DEVELOPMENT OF A METHOD FOR HETEROLOGOUS EXPRESSION OF MOSS METHYLTRANSFERASES IN *PICHIA PASTORIS* YEAST

By

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Abstract

The abundance of *O*-methyl-ether sugar residues in plant cell wall polysaccharides varies between plant taxa and could influence the efficiency with which plant biomass is converted into biofuels for transportation. In previous experiments, two methyltransferase genes were found in the moss *Physcomitrella patens* that, when expressed in tobacco, caused production of 3-*O*-methyl-galactosyl residues in tobacco cell wall polymers. This finding is curious, however, since the moss natively contains 3-*O*-methyl-rhamnosyl residues, but little, if any, 3-*O*-methyl-galactosyl residues in its cell wall polymers. The goal of this project is to develop a method for expressing the moss methyltransferase genes *MT1* and *MT6* in the yeast *Pichia pastoris*, which contains no endogenous substrates on which the MT1 and MT6 enzymes can act. Extracts from such transgenic *Pichia* would be useful in testing the substrate specificities of MT1 and MT6 to determine whether these enzymes might synthesize 3-*O*-methyl-galactosyl residues in some conditions, but 3-*O*-methyl-rhamnosyl residues in other conditions. This report focuses on progress towards this goal, including: design and linearization of *E. coli* plasmids carrying the *MT1* or *MT6* gene and a Zeocin resistance gene; electroporation of these linearized plasmids into wild-type *Pichia* cells; primary screening of electroporated cells for growth in the presence of Zeocin; and secondary screening by polymerase chain reaction for proper insertion of the plasmid into the *AOX1* promoter of *Pichia*. Colonies that passed the primary and secondary screens are being tested for methyltransferase activity in ongoing experiments with a variety of polysaccharide substrates.

Acknowledgements:

I would like to thanks to Anjin Huang for her extensive work on designing and leading a significant part of the transformation of new *Pichia* cultures. I would also like to thank Dr. Eugene A. Nothnagel, my faculty mentor, for his unending dedication, guidance, and support throughout both this capstone project and my time as an undergraduate student at UCR. Additionally, I would like to thank Dr. Martha L. Orozco-Cárdenas for aiding in this project by donating both lab space and time to developing this project. Finally, I would like to thank other student collaborators, Carlos Aguilar, James Yen, Maivy Ngoc Le, and others in assisting with conducting many of the experiments that culminated in this capstone project.

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Introduction:

The plant cell wall contains many varied complex polymers, including especially cellulose, hemicelluloses, and pectin polysaccharides that together provide crucial cellular functions like support, growth, defense, etc. (Popper et al. 2004). While the presence of cellulose, hemicelluloses, and pectins is generally conserved across land plants, the relative abundances of these polysaccharides can vary both between difference species and between different cell types within one species. Hemicelluloses and pectins carry some amounts of methyl-ether and methyl- and acetyl-ester substituents on their glycosyl residues, and the abundances of these substituents also vary both between different species and between different cell types within one species.

The general focus of this project is methyl-ether substituents on cell wall glycans. Some types of glycosyl residues carrying methyl-ether substituents, particularly the 4-O-methyl-glucuronosyl residues of hemicelluloses and the 2-*O*-methyl-xylosyl and 2-*O*-methyl-fucosyl residues of pectins, occur in most lower plants and essentially all higher plants. The specific focus of this project is other glycosyl residues carrying methyl-ether substituents, namely 3-*O*-methyl-rhamnosyl (3-*O*-Me-Rha) or 3-*O*-methyl galactosyl (3-*O*-Me-Gal) residues (Fig. 1) as components of pectins and related polymers. These residues are at low abundances, if present at all, in the most derived group of land plants, the angiosperms,

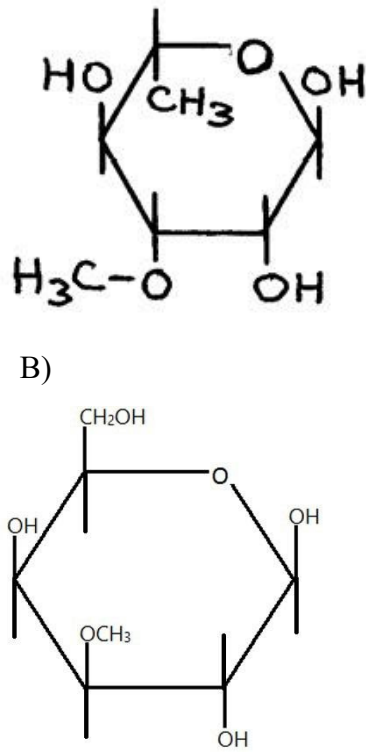

Figure 1. Haworth projections of A) 3-*O*-methyl-α-L-rhamnose, and B) 3-O-methyl-β-D-galactose

but are present at larger abundances going back in evolution through gymnosperms, pteridophytes, lycophytes, and bryophytes (Popper et al. 2004).

The initial work of this laboratory in this field of research was the discovery that the moss *Physcomitrella patens*, a bryophyte, has up to 15 mole percent of 3-*O*-Me-Rha residues in the arabinogalactan proteins of its cell wall space (Fu et al. 2007). Continuing work by this laboratory is the discovery of the *Physcomitrella* moss methyltransferase genes *MT1* and *MT6*. When these methyltransferase genes *MT1* and *MT6* were heterologously expressed in tobacco, 3-*O*-Me-Gal residues were found in the cell wall polymers of the transgenic tobacco plants. This finding is curious because while the abundance of 3-*O*-Me-Rha residues is significant in the cell wall of *Physcomitrella*, the abundances of 3-*O*-Me-Gal residues in the cell walls of both *Physcomitrella* and wild-type tobacco is not above noise level and might be zero. This result invites the question: Could the methyltransferase enzymes MT1 and/or MT6 cause synthesis of 3-*O*-Me-Rha residues in moss cell wall polymers but 3-*O*-Me-Gal residues in tobacco cell wall polymers? The goal of the current project is to develop a method for expressing the moss methyltransferase genes *MT1* and *MT6* in the yeast *Pichia pastoris*, which would have little interference from endogenous substrates and would facilitate studies of the substrate specificities of the MT1 and MT6 methyltransferases.

Hypothesis:

Using such a heterologous expression system allows testing of the following hypothesis: If the active sites of the MT1 and MT6 methyltransferase enzymes are not highly specific, and if various substrates and conditions are used in test-tube biochemical assays, then in some situations methyltransferases MT1 and MT6 will synthesize 3-*O*-Me-Gal residues, while in other conditions MT1 and MT6 will synthesize 3-*O*-Me-Rha residues.

