°C for 9 hours. The Guar and Konjac carbohydrate samples were digested at 42°C for 1 hour. All digests were terminated by boiling for 5 minutes. These samples were dried for 9 hours using a speed vac. Dried samples were analyzed using mass spectroscopy at the Michigan State University Mass Spectrometry and Metabolomics Core Facility.

Table 4 Composition of reagents used in this study

<table>
<thead>
<tr>
<th>YPD</th>
<th>10 g Yeast Extract, 20 g Peptone, 20 g Glucose, Distilled water to 1 L. 20g/L of Agar if making plates.</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 x TBS-Tween</td>
<td>12.11 g 100 mM Tris, 87.66 g 1.5 M NaCl, 10 ml 1% Tween-20. Bring to 1 L with distilled water.</td>
</tr>
<tr>
<td>LB media</td>
<td>10 g Tryptone, 5 g Yeast extract, 10 g NaCl. Bring to 1 L with distilled water.</td>
</tr>
<tr>
<td>Extraction Buffer</td>
<td>50 mM Hepes-KOH pH 7.5, 0.4 M Sucrose, 1 µl/ml of protease inhibitor cocktail</td>
</tr>
<tr>
<td>4X ManS assay buffer</td>
<td>200 mM Hepes-KOH pH 7.5, 10 mM DTT, 10 mM MgCl₂, 20 mM MnCl₂, 24% glycerol</td>
</tr>
</tbody>
</table>

Results

Restriction Enzyme Digests to Confirm Proper Gibson Assembly Product

To confirm the *E. coli* were transformed with the correct Gibson assembly product, the purified plasmid from the transformed *E. coli* lines was digested using
restriction enzymes and successful construction of desired recombinant DNA molecules was confirmed. A pPICZ-CcMANS1 digest and a pYES-CcMANS1 digest was compared to undigested samples confirming that the digests were successful and that each contained the correct plasmid (Fig 7). KpnI and HindIII digests of pPICZ-CcMANS1 and the HindIII digest of pYES-CcMANS1 produced restriction fragments that corresponded to the expected fragment sizes (Fig. 7). Subsequent DNA sequencing confirmed that each plasmid was error-free.

**Table 5** Restriction enzymes used to confirm the successful transformation of *E. coli* for each desired vector/transgene combination.

<table>
<thead>
<tr>
<th>Vector/Transgene</th>
<th>Restriction Enzyme</th>
<th>Expected Bands, in base pairs (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pYES-CcMANS1</td>
<td>HindIII</td>
<td>6060, 1368 bp</td>
</tr>
<tr>
<td></td>
<td>Ncol</td>
<td>7428 bp</td>
</tr>
<tr>
<td></td>
<td>BseII</td>
<td>7428 bp</td>
</tr>
<tr>
<td>pPICZ-CcMANS1</td>
<td>HindIII</td>
<td>2194, 1778, 1403 bp</td>
</tr>
<tr>
<td></td>
<td>KpnI</td>
<td>4532, 843 bp</td>
</tr>
<tr>
<td>pYES-AiCSLA9</td>
<td>HindIII</td>
<td>6835, 804 bp</td>
</tr>
<tr>
<td></td>
<td>Sphi</td>
<td>7639 bp</td>
</tr>
<tr>
<td></td>
<td>BseII</td>
<td>Uncut</td>
</tr>
<tr>
<td></td>
<td>Psfl</td>
<td>7151, 488 bp</td>
</tr>
<tr>
<td></td>
<td>Ncol</td>
<td>4680, 2959 bp</td>
</tr>
<tr>
<td>pPICZ-C418</td>
<td>PstI</td>
<td>Uncut</td>
</tr>
<tr>
<td></td>
<td>Kpnl</td>
<td>3749 bp</td>
</tr>
<tr>
<td></td>
<td>HindIII</td>
<td>2194, 1555 bp</td>
</tr>
<tr>
<td></td>
<td>BamHI</td>
<td>Uncut</td>
</tr>
</tbody>
</table>

**Figure 7** KpnI and HindIII were used to digest the pPICZ-CcMANS1 and pYES-CcMANS1 plasmids purified from *E. coli*. These digests were used to confirm the desired transgenic vector.
Large Scale Plasmid Prep and Linearization of DNA for Pichia pastoris

Transformation

Because the mini-prep purification did not yield enough plasmid for yeast transformation, a large-scale preparation of transformed E. coli was completed. The digest produced restriction fragments that corresponded to the expected fragment sizes (Fig. 8).