Significance:

What is the motivation to study synthesis of methylated sugar residues in plant cell walls? The hypothesis to be investigated in the current project addresses details of plant cell wall synthesis that are not yet understood, and thus the project has significance towards achieving a better understanding of the fundamental biology of plants. The project also has at least two areas of potential practical significance. One such area is production of alternatives to petroleum fuels. For plants engineered to have greater cell wall methyltransferase activity, an increase from 6 to 7 carbons in a given hexose monomer could increase the yield of biomass conversion to synthetic diesel fuel. Another area of potential practical significance is engineering plants to be more resistant to drought stress. Mosses are more desiccation tolerant than most angiosperms. Tobacco plants transformed to express *Physcomitrella* methyltransferase MT1 or MT6 have leaf tissue that has been shown to be more desiccation tolerant than leaf tissue from wild-type tobacco plants (Cid et al. 2015), which indicates that such an approach might lead to food plants with better productivity in drought stress conditions.

Materials and Methods:

In previous work in this laboratory, the approach was to express the *Physcomitrella MT1* and *MT6* cDNAs in *E. coli* bacteria to obtain MT1 and MT6 methyltransferases for use in test-tube biochemical enzyme assays to test the stated hypothesis. Although the transformed *E. coli* synthesized high levels of the transgenic polypeptides, these polypeptides accumulated in denatured form in inclusion bodies inside the *E. coli* cells, a common problem when expressing eukaryotic proteins in *E. coli* (Williams et al. 1989, Freedman and Wetzel 1992, Singh et al. 2015). Methods were eventually developed for solubilizing the denatured polypeptides, but various attempts to refold the solubilized, denatured polypeptides to active enzymers were all

unsuccessful. After more than one year of effort, expression in *E. coli* was abandoned as unworkable, and the approach turned to expression of MT1 in MT6 in *Pichia pastoris*, a yeast successfully used for expression of many eukaryotic proteins (Higgins 1995).

The VectorBuilder company was contracted to construct *E. coli* plasmids (Fig. 2) carrying a cassette for expression of MT1 or MT6 in *Pichia pastoris* (Fig. 2). VectorBuilder additionally used these expression cassette plasmids to generate three strains of *Pichia* transformed with the MT1 plasmid and three strains of *Pichia* transformed with the MT6 plasmid. Finally, VectorBuilder determined and reported the nucleotide sequences obtained when using oligonucleotide primers that spanned the alphaFactor/{MT1 or MT6} region in each of the six transformed *Pichia* strains. Upon receipt of these sequences from VectorBuilder, pairwise sequence alignment using the EMBOSS Needle algorithm (https://www.ebi.ac.uk/Tools/psa/emboss_needle/) was performed and confirmed that

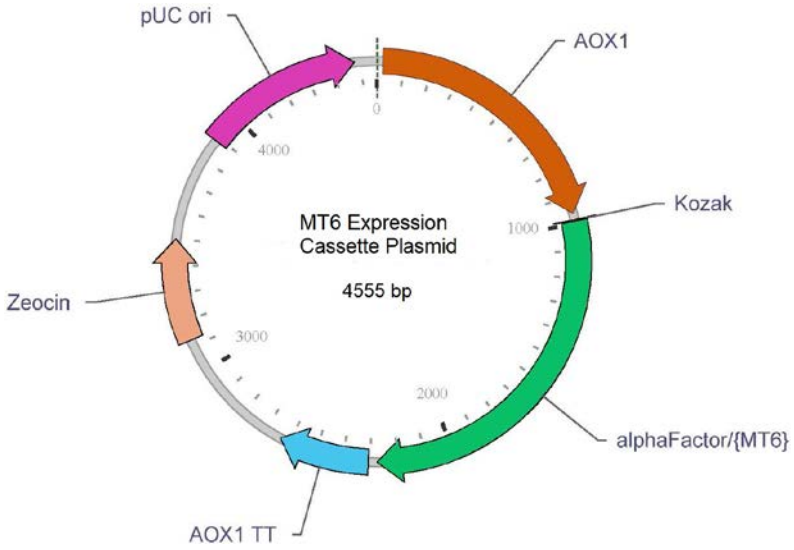

Figure 2. The MT6 expression cassette plasmid constructed by VectorBuilder under contract for this project. The principal components are: AOX1, the methanol-induced alcohol oxidase 1 promoter from *Pichia*; Kozak, protein translation initiation sequence; alphaFactor/{MT6}, the *Physcomitrella* MT6 cDNA sequence preceded by the *Saccharomyces* alpha secretion signal; AOX1 TT, alcohol oxidase 1 transcription terminator sequence from *Pichia*; Zeocin, the promoter and gene encoding Zeocin antibiotic resistance that enables selection of transformants; pUC ori, origin of replication of the plasmid in *E. coli*. For transformation of *Pichia*, the plasmid is linearized by a unique restriction endonuclease cut in the AOX1 promoter region and then electroporated into the *Pichia* cells.

each of the six strains contained the expected MT1 or MT6 sequence.

The six transgenic *Pichia* cell lines received from VectorBuilder were grown on several media recommended by Invitrogen (2010), as well as on a medium of our own formulation. No methyltransferase activity producing either 3-*O*-Me-Rha or 3-*O*-Me-Gal residues were detected when homogenates from any of the six transgenic *Pichia* cell lines were used in test-tube biochemical enzyme assays. It was also observed that, when grown on agar-solidified medium, five of the six lines had a somewhat slimy appearance that did not occur with *Pichia* X-33 wild-type cells, suggesting that the transgenic *Pichia* lines from VectorBuilder might actually be co-cultures growing with the MT1- or MT6-plasmid-carrying *E. coli* cells that were used to generate the plasmids for transforming wild-type *Pichia* cells. This apparent contamination of the VectorBuilder-supplied transformed *Pichia* cultures led to a need to generate new transgenic *Pichia* cell lines.

To generate these new transgenic *Pichia* cell lines, *E. coli* strains carrying the MT1 or MT 6 expression cassette plasmid were grown in 50 mL shake cultures in low salt LB medium with 25 μg/mL Zeocin (Invitrogen 2010). The cells from two such 50 mL cultures were pooled, collected by centrifugation, and amounted to approximately 0.75 g fresh weight. Plasmids from this mass of cells were purified using a Qiagen EndoFree Plasmid Maxi Kit, following the instructions from the manufacturer (Qiagen 2015). The purified plasmids were then linearized using *PmeI* restriction endonuclease that cuts the plasmid at a unique site near the center of the *AOX1* promoter sequence in the plasmid (Fig. 2). After analysis by agarose gel electrophoresis to verify complete linearization of the plasmid, the preparation was extracted with chloroform-phenol to remove protein, ethanol precipitated, and then dissolved in 100 μL of TRIS-EDTA (TE) buffer. This solution was then filter-sterilized using a Merck Millipore Ultrafree MC

centrifugal filter unit with a GV Durapore membrane having 0.22 μm pores (UFC30GV0S), to eliminate any surviving *E. coli* cells from the solution of linearized plasmid and to prevent contamination of the *Pichia* cells during electroporation. The filter sterilized plasmid solution was then ethanol precipitated under sterile conditions and redissolved in a small volume of sterile distilled water to give a final concentration of approximately 700 ng/µL, as judged by Nanodrop spectrophotometry. Several batches of linearized plasmid were prepared and pooled to provide enough for electroporation into wild-type *Pichia*.

In preparation for electroporation, *Pichia* X-33 wild-type cells were grown in shake culture to a density of OD_{600} =1.0 in 250 mL of liquid YPD medium (Invitrogen 2010), or to a higher density in a correspondingly smaller volume. These cells were made competent for electroporation by a modification of the procedure of Cregg (2007). Working under sterile conditions, the culture was transferred to an autoclaved 250 mL Nalgene centrifuge bottle with screw cap, and centrifuged at 1500 × g for 10 minutes at 4ºC. The resulting pellet of cells was uniformly resuspended in 100 mL of sterile YPD/HEPES (mixture of 100 mL YPD with 20 mL of 0.22 μm filter-sterilized 1 M HEPES buffer, pH 8.0). Dropwise addition of 2.5 mL of 1 M dithiothreitol (DTT) solution was made to the cell suspension by using a 3 mL disposable syringe fitted with a sterile 0.22 μm Durapore Hydrophilic PVDF Millex-GV syringe filter assembly (Millipore SLGV033RS) so the DTT solution was filter sterilized as it was added to the cells. The bottle was then capped and incubated on a gyratory shaker at 80 RPM for 15 minutes at 28-30 °C. Next, 125 mL of ice-cold, autoclaved distilled water was added to the cells in the Nalgene bottle, which was then centrifuged at 1500 × g for 10 minutes at 4ºC. The resulting pellet was resuspended in 225 mL of ice-cold, autoclaved distilled water and centrifuged under the same parameters. The tube following this centrifugation step was kept cold

on ice. The supernatant was again discarded, and the pellet was gently resuspended in 20 mL of ice-cold autoclaved1 M sorbitol and transferred to a sterile 50 mL conical polypropylene tube with screw cap. This tube was centrifuged 1500 × g for 10 minutes at 4°C. The supernatant was discarded, and the pellet was resuspended by adding 0.5 mL of ice-cold 1 M sorbitol. The suspended cells were competent at that point and held on ice under sterile conditions until electroporation.

Electroporation of the linearized expression cassette plasmid into the competent wild-type *Pichia* cells was done with a Bio-Rad Gene Pulser Xcell Electroporation System model 165-266 with CE Module. Competent wild-type *Pichia* cell suspension (40 μL) and either 0.5 μg (low amount) or approximately 3.5 µg (high amount) of the MT1 or MT6 linearized expression cassette plasmid in sterile, distilled water were added to a 1.5 mL autoclaved microcentrifuge tube, mixed gently by pipetting up and down, and chilled on ice. This entire mixture was then transferred by a sterile Pasteur pipet into a sterile, 0.2 cm gap Gene Pulser cuvette (Bio-Rad 165-2082), which as then capped and held on ice until placed into the Gene Pulser ShockPod module and immediately delivered one square wave, 1500 volt pulse of 5.0 ms duration. Immediately after the pulse, the cuvette was removed from the ShockPad module, and 0.75 mL of ice-cold, autoclaved 1 M sorbitol was added to the cuvette. A sterile Pasteur pipet was then used to gently transfer the suspension of electroporated cells from the cuvette to a sterile 17 ×100 mm round-bottom polystyrene culture tube with snap cap. The electroporation cuvette was rinsed with an additional 0.75 mL of ice-cold, autoclaved 1 M sorbitol which was then also transferred to the culture tube and held on ice. Electroporation was done with the low and high amounts of the MT1 linearized expression cassette plasmid, and with the low and high amounts of the MT6 linearized expression cassette plasmid, to result in a total of 4 culture tubes from the 4

electroporation events: low amount MT1 plasmid (MT1L), high amount MT1 plasmid (MT1H), low amount MT6 plasmid (MT6L), and high amount MT6 plasmid (MT6H). These 4 culture tubes were then moved from ice to 30 °C where they were incubated without shaking for 1-2 hours in the dark. Aliquots of 30, 100, or 200 µL of the electroporated cell suspension from a tube were then spread on a total of 10 Petri plates with various agar media detailed in Table 1. The plates were then incubated, inverted, at 28-30 °C in dark for 10 days. An additional 100 μL of sterile 100% methanol was added to the lid of MMSZ plates every 24 hours to replace

30 µL of cell suspension on 1 Petri dish with YPDSZ100 [-30-(MT1L, MT1H, MT6L, MT6H)]
100 µL of cell suspension on 1 Petri dish with YPDSZ100 [-100-(MT1L, MT1H, MT6L, MT6H)]
200 µL of cell suspension on 1 Petri dish with YPDSZ100 [-200-(MT1L, MT1H, MT6L, MT6H)]
30 µL of cell suspension on 1 Petri dish with MMSZ100 [-30-(MT1L, MT1H, MT6L, MT6H)]
100 µL of cell suspension on 1 Petri dish with MMSZ100 [-100-(MT1L, MT1H, MT6L, MT6H)]
200 µL of cell suspension on 1 Petri dish with MMSZ100 [-200-(MT1L, MT1H, MT6L, MT6H)]
200 µL of cell suspension on 1 Petri dish with YPDSZ300 [-200-(MT1L, MT1H, MT6L, MT6H)]
200 uL of cell suspension on 1 Petri dish with MMSZ300 [-200-(MT1L, MT1H, MT6L, MT6H)]
200 µL of cell suspension on 1 Petri dish with YPDSZ1000 [-200-(MT1L, MT1H, MT6L, MT6H)]
200 uL of cell suspension on 1 Petri dish with MMSZ1000 [-200-(MT1L, MT1H, MT6L, MT6H)]
1460 µL total plated from available 1500 µL of cell suspension from each electroporation event

Table 1. Ten Petri dish cultures started from each culture tube of 1.5 mL of *Pichia* cell suspension from one of the four electroporation events (MT1L, MT1H, MT6L, or MT6H). The media were formulated as described by Invitrogen (2010) and were: Yeast Extract Peptone Dextrose Sorbitol Medium with Zeocin (YPDSZ) or Minimal Methanol Sorbitol Medium with Zeocin (MMSZ). The 100, 300, or 1000 following YPDSZ and MMSZ indicates the Zeocin concentration (μg/mL) in the medium.

evaporated and metabolized methanol.

By the end of the 10 days of incubation, a total of 93 colonies appeared on the total of 40 plates. Ten of the 93 colonies appeared on YPDSZ plates, while 83 colonies appeared on MMSZ plates. Of the 93 colonies, only 1 appeared on a Z1000 plate, 3 appeared on Z300 plates, and the other appeared on Z100 plates. Some colonies appeared on plates at all 3 levels of cell suspension inoculation, although the largest number of colonies appeared on plates inoculated with 200 µL of cell suspension, as might have been expected.

At the end of the initial10 days of incubation, each of the 93 colonies was streaked onto

its own plate where the medium was the same as that on which the colony initially grew, except that the 1 M sorbitol was no longer present. The Zeocin level was the same as that in the medium where colony initially grew. These 93 plates were incubated, inverted, at 28-30 °C in dark for 3-10 days, as needed for new colonies to appear in the streak path. This streak plating step is important to prevent mixed colonies, ensuring that each subsequent colony is composed of a single transformed strain (Cregg 2007). Of the 93 plates, 91 showed growth of new colonies within 3-10 days. Those 91 plates were edge-wrapped with Parafilm and moved to storage at 2-3 °C until further analysis. None of the 91strains had a slimy appearance.