![Figure 8](image)

Figure 8 Samples were linearized with SacI and confirmed using gel electrophoresis.

Pichia pastoris Transformation Using Electroporation

To transform Pichia pastoris with the linearized plasmid, electroporation was used. The transformed yeast cells were plated on YPDS +Amp +G418 media and many colonies grew, showing that the transformation was successful.
**PCR Amplification of Desired Transgene**

The purified yeast DNA served as a template for a PCR verification of 77-
CcMANSJ transgene presence. Bands representing the PCR product corresponding to the transgene were present. These PCR products were expected to be the size of the CcMANSJ transgene which is confirmed by the location of the bands, around 1,600 base pairs (Fig. 9).

**Figure 9** Lanes 1-25 correspond to different X33 lines that transformed with the pPICZ-CcMANSJ construct. Lanes 25 and 52 are untransformed X33 and the negative controls can be seen in the far right lanes of each gel.

**Transformation of Saccharomyces cerevisiae Competent Cells Via Heat Shock**

The second yeast model system used in this study was *Saccharomyces cerevisiae*. Transformed *S. cerevisiae* cells were plated on SD-Ura media, and many colonies grew, indicating that the transformation was successful. The yeast DNA was purified from the cultures to serve as a template for a PCR verification of 77-CcMANSJ transgene presence.

Bands representing the amplified transgene were present in all transformed samples (Fig. 10). These PCR products were expected to be the size of the GBlock, and
this can be seen by comparison to the positive control. There appears to have been contamination of the InvSC1 untransformed sample even after repeating the analysis, suggesting it may have occurred during the boil prep of these samples. Though there was no proper negative control of the InvSC1, these results provided enough confirmation to move onto the expression of CcMANS1.

Figure 10 Lanes 1-13 correspond to different InvSC1 lines that have been transformed with the pYES-CcMANS1 construct. The + control is the amplified GBlock and the – control is the untransformed InvSC1.

**SDS PAGE/Immunoblot of S. cerevisiae and P. pastoris samples**

To determine if the microsomal membrane samples from transgenic yeast cultures contained the recombinant T7-tagged CcMANS1 protein, the samples were prepared and analyzed using SDS-PAGE and immunoblot. *Pichia* samples that express the T7-OsCSLA1 protein, from rice, were used as a positive control for this experiment, and
untransformed X33 Pichia cells were used as a negative control. The calculated mass of T7-CcMANS1 is approximately 62 kDa, similar to that of T7-OsCSLA1.

The T7 tagged CcMANS1 protein was found in the microsomes and pellets of all analyzed samples (Fig. 11, Fig. 12). The bands observed were similar in size to CSLA1 from *Oryza sativa* and the expected size of T7 tagged CcMANS1. This result prompted us to proceed with the biochemical assays of both the *Pichia pastoris* as well as the *Saccharomyces cerevisiae* expressed CcMANS1 samples.

**Figure 11** T7-tagged proteins were present in both the microsomal membrane fractions (µ1-µ4) as well as the cell debris pellet (P1-P4). Samples chose for analysis are noted as #1, #6, #7, and #10. *S. cerevisiae* microsomes containing T7-tagged OsCSLA1 served as the positive control.
Figure 12 T7 tagged proteins were present in the *P. pastoris* microsomal membrane fractions. *P. pastoris* microsomes containing T7-tagged OsCSLA1 served as the positive control and X33 served as the negative control.