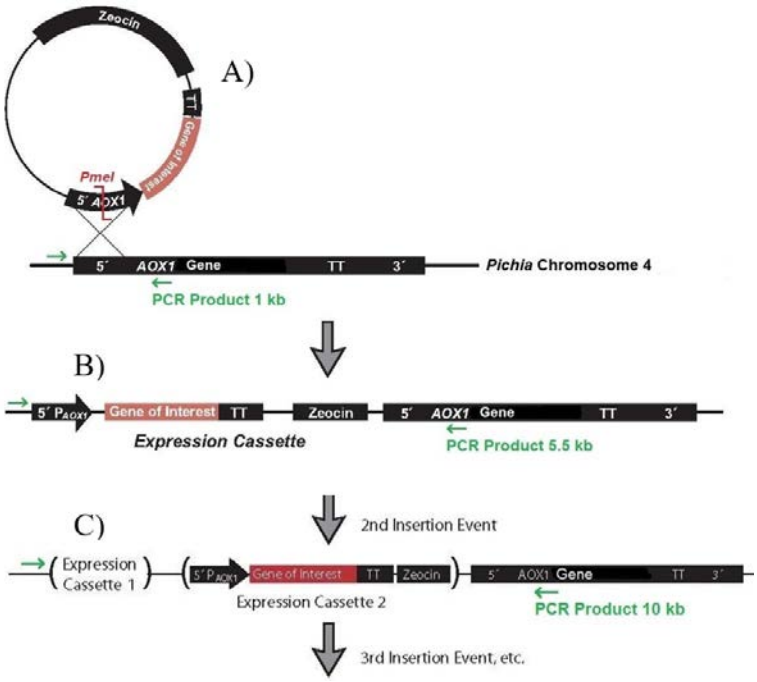

Figure 3. Single or multiple insertions of expression cassette plasmids into the *AOX1* promoter in *Pichia* chromosome 4. A) *MT1* or *MT6*-expression cassette plasmid (4.5 kb) where the Gene of Interest refers to either *MT1* or *MT6* cDNA (essentially same as Fig. 2, except slightly different artist's interpretation). The *AOX1* promoter region is labeled as 5'*AOX1* or 5'P_{AOX1}. The *PmeI* restriction endonuclease cut (red) in the middle of 5'*AOX1* is used to linearize the plasmid prior to electroporation into wild-type *Pichia* cells. Green arrows indicate PCR primers selected to base pair either just upstream or just downstream of the *AOX1* promoter on *Pichia* chromosome 4. With no insertion of the expression cassette plasmid, the PCR product size is 1 kb. B) With one insertion of the expression cassette plasmid, the anticipated PCR product size is 5.5 kb. C) With two insertions of the expression cassette plasmid, the anticipated PCR product size is 10 kb. Figure adapted from Invitrogen (2010).

The transformation of these 91 strains with the 4.5 kb

linearized plasmid containing either MT1 or MT6 was then analyzed by a secondary screen involving a PCR-based assay, using primers selected to base pair either just upstream or just downstream of the *AOX1* promoter on *Pichia* chromosome 4 (Fig. 3). The size of the anticipated PCR product using these primers is 1 kb if there has been no insertion of the expression cassette plasmid, 5.5 kb if there has been two insertions of the expression cassette plasmid, and 10 kb if there has been two insertions of the expession cassette plasmid (Fig. 3).

To start the PCR screening process, a colony was picked up from one of the Petri dish streak cultures stored at 2-3 °C and used to inoculate 3 mL of liquid YPDZ100 medium (YPD used for wild type) in a culture tube. These liquid cultures were grown on a 300 RPM shaker for 3 days at 28-30 °C in the dark, and then harvested for genomic DNA preparation by using the YeaStar Genomic DNA Kit from Zymo Research. In the initial experiments of this type, Protocol I recommended by the manufacturer was used (Zymo Research 2016). As designed for *Saccharomyces cerevisiae* yeast but stated to be applicable to *Pichia pastoris*, this method involves incubating 5 x 10^7 yeast cells with R-Zymolyase (an enzyme mixture of Zymolyase, which degrades cell wall β-1,3-glucan, and ribonuclease, which degrades RNA, lysing the resulting spheroplasts (yeast cells without cell wall) with YD Lysis buffer, removing lipids by chloroform extraction, and purifying the DNA on a Zymo-spin III silica membrane column with DNA wash buffer (80% ethanol) followed by elution with TE buffer. The concentration of the resulting genomic DNA was measured by NanoDrop UV spectrophotometry. Because the yield of DNA obtained from Pichia cells by using Protocol I was rather low, several variations of Protocol I were later tested and led to a method that gave improved DNA yields (see Results). Genomic DNA prepared from the Picha strains was used in PCR experiments with the described primers (Fig. 3) and LongAmp Hot Start *Taq* DNA polymerase (supplied by New England

Biolabs). From the secondary screening using the PCR-based assay, colonies that indicated proper transformation by presence of a 5.5 kb band (Fig. 3) were used in test-tube biochemical assays to assess the presence of methyltransferase activity.

Pichia strains to be tested for methyltransferase activity were grown in liquid culture. For the first 3 days of growth, 15 mL of buffered minimal glycerol (BMG) medium was used, and this was followed by transfer to 50 mL of buffered minimal methanol (BMM) medium for an additional 3 days. These media are recommended by Invitrogen (2010) because BMG supports increase of cell mass and then BMM induces the AOX1 promoter to produce the alcohol oxidase needed for growth on methanol but should also induce expression of the desired transgene which is also controlled by the AOX1 promoter.

After 3 days in BMM, the *Pichia* cells were harvested and transferred to 250 mL Nalgene centrifuge bottles where they were washed by centrifugation using a lysis buffer containing protease inhibitors (Fig. 4). At this point, one of two different methods was used to open the *Pichia* cells for extraction of proteins. The first method to open *Pichia* cells was to vortex the cells with acid-washed glass beads (0.5 mm dia.). The mixture of cells and glass beads was vortex at maximum speed 7 times, alternating 1 minute of vortexing with 1 minute of cooling on ice (Pedro et al. 2015). After vortexing, low speed centrifugation pelleted the glass beads, unbroken cells, and cell debris, allowing collection of proteins and organelles in the supernatant (Fig.4).

The second method used to open *Pichia* cells was to incubate the cells for 3.5 hours with Zymolyase enzyme (Zymo Research) to digest the cell walls and release spheroplasts, and then use a Dounce homogenizer three times to lyse the spheroplasts in breakage buffer (Fig. 4). After each use of the Dounce homogenizer, the homogenate was centrifuged, the supernatant was

poured off and saved, the pellet was resuspended in breakage buffer, and then it was run through the Dounce homogenizer again. The supernatants from the three passes through the Dounce homogenizer were pooled and move forward to the next step.

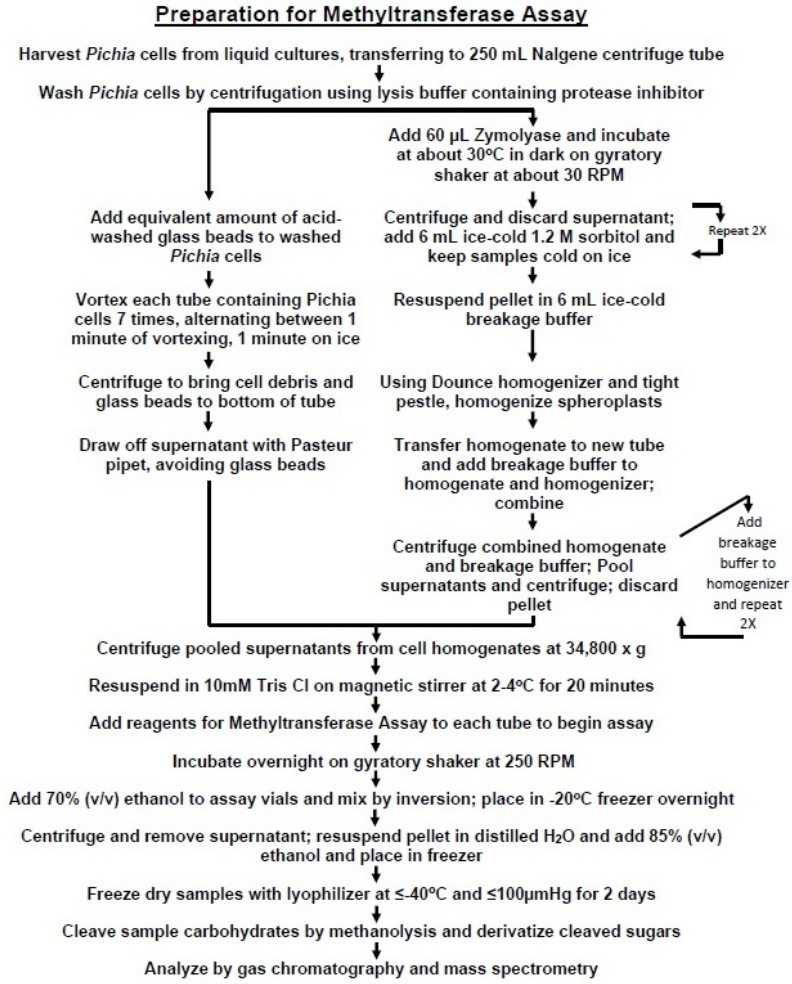

Figure 4. Flowchart summary of methods for preparing protein extracts from Pichia strains and assaying those extracts for methyltransferase activity.

The supernatants from both the glass bead and the Dounce homogenizer procedures were centrifuged at 34,800 × g for 30 minutes to yield a pellet of organelle membranes and a supernatant of soluble proteins plus lighter membrane vesicles (Daum et al. 1982, Zinser and Daum 1995, Wriessnegger et al. 2007, Wriessnegger et al. 2009, Ivashov et al. 2013, Grillitsch et al. 2014, Klug et al. 2014). The supernatant fraction was designated S34800g. The pellet from this centrifugation step was resuspended in buffer, with 1% Triton X-100 detergent in some experiments, on a magnetic stirrer at 2-4ºC for 20 minutes. This resuspended pellet was designated P34800g. Both the S34800g and P34800g fractions were analyzed by SDS-PAGE to check whether synthesis of the

74 kDa alcohol oxidase had been abundantly induced, which would indicate that either *MT1* or *MT6* should also be induced since they are controlled by the same AOX1 promoter.

The test tube biochemical assay for methyltransferase activity (Fig. 4) was performed by combining the S34800g fraction or the P34800g fraction with the methyl donor S-adenosyl-L-methionine (SAM) and one or more of the following potential polysaccharide substrates: tobacco pectin, oxalic acid-hydrolyzed gum arabic, oxalic acid-hydrolyzed gum ghatti, potato galactan, guar galactomannan, and larch arabinogalactan. The reaction mixtures were incubated overnight at 28-30 °C, and then the polysaccharide substrates were recovered from the reaction mixture by ethanol precipitation and lyophilization. The freeze-dried polysaccharides were then cleaved by methanolysis, derivatized by trimethylsilylation, and analyzed by gas chromatography-flame ionization detection (GC-FID) and by gas chromatography-mass spectrometry (GC-MS) (Fig. 4).

Results:

After electroporation of the *MT1*- or *MT6*-expression cassette plasmids into wild-type *Pichia*, 93 colonies were recovered in the first screening with Zeocin antibiotic. Of these initial 93 surviving colonies, 89 survived all subsequent selections on Zeocin-containing media. These 89 colonies were then assessed by a secondary screen using a PCR assay to confirm proper insertion of the MT1 or MT6 plasmid into the Pichia *AOX1* promoter.

The manufacturer's Protocol I (Zymo Research 2016) was initially used with the YeaStar Genomic DNA Kit for purifying *Pichia* DNA as needed for the PCR assay. Because the yield of DNA was low by this procedure, several modifications to Protocol I were made in attempt to increase the DNA yield. These medications included the amount of cells used, how the cells were broken open, and how the DNA was extracted from the cell homogenate, and how the DNA was purified and released from the silica membrane spin column. The mechanism by which the

silica membrane spin column purifies DNA is illustrated in Figure 5.

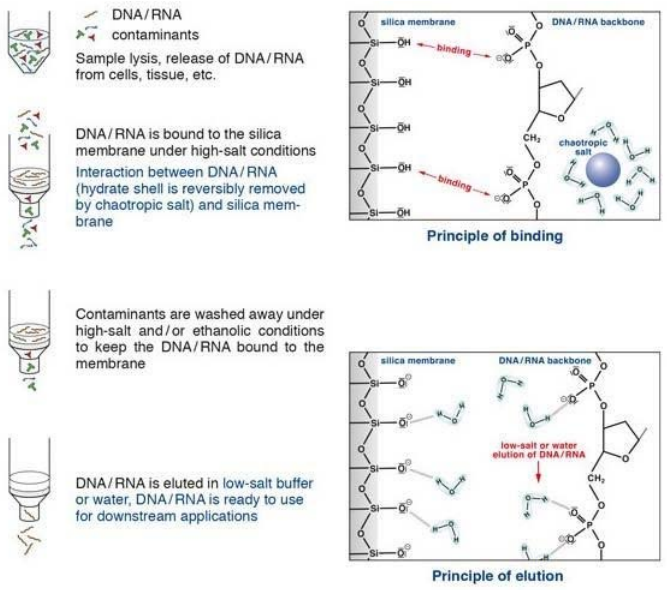

Figure 5. Principles of DNA binding to and elution from a silica membrane spin column. In high-salt conditions, such as guanidinium chloride and other proprietary agents in YD Lysis buffer, DNA adheres to the silica membrane. DNA also stays on the silica membrane in wash buffers that contain 80% ethanol. After the washes, DNA is eluted by low salt buffers with no ethanol. Figure from Macherey-Nagel (2019.