**Biochemical Assay**

To determine the enzymatic activity of CcMANS1, $^{14}$C-labeled nucleotide sugars were used as substrates for *in vitro* biochemical assays. AkCSLA, TfMANS and OsCSLA1, enzymes with known mannan synthase and glucomannan synthase activity were used as positive controls for each assay and the untransformed yeast samples were used as the negative controls to determine the baseline incorporation levels in yeast samples.

Negative controls yielded an average of 560 DPM of ethanol insoluble products. Positive controls AkCSLA, TfMANS, and OsCSLA yielded an average of 9028 DPM, 2419 DPM and 12,231 DPM, respectively. All of the CcMANS1 samples had an average
between 499 and 713 DPM. The amount of radioactivity found in the 70% ethanol insoluble fractions from the CcMANS1 samples was approximately the same as that of the non-transgenic X33 samples (Fig. 13, Fig. 14). The negative control for the Saccharomyces cerevisiae samples was the non-transgenic InvSc1 which had an average of 845 DPM. All of the CcMANS1 samples in Saccharomyces cerevisiae had an average DPM that was less than the baseline activity measured in InvSc1 (Fig. 16). CcMANS1 samples all had an average DPM that was similar to that of the negative controls and all positive controls showed activity at least four times that of the negative controls.

Figure 13 Mannan synthase assays of P. pastoris samples. OsCSLA1, a known mannan synthase, served as a positive control for this assay. Microsomal membranes from non-transgenic X33 were used as the negative control.
Figure 14. Mannan synthase assays of *S. cerevisiae* samples. The non-transgenic InvSc1 was used as a negative control to determine activity of *Saccharomyces cerevisiae* CcMANS1 samples. Similar results were obtained from the glucomannan synthase assay (Fig. 15, Fig. 16).

CcMANS1 samples did not exhibit any glucomannan synthase activity (Fig. 15, Fig. 16). OsCSLA1 and AkCSLA are known glucomannan synthases and showed more activity than TfMANS, a known pure mannan synthase. This can be seen by its low average DPM in the glucomannan activity assay (Fig. 15.) (Liepman et al. 2007, Gille et al. 2011, Wang et al 2012).
Figure 15 Known glucomannan synthase AkCSLA in *Pichia pastoris* served as a positive control for this assay. The non-transgenic X33 was the negative control to determine activity of CcMANS1 samples.

Figure 16 The non-transgenic InvSc1 was the negative control to determine activity of *Saccharomyces cerevisiae* CcMANS1 samples.
Sequencing of Pichia gDNA

Due to the lack of in vitro mannan synthase activity shown by any of the CcMANS1 samples from either yeast, genomic DNA was isolated from Pichia pastoris and the CcMANS1 transgene was amplified to verify that the sequence corresponding to the CcMANS1 protein was not mutated in the transgenic yeast lines. No mutations within the protein coding sequence were found, indicating that this was not the cause of the lack of observed enzymatic activity.

Fluorophore Assisted Carbohydrate Electrophoresis (FACE)

To determine if the lack of in vitro activity of CcMANS1 was due to inactivation of the protein during microsome isolation and analysis, samples of carbohydrates from transgenic yeast were analyzed using fluorophore assisted carbohydrate electrophoresis. This method has not been used to determine CSLA enzyme activity in vivo so the method was optimized and the standards were chosen according to how well each oligosaccharide chain was separated on the gel. Different digests of carbohydrate standards were compared prior to analyzing any samples (Fig. 17, Fig. 18). 1,4-β-mannanases BMABS and BMACJ were compared for effectiveness (Fig. 17, Fig. 18). Guar galactomannan (A), Konjac glucomannan (B) and Pure mannan from Carob (C) were digested using BMACJ and BMABS, and BMABS was chosen because of its ability to digest all three carbohydrate standards (Fig. 18). Concentration of 1,4-β-mannopentaose was also tested on this gel, as seen by the M5 and M10 lanes which are samples in 5 nmol and 10 nmol.
concentrations, this allowed us to determine that 5 nmol and 10 nmol were both too much carbohydrate to obtain a clear band. Digests of these different carbohydrates were carried out and these optimized parameters were then used to digest and analyze carbohydrates from transgenic yeast cells expressing CcMANS1.