A summary of the results obtained from the medications to the manufacturer's Protocol I is presented in Table 2. From those results, it is apparent that increasing the number of cells extracted, increasing the incubation time of the cells with the R-Zymolyase enzyme mixture, using a more gentle method to disrupt the cells in YD lysis buffer following the R-Zymolyase incubation, and using modified buffers to wash and elute the DNA from the silica membrane spin column resulted a significantly increased DNA yield without sacrificing DNA quality, as judged by Nanodrop UV spectrophotometry, including the A_{260}/A_{280}, and A_{260}/A_{230} purity indicators. The A_{260}/A_{280} ratio checks for protein contamination (measured at A_{280}) in the DNA preparation, with pure DNA typically measuring between 1.85-1.88 (Koetser and Cantor 2019). The results in Table 2 show that the A_{260}/A_{280} ratio varied between 1.63 – 2.02 and finishing around 1.74 after the modifications. The A_{260}/A_{230} measurement assesses contamination from other sources in the DNA preparation, including compounds in the lysis, wash, and elution buffers, and is around 2.1-2.3 for pure dsDNA (Koetser and Cantor 2019).

From Table 2 it is clear that the A_{260}/A_{230} ratio, even after the modifications to Protocol I, is, is still well below the ratio expected for pure DNA, possibly due to some residual guanidinium chloride remaining from the YD Lysis Buffer in the YeaStar kit.

Parameter Adjustments to Improve the DNA Yield from *Pichia pastoris* Using the YeaStar Genomic DNA Kit from Zymo Research

Zymo Research recommends Protocol I (essentially row 1 in below table) for *Saccharomyces cerevisiae*, where the yield is stated to be 7000 – 20000 ng DNA, and also for other fungi, including *Pichia*. We found the DNA yield for *Pichia* to be much lower, at least in part because the DNA content per cell is lower for *Pichia* than for *Saccharomyces*:

Saccharomyces has 12.1 Mbp genome size (haploid) but is usually in the diploid state as grown in labs.
Pichia has 9.43 Mbp genome size (haploid) and is usually in the haploid state as grown in labs [De Schutter et al. (2009) Nature Biotech 27: 561-566]

Thus: $\frac{\text{DNA content per } \textit{Saccharomyces} \text{ cell}}{\text{DNA content per } \textit{Pichia} \text{ cell}} = \frac{(2)(12.1\text{Mbp})}{(1)(9.43\text{ Mbp})} = 2.57$

Number of cells extracted	R-Zymolyase (25 units) digestion at 37 °C	Agitation-incubation after addition of YD Lysis Buffer	Washes (300 µL ea) after extract loaded on Zymo-Spin III column	Elution solvent (60 µL) and incubation prior to spin out from column	DNA yield (ng) Ave ± SEM	A_{260}/A_{280} Ave ± SEM	A_{260}/A_{230} Ave ± SEM
5×10^7	1 hr	5 sec gentle vortex, 6 tube inversions	DNA Wash Buffer + 80% EtOH (2X)	Dist. H_20; 1 min 23°C incubation	487 ± 38	1.63 ± 0.02	0.56 ± 0.04
5×10^7	4 hr	5 sec gentle vortex, 10 tube inversions; 15 min 23 °C incubation	DNA Wash Buffer + 80% EtOH (2X); 10 mM TRIS•Cl pH 8.5 + 80% EtOH (1X)	10 mM TRIS•Cl pH 8.5; 1 min 23°C incubation	630 ± 33	1.88 ± 0.03	0.52 ± 0.07
5×10^7	4 hr	1 min hard vortex, 10 min 23 °C incubation	DNA Wash Buffer + 80% EtOH (2X); 10 mM TRIS•Cl pH 8.5 + 80% EtOH (1X)	10 mM TRIS•Cl pH 8.5; 1 min 23°C incubation	594 ± 60	2.02 ± 0.10	0.13 ± 0.02
15×10^7	4 hr	1 min hard vortex, 10 min 23 °C incubation	DNA Wash Buffer + 80% EtOH (2X); 10 mM TRIS•Cl pH 8.5 + 80% EtOH (1X)	10 mM TRIS•Cl pH 8.5; 1 min 23°C incubation	909 ± 129	1.85 ± 0.11	0.34 ± 0.01
15×10^7	4 hr	4 X 30 sec hard vortex, 10 min 23 °C incubation	DNA Wash Buffer + 80% EtOH (2X); 10 mM TRIS•Cl pH 8.5 + 80% EtOH (1X)	10 mM TRIS•Cl pH 8.5; 1 hr 55°C incubation	1085 ± 58	1.76 ± 0.02	0.59 ± 0.07
15×10^7	4 hr	No vortex, 15 tube inversions; 10 min 37 °C incubation	DNA Wash Buffer + 80% EtOH (2X); 10 mM TRIS•Cl pH 8.5 + 80% EtOH (1X)	10 mM TRIS•Cl pH 8.5; 1.5 hr 55°C incubation	1326 ± 45	1.74 ± 0.01	0.74 ± 0.05

Table 2. Results of parameter adjustments to improve DNA yield from *Pichia pastoris* using the YeaStar Genomic DNA Kit from Zymo Research.

The PCR-based secondary screen of the 89 Pichia strains that survived the Zeocin primary screen produced remarkable results. Only 16 of the 89 strains yielded a PCR product of the 5.5 kb size expected for the case of one copy of the expression cassette plasmid inserted into the AOX1 promoter on Picha chromosome 4. The other 73 strains all yielded only a 1kb PCR product, indicating no insertion into the *AOX1* promoter. All of those 73 strains survived repeated screenings on media containing 100 μg/mL Zeocin, however, which seems to imply that expression cassette inserted into some location in the genome other than the *AOX1* promoter on

chromosome 4. Because such insertion would likely result in an incomplete promoter in front of

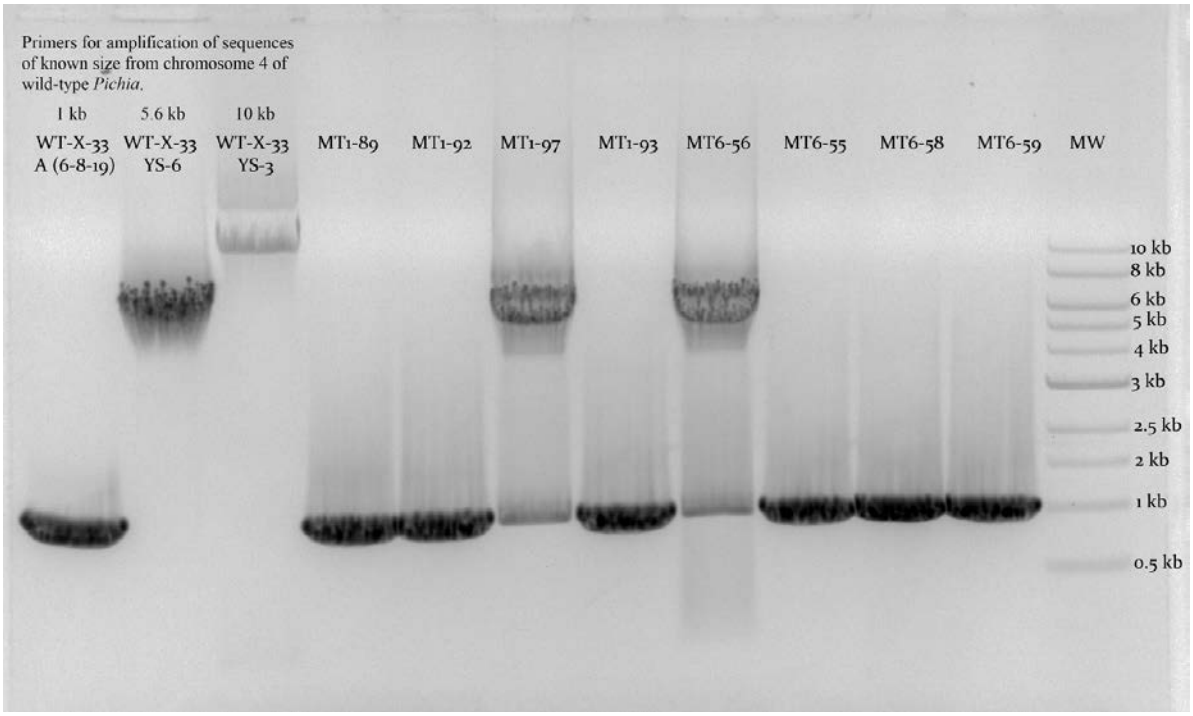

Figure 6. Agarose electrophoretic gel showing PCR products from wild-type and several transgenic *Pichia* strains. The three wild-type lanes at left are positive controls using primers designed to basepair to portions of the *AOX1* sequence or nearby sequences on chromosome 4 and yield PCR products of sizes 1 kb, 5.5 kb, and 10 kb. Molecular weight markers appear in the far right lane. The other lanes on the gel show PCR products produced using the primer pair described in Figure 3. Among these other lanes, strains MT1-97 and MT6-56 produced a 5.5 kb PCR product indicating the anticipated incorporation of one copy of the expression cassette plasmid into the *AOX1* promoter on chromosome 4, while the other four strains produced only a 1 kb PCR product, likely indicating insertion of the expression cassette plasmid elsewhere in the genome.

the *MT1* or *MT6* transgene, these 73 strains are probably not good candidates for finding methyltransferase activity.

It is noteworthy that none of the 89 transgenic Pichia strains produced a 10 kb PCR product that would indicate insertion of two copies of the expression cassette plasmid into the *AOX1* promoter, even though the positive control lanes in Figure 6 indicate that the PCR system

used in this project was capable of producing a 10 kb PCR product.

For the remainder of this project, attention was focused on the 16 transformed Pichia strains that yielded the expected 5.5 kb PCR product. Figure 7 shows SDS-PAGE protein electrophoretic analysis of the S34800g and P34800g subcellular fractions from X-33 wild-type and five of the 16 selected transgenic *Pichia* strains. All six of the S34800g lanes show an abundant polypeptide of apparent size 74 kDa, which is the size of the *Pichia* alcohol oxidase. This observation is consistent with strong induction of the *AOX1* promoter, as expected in the BMM medium (Invitrogen 2010). None of the lanes from the transformed *Picha* strains show a band of

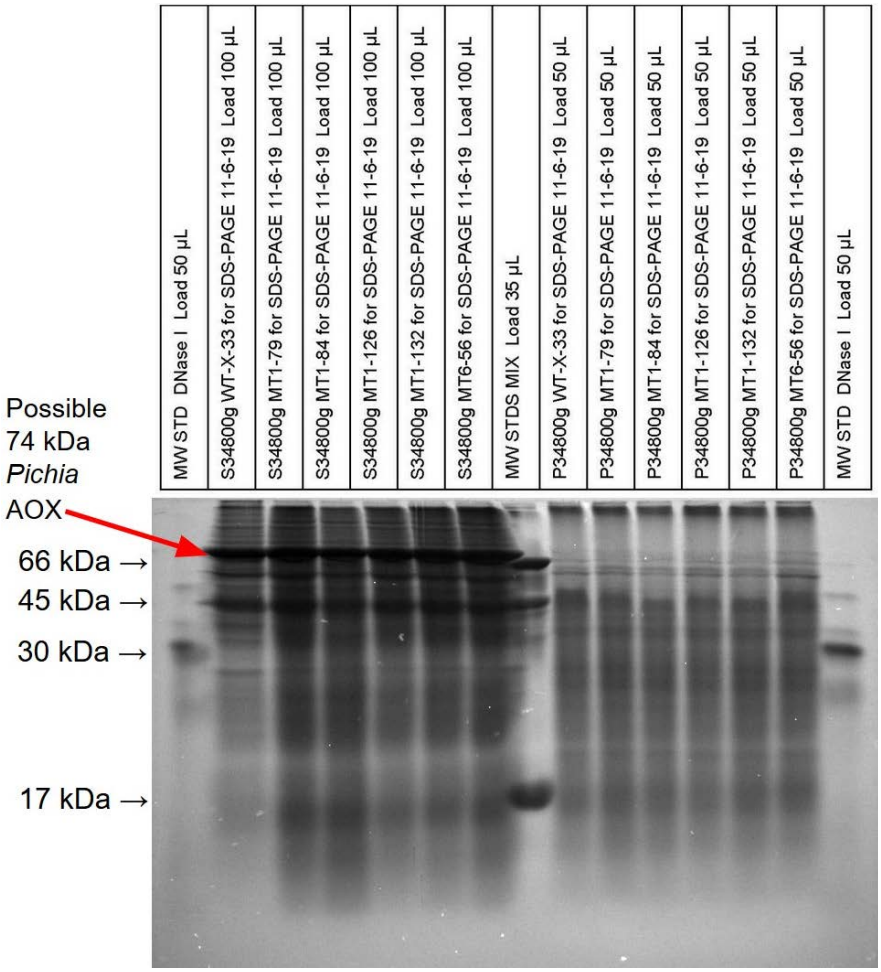

Figure 7. SDS-PAGE electrophoretic gel of supernatant and pellet fractions (S34800g and P34800g, respectively) from the 34,800 × g centrifugation of *Pichia* homogenate. Molecular weight standards are BSA (66 kDa), ovalbumin (45 kDa) and horse myoglobin (17 kDa) in the center lane and bovine pancreatic DNase I (30 kDa) in edge lanes.

size appropriate to be MT1 or MT6, and a band that does not appear in the wild type lanes. It is typical, however, that bands from transgenic proteins expressed in *Pichia* are not abundant enough to be seen on gels stained with Coomassie Blue (Invitrogen 2010).