Figure 17 FACE analysis of digested Guar galactomannans, Konjac glucomannans and pure mannans derived from Carob galactomannans. Konjac glucomannan samples provided the only clear representation of a successful digest with BMACJ. 5 nmol and 10 nmol of 1,4-β-mannopentaose appears to have been too high of a concentration to produce a clear band. The digest time interval indications are as follows: 0'- Sample 1, 30'- Sample 2, 60'- Sample 3, Overnight – Sample 4. The negative control was the enzyme without any added carbohydrate as well as negative digest controls of the carbohydrates undigested, seen in lanes A B and C.
Figure 18 FACE analysis of digested Guar galactomannans, Konjac glucomannans and pure mannans derived from Carob galactomannans. All three digested carbohydrates showed clear varying sized products with the BMABS enzyme. The digest time interval indications are as follows: 0'- Sample 1, 30'- Sample 2, 60'- Sample 3, Overnight – Sample 4.

Transgenic yeast samples were compared using the optimized parameters found in this study. AtCSLA9, a known mannan synthase was compared to X33 and there were no noticeable bands of carbohydrate observed using FACE (Fig. 19). AtCSLA9 was used as a positive control for this assay because it has previously been shown, using carbohydrate linkage analysis, to produce 1,4-β linked mannans in vivo, in *Pichia pastoris* (Liepman, Personal Communication 2016). Because we were unable to detect a difference between untransformed, AtCSLA9 transformed, and CcMANSl transformed samples with FACE mass spectroscopy was used (data not shown).
Figure 19 FACE analysis of Carbohydrates from *Pichia pastoris* lines expressing T7-AtCSLA9 and nontransgenic X33 produced carbohydrates. BMABS concentrations varied between the five X33 and the 5 AtCSLA9 samples. Mannooligosaccharides were also compared and some bands were identified.

**Mass Spectroscopy**

Nontransgenic X33 was compared to known carbohydrate samples. Konjac glucomannan was used and noticeable peak differences were present between the X33 insoluble digested samples and the Konjac digested samples (Fig. 20). The samples containing both X33 and Konjac confirmed that the *in vivo Pichia* carbohydrates present in the X33 samples, would not mask the presence of other carbohydrates.
Figure 20 Mass spectroscopy results of X33 insoluble fraction digested, X33 insoluble fraction undigested, Konjac digested, and X33 insoluble fraction +Konjac digested. Peaks identified in both the digested X33 insoluble fraction and the digested Konjac sample were both seen the digested X33 insoluble fraction +Konjac as expected.

CcMANS1 samples were compared to X33 produced carbohydrates as well as AtCSLA9 produced carbohydrates. Analysis of digested AtCSLA9 samples yielded identifiable peaks with 3 and 4 degrees of polymerization (DP) (Fig. 21). AtCSLA9 samples compared to X33 nontransgenic samples confirmed the presence of an active AtCSLA9 protein, which is a known mannan synthase (Liepman et al. 2005, Liepman et al. 2007). AtCSLA9 is known to produce 1,4-β linked mannans which is why this served as the positive control. Non-transgenic X33 contains other mannose containing carbohydrates with varying linkages. The absence of any identifiable peaks in the undigested samples leads us to believe that the digestion of the carbohydrates into smaller chains allows for mass spectroscopy identification of these small oligosaccharides.
Figure 20 Mass spectroscopy results of X33 insoluble digested, AtCSLA9 insoluble digested and undigested, and two samples of CcMANS1 insoluble digested and undigested. Peaks seen at 100% in the CcMANS1 samples represent a value less than half of the value of the peaks seen in the X33 sample.
CcMANS1 #5 and CcMANS1 #8 had peaks with masses corresponding to carbohydrates with 3, 4, and 5 DP. These peaks appear to have the same elution time as the carbohydrates present in the X33 samples (Fig. 21). Though they appear larger, these peaks are at approximately 92,500, compared to the shorter X33 peaks which appear to be at approximately 200,000 (Fig. 21). There is a lack of independently unique peaks in the CcMANS1 samples, while the positive control AtCSLA9 displays multiple peaks that are unique to that sample.

**Discussion**

This study attempted to characterize the enzymatic activity of CcMANS1, a putative mannan synthase from *Coffea canephora*. The vectors used in this study were successfully constructed and confirmed to be error free. Plasmid DNA purified from *E. coli* was transformed into both *Pichia pastoris* and *Saccharomyces cerevisiae*. These yeast expression systems were made to express the CcMANS1 protein and its presence was verified using SDS-PAGE and immunoblot. The protein-rich Golgi membrane was purified and *in vitro* activity of CcMANS1 was compared to known mannan and glucomannan synthases. However, *in vitro* and *in vivo* activity of recombinant CcMANS1 expressed in *Pichia pastoris* or *Saccharomyces cerevisiae* could not be verified using *in vitro* radiochemical assays and *in vivo* analyses (FACE and mass spectrometric analysis of carbohydrates).
Expression of recombinant CSL proteins in yeast has produced active proteins in several other characterization studies (Cocuron et al. 2007, Liepman et al. 2007, Gille et al. 2011, Wang et al. 2011). *In vitro* activity has been successfully measured using radioactive enzymatic assays and similar protocols were followed during this study (Liepman et al. 2005, Cocuron et al. 2007, Liepman et al. 2007, Gille et al. 2011, Wang et al. 2011). Methods outlined in this study have produced functional CSL proteins in other studies and lack of function does not appear to be due to experimental design. Attempts to identify the cause of the lack of function were inconclusive.

Lack of function of the CcMANS1 protein could be due to many factors including the addition of the T7 tag, the need for addition proteins or post translational modification. The addition of the T7 tag may have compromised the activity of the recombinant CcMANS1 protein. Many other CSLA proteins have been studied with similar tags and remain active (Liepman et al. 2005, Liepman et al. 2007, Gille et al. 2011). These possible causes of loss of function may serve as a foundation of research focus in future studies.

It is possible that the activity of CSL proteins can be affected by need for a multiprotein complex (Cocuron et al 2007, Wang et al. 2013, and Chou et al. 2015). The mutation of mannan synthase related 1 (msr1) and msr2 decreased the levels of mannans produced in the fenugreek endosperm (Wang et al. 2013). These enzymes may be part of a multiprotein complex, or may be responsible for the bringing together of enzymes into a multiprotein complex. Expression of a single plant enzyme in a yeast system may show
no activity because of the lack of necessary coenzymes. Studying proteins isolated from plant tissue is difficult because it is often difficult to differentiate when a protein is necessary for functionality because of the vast number of additional proteins present, along with the high number of CSLA and related proteins. Two putative mannan synthases from *Coffea canephora* were identified and it is possible that both need to be in expressed for any mannan synthase activity. In future studies, identification of a *Coffea canephora msr* gene may provide the necessary coenzymes to express a functioning protein in *Pichia pastoris* or *Saccharomyces cerevisiae*. It is worth investigating the need for two coexpressed *Coffea canephora* mannan synthases. The requirement of a coenzyme or multiprotein complex is one of the theories that could serve as future research foundations.

Other possible reasons for the lack of enzymatic function include possible post-translational modifications that render the active site inaccessible or cause conformational changes resulting in non-functional recombinant CcMANS1 produced by yeast. This type of modification could involve phosphorylation or glycosylation that result in the CcMANS1 protein becoming non-functional. In the future, it would be worth investigating the possibility of these modifications being present.