All of the 16 PCR-selected transformed *Pichia* strains were assayed for methyltransferase activity towards various polymeric substrates. Detection of the anticipated methylated sugars, 3-*O*-Me-Rha and 3-*O*-Me-Gal, was done by GC-FID/GC-MS analysis. Neither of the anticipated methylated sugars was detected in high abundance in the polymeric products from any of the assay trials. Hints of very weak 3-*O*-Me-Rha or 3-*O*-Me-Gal were observed in a few of the methyltransferase assays. In Figure 8, the MT6-56 polymeric products (Figure 8C and 8D) show the strongest peak observed thus far with GC retention time, MS ion chromatograms, and full mass spectrum all matching well to those of of authentic 3-*O*-Me-Rha from *Physcomitrella* cell wall (Figure 8A and 8B). The corresponding region of the graphs from wild-type *Pichia* (Figure 8E and 8F) show much weaker signals that are little stronger than noise level. Although the results in Figure 8 might be encouraging, they are not yet satisfactorily reproducible. More experiments are required before these results can be considered to be established.

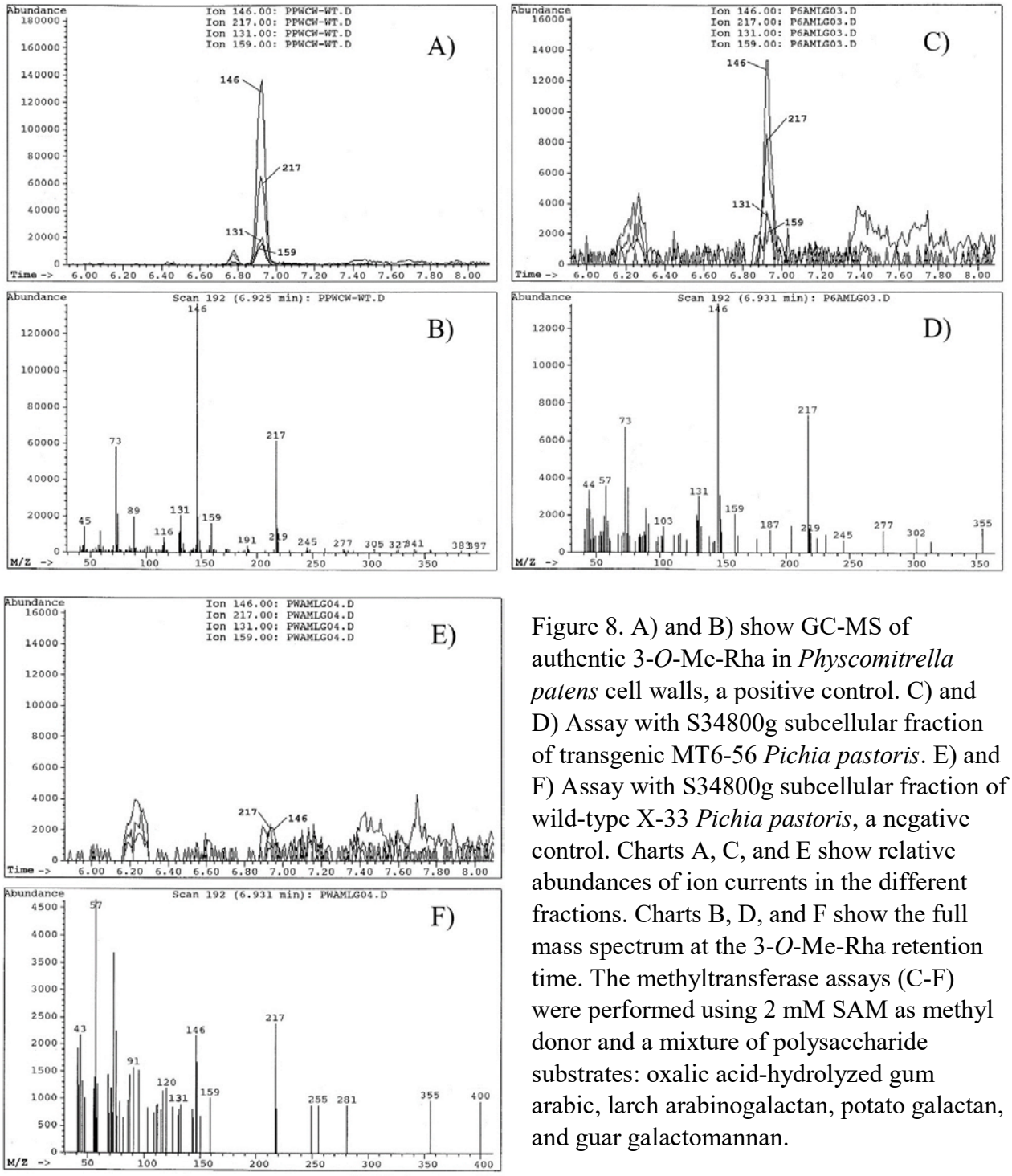

Figure 8. A) and B) show GC-MS of authentic 3-*O*-Me-Rha in *Physcomitrella patens* cell walls, a positive control. C) and D) Assay with S34800g subcellular fraction of transgenic MT6-56 *Pichia pastoris*. E) and F) Assay with S34800g subcellular fraction of wild-type X-33 *Pichia pastoris*, a negative control. Charts A, C, and E show relative abundances of ion currents in the different fractions. Charts B, D, and F show the full mass spectrum at the 3-*O*-Me-Rha retention time. The methyltransferase assays (C-F) were performed using 2 mM SAM as methyl donor and a mixture of polysaccharide substrates: oxalic acid-hydrolyzed gum arabic, larch arabinogalactan, potato galactan, and guar galactomannan.

Discussion:

After optimization of the procedures (Table 2) used with the YeaStar Genomic DNA Kit from Zymo Research, the quantity and quality of *Pichia* DNA obtained was adequate for the long amplicon PCR needed for the PCR-based secondary screening of transformed *Pichia* strains (Fig. 6). This PCR method appears to be a reliable test for multiple insertions of expression casette plasmids, such as the *MT1* or *MT6* linearized plasmids used here, into the *AOX1* promoter region on *Pichia* chromosome 4. The PCR test indicated that only 16 of 89 transgenic *Pichia* strains demonstrating Zeocin antibiotic resistance had the expected expression casette insertion into the middle of the *AOX1* promoter. This result seems to indicate that the plasmids are incorporating somewhere else in the *Pichia* genome, where the moss methyltransferase genes *MT1* or *MT6* might not have a functioning promoter to enable production of methyltransferase protein.

All of the 16 PCR-selected transformed *Pichia* strains have no, or at best very weak and not yet satisfactorily reproducible methyltransferase activity. Further experimentation is required to give consistently replicable results to confirm methyltransferase activity in these transgenic *Pichia* strains. If the apparent synthesis of 3-*O*-Me-Rha hinted at by the very weak activity observed so far in methyltransferase assays can be confirmed, then the next step of this experiment would be to invesitgate the factors that control which methylated sugar, 3-*O*-Me-Rha or 3-*O*-Me-Gal, is synthesized.

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