CSLA proteins have been extensively studied in *Pichia pastoris* so its use as an expression system for CcMANS1 was backed by prior successful studies (Gille et al. 2011, Wang et al. 2012). On the other hand, efforts to measure *in vitro* activity of CSLC proteins expressed in *Pichia pastoris* were unsuccessful, in this case, analyses of *in vivo*-
produced carbohydrates from transgenic yeast lines expressing CSLC proteins revealed that these proteins synthesize cellodextrins (1,4-β-glucan oligosaccharides; Cocuron et al. 2007). As shown by the comparable expression levels in both yeasts used in this study (Saccharomyces and Pichia), Saccharomyces cerevisiae may be a useful expression system to study CSLA proteins that have not been successfully expressed using other expression systems. Further optimization of CSLA expression in Saccharomyces cerevisiae may lead to better expression methods for CSLA or related proteins, and may open up new options for researchers.

AtCSLA9 is a CSLA protein that has shown in vivo mannan synthase activity (Liepman et al. 2005, Liepman et al. 2007). The lack of in vivo mannan synthase activity for our positive control as well as the CcMANS1 samples as seen by the FACE assay, leads us to conclude that the sensitivity of the FACE method may not be sufficient to measure activity of CSLA or related CSL proteins in yeast. In order to determine the sensitivity capabilities of FACE, TmCSLC or AtCSLC expressed in Pichia could be tested because in vivo activity of these two CSL proteins has been previously confirmed (Cocuron et al. 2007.)

The lack of 1,4-β linked mannans found in the CcMANS1 samples analyzed by mass spectroscopy could be due to the high level of background carbohydrates present in the crude yeast samples sent for analysis. This could be resolved if methods for purifying and isolating the desired carbohydrates from yeast are optimized, or the amount of
desired carbohydrate is increased. This method of carbohydrate analysis may prove beneficial for future studies attempting to identify \textit{in vivo} activity of CSL proteins.

Despite the lack of functionality of CcMANS1, this study has led to findings that will help future researchers optimize their research into CSLA and other related enzymes. There are other \textit{Coffea canephora} putative mannan synthases such as CcMANS2 (Pré et al. 2008), and this protein could be studied by other researchers. The expression of both CcMANS1 and CcMANS2 may be necessary for proper functioning of mannan synthases from \textit{Coffea canephora}.

Investigating why CcMANS1 did not show \textit{in vitro} mannan synthase activity has led to the exploration of different methods of analyzing \textit{in vivo} activity of yeast expressed CSLA proteins. Fluorophore Assisted Carbohydrate Electrophoresis (FACE) may serve as a new method for analyzing recombinant protein activity if there is enough enzyme activity. The lack of additional CSL proteins and the lack of naturally occurring 1,4-\beta mannan synthases in \textit{Pichia pastoris} make this a promising strategy for measuring the activity of CcMANS1, as well as other 1,4-\beta mannan synthases that may not have been successfully characterized before.

Mass spectroscopy analysis of transgenic yeast produced mannans has been shown to produce promising results (Cocuron et al. 2007). In this study, the confirmation of 1,4-\beta linked mannans in AtCSLA9 transgenic yeast samples, by use of mass spectroscopy, has led us to believe that this type of assay may be beneficial for future carbohydrate analysis of other CSLA proteins expressed in yeast.
Investigation into the loss of function in CcMANS1 may help optimize how researchers approach characterization of CSL proteins in the future. As more genes responsible for cell wall component synthases from different plants are identified, it will be important to have a reliable method for characterizing and confirming the activity of these proteins.

Building our understanding of plant cell wall biosynthesis will prove invaluable as we begin to utilize this information to optimize plant cell wall use. Plant cell wall tissue is the most abundant source of renewable biomass, yet it is grossly underused (Liepman & Cavalier 2012). Research into how plant cell walls are synthesized is important in helping biologists understand plant biomass characteristics and will eventually lead to engineering plant materials better suited for specialized applications.